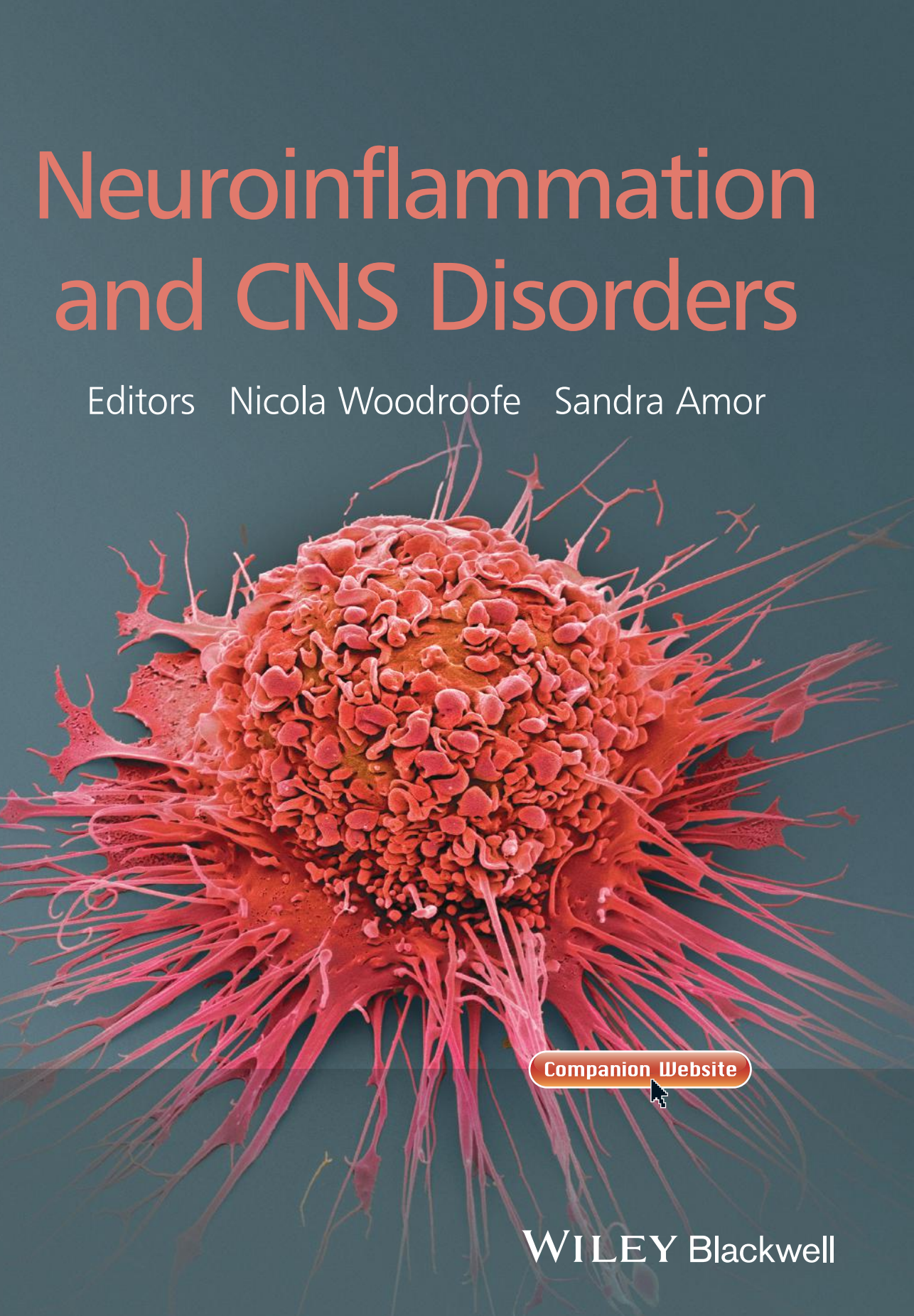
Neuroinflammation and CNS Disorders

Editors Nicola Woodroofe Sandra Amor



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Preface

It is widely assumed that the central nervous system is an immune-privileged site, suggesting that antigens gaining entry to the brain and spinal cord do not invoke an immune response.

While this idea was first discussed over 70 years ago, it is clear that immune privilege is not absolute since immune responses do take place in the central nervous system and are crucial for shaping the brain during development and for controlling infections in the brain. As well as these examples, in the last decade there has been an explosion of information on the role of immune responses in neurodegenerative disorders. In many of these diseases, it is still unclear whether the innate and adaptive responses are pathogenic or play a role in repair, and thus understanding their precise roles is key to controlling these diseases by designing immune-therapeutic approaches.

It is for this reason that we undertook the task of compiling the latest information on the interactions between the immune system and central nervous system.

In the first section of this book, the chapters are dedicated to the communication between the immune system and the central nervous system that is best exemplified by cross-talk between glia and neurons shown to be essential for maintaining homeostasis. This section is specifically designed as an introduction to the topic and forms the basis for the second section devoted to specific neurological diseases.

We are indebted to our many colleagues who have taken time from their busy schedules to help us compile this book. In particular, we would like to especially thank Stan Appel and his team, who underwent the hardship of tragically losing a colleague, Jenny Henkel, during the production of the chapter. Likewise, we also sincerely thank Andrew Harkin, who took over from Tom O'Connor who lost his life during the writing of their chapter. We hope that their memory will live on through their work and help inspire new generations in their fields.

We are not unaware that this will not be the last work on how the immune system interacts with the central nervous system, but we are confident that this book forms the basis of what is to come in the field.

Introduction: Interactions between the Immune and Central Nervous Systems

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In this introductory chapter, we briefly trace the history of the field and highlight the important influence that research in neuroimmunology has had on modern immunologic and neurological ideas. The link between many neurological diseases is the realisation that the immune and nervous systems are inextricably linked, and that perturbations in this delicate balance are involved in many disorders. This has opened up new avenues for therapeutic approaches to the treatment of central nervous system (CNS) inflammatory and neurodegenerative and neoplastic disorders. In this introduction to the book, we provide links to other chapters in the book that expand upon these key features. For those new to the field we have included a section (Chapters 1–5) highlighting the key basic concepts in the field, while the second section (Chapters 6–15) covers the role of the immune response in specific disorders of the CNS.

Origins

The field of neuroimmunology developed from sub-specialities in immunology and neurology into a rapidly expanding discipline of its own. While the first international congress of neuroimmunology was held in Stresa, Italy in 1982, the International Society of Neuroimmunology (ISNI) was only founded after the second congress in 1987 in Philadelphia, United States. The *Journal of Neuroimmunology* had been launched in March 1981, and the *Journal*

of Clinical and Experimental Neuroimmunology in 1988. The origins of neuroimmunology predate the establishment of the society by nearly a century, and the discipline has its roots in several interdisciplinary topics. It is a discipline that now encompasses a wide range of disorders including peripheral neuropathies and those affecting the CNS (Table I.1) (Amor *et al.*, 2010, Amor and Woodroofe, 2013; Peferoen *et al.*, 2013). The milestones in the history of neuroimmunology are outlined in Table I.2.

By invitation only

It is widely assumed that the CNS is an immune-privileged tissue, suggesting that antigens gaining entry to the brain and spinal cord do not invoke an immune response (Chapter 1). While this idea was first discussed over 70 years ago, it is clear that immune privilege is not absolute since immune responses do take place in the CNS and are crucial for shaping the brain during development and controlling infections in the brain. However, immune cells do not freely patrol the brain as with other organs, and those that enter are by invitation only. The gatekeeper of the CNS is the blood–brain barrier, which when compromised is unable to control such selection, thereby contributing to the tissue damage. In many neurological disorders there is evidence that the blood–brain barrier, and indeed the barriers that maintain immune privilege in the spinal cord and optic nerve, are less effective. However, whether such damage precedes or is the result of inflammation is still a 'chicken and egg' topic of discussion. As well as the physical barriers, the CNS attempts to maintain control by expression of immunomodulatory molecules on neurons and oligodendrocytes (Peferoen *et al.*, 2013). Thus, as well as damaging the CNS, both the innate and adaptive immune responses regulate and suppress inflammation (Chapters 2 and 3) and aid repair (Chapter 5). While such approaches are very effective in controlling immune responses in the CNS, this strategy is also exploited by tumours to interfere with or evade the immune system, thereby establishing a permissive environment in which to expand (Chapter 15). As well as these examples, in the last decade there has been an explosion of information on the role of immune responses in neurodegenerative disorders. In many of these diseases it is still unclear whether the innate and adaptive responses are pathogenic or play a role in repair, and thus understanding their precise roles is key to controlling these diseases by designing immune-therapeutic approaches.

Cross-talk between the immune system and CNS

As discussed in this introduction, neurons have a profound influence on the immune system, which is called neuroimmunomodulation. This influence is shaped by neurotransmitters, such as serotonin, histamine and gamma-aminobutyric acid; neuropeptides, such as adrenocorticotropin, vasoactive

Table I.1 Neuroimmunological aspects of disorders of the central nervous system (CNS)

Disorder	Clinical characteristics and immune involvement	Chapter
Alzheimer's disease (AD)	Pathology of human tissues, *in vitro* studies and animal models of AD provide evidence for involvement of immune activation pathways. Long-term use of anti-inflammatory drugs is linked with reduced risk of developing the disease.	6
Parkinson's disease	Movement disorder due to deterioration of the nigrostriatal system. Chronic activation of microglia is observed to be associated with neurodegeneration.	6
Huntington's disease and other polyglutamine expansion diseases	Microglia expressing a mutant huntingtin protein are blunted in their ability to migrate, leading to immune dysfunction.	6
Infections	Encephalitis, encephalomyelitis, meningitis, polyradiculitis or polyneuritis. Characteristics depend on infectious agents [e.g. human T-lymphotropic virus type 1 (HTLV1)-associated myelopathy (HAM)]. Immune responses depend on infectious agents.	7
Amyotrophic lateral sclerosis (Lou Gehrig's disease)	Immune abnormalities in the CNS and peripheral immune responses. Microglia activation is associated with the production of neurotoxic as well as neurotrophic factors.	8
Multiple sclerosis (MS)	Demyelination and neurodegeneration in brain, spinal cord and optic nerve. Innate and adaptive immune activation. Oligoclonal immunoglobulin in cerebrospinal fluid.	9
Acute demyelinating encephalomyelitis (ADEM)	Usually associated with or following a viral infection or following vaccination. Most cases are in children and adolescents (average ages 5–8). Demyelinating lesions are associated with immune activation (like MS).	9
Neuromyelitis optica (NMO)	Inflammatory disorder of the CNS predominantly affecting the optic nerves and spinal cord. Most patients have antibodies to aquaporin-4 (AQP4) which are thought to directly attack astrocytes.	9
Systemic lupus erythematosus (SLE), diabetes and gluten ataxia	Neurodegeneration and inflammation affect a large number of patients with SLE. Persons with gluten ataxia display a loss of Purkinje cells associated with immune activation in the CNS.	10
Depression	Link between levels of pro-inflammatory cytokines and depression in susceptible individuals. Changes in serotonergic and/or glutamatergic transmission in the CNS and reduced neurotrophic factor expression.	11

(*continued*)

Table I.1 (*Continued*)

Disorder	Clinical characteristics and immune involvement	Chapter
Epilepsy	A predisposition to develop seizures is frequently associated with cognitive and psychological sequelae. Both the innate and adaptive immune responses have been linked with disease. Anti-inflammatory agents are used to control some forms of epilepsy.	12
Stroke and intracerebral haemorrhage	Dramatic increase in the systemic inflammation and innate immune activation triggered to resolve debris as well as neutrophil traffic into infarcted brain tissue.	13
Spinal cord injury	Direct damage to axons, neuronal cell bodies and glia causes functional loss. The injury triggers an inflammatory response that contributes to secondary tissue damage.	14
Primary brain tumours	Cellular and molecular mechanisms that mediate tumour escape from natural immune surveillance (e.g. tumours down-regulate major histocompatibility complex expression).	15

Table I.2 Milestones in the history of neuroimmunology

Year	Milestone
1825–1893	Jean-Martin Charcot was a French neurologist and professor of anatomical pathology. He recognized the neurological diseases multiple sclerosis (MS), Charcot–Marie–Tooth disease and amyotrophic lateral sclerosis.
1949	Induction of experimental autoimmune encephalomyelitis in mice
1960	Nobel Prize: Peter B. Medawar (1915–1987) and Frank Macfarlane Burnet (1899–1985). The immune system can distinguish between self and non-self, and the brain is immune-privileged.
1980	Nobel Prize: Baruj Benacerraf (1920–2011), Jean Dausset (1916–2009) and George Davis Snell (1903–1996), "for discovery of the Major histocompatibility complex genes which encode cell surface molecules important for the immune system's distinction between self and non-self".
1981	Launch of *Journal of Neuroimmunology*
1982	First international neuroimmunology meeting
1996	Nobel Prize: Peter C. Doherty and Rolf M. Zinkernagel. Importance of major histocompatibility complex molecules in the detection, removal and killing of virus-infected cells
1999	Alemtuzimab (antibody to CD52; Campath 1H) effective in suppression of active inflammation in MS
2005	Recognition that antibodies to aquaporin-4 (AQP4) are present in people with optic-spinal MS (now classified as neuromyelitis optica) and bind to the AQP4 water channel
2011	Effective use of Rituximab (to deplete B cells) in MS to reduce relapses
2011	Nobel Prize in Physiology or Medicine: Bruce Beutler, Jules Hoffman and Ralph M. Steinman. Identification of dendritic cells and importance in T cell activation and specifically the role of the innate immune response

intestinal peptide, neuropeptide Y, endorphins and substance P; and neurotrophic growth factors, such as nerve growth factor and ciliary neurotrophic growth factor. In return, the immune system influences neuronal functions by producing immune and inflammatory mediators, such as cytokines and chemokines, leading to conditions such as sickness behaviour and depression (Chapter 11). That many autoimmune disorders are influenced by hormones is reflected by the high association of autoimmune disorders in females compared to males, including MS. These studies have now led to therapeutic approaches (so-called neuroendocrine immunomodulation) that may also be applicable to other CNS disorders in which the immune system is involved.

Of mice and men

The field of neuroimmunology has contributed to advancements in modern neuroimmune disorders largely through discoveries made in experimental models. Nevertheless, these concepts that have emerged from *in vivo* animal studies must also be valid in humans. These studies, including tracking immune responses in the brain, have yielded important insights into the mechanisms of damage as well as immunoregulation in neuroimmune disease. With the advent of powerful tools such as multifunctional flow cytometry, gene expression profiling, proteomics and mass spectrometry imaging, these studies now offer increased insight into how the immune system and CNS interact and indeed how such cross-talk can be manipulated. The new developments in human cellular immunology have also advanced the application of immune therapies to target specific arms or pathways involved in these immune-mediated disorders. This field will allow the development of novel approaches to treatment of neurodegenerative disorders, although it must be borne in mind that effective therapies can only arise from the correct use, application and interpretation of data arising from animal models and, most critically, the translation of these data to humans.

Immune responses and neurodegenerative disorders

The role of the innate and adaptive immune responses in neurodegenerative diseases has become a major focus of neuroimmunologists. This is partly due to the increasing ageing community, since the average life expectancy now extends late into the eighth decade in the Western world. Many neurodegenerative disorders occur more frequently in people of advanced age. In 2000, the number of persons with dementia was estimated at 25 million worldwide, but this figure does not include neurodegenerative diseases that are not classically associated with cognitive decline, such as traumatic brain injury and systemic lupus erythematosus (Chapter 10).

The major challenge in this area is to understand why and how the immune system is activated and the precise roles of immune responses in neuronal damage and dysfunction and in cognitive decline. Clearly, ageing plays a key role in neurodegenerative disorders, and this may partly rely on the decreased effectiveness of the ageing immune system (Chapter 4). It is probable that while subtle differences between diseases are observed, common pathways may imply that broad therapeutic approaches may be applied to these diseases. Such an understanding will be a key to developing therapeutics targeting the relevant component of the immune system.

In summary, this introductory section has chronicled the emergence of neuroimmunology in the latter part of the 20th century as well its contributions to modern immunology. More details are provided in the separate chapters in this book by experts in their fields.

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About the Companion Website

This book is accompanied by a companion website:

www.wiley.com/go/woodroofe/neuroinflammation

The website includes:

- Powerpoints of all figures from the book for downloading
- PDFs of all tables from the book for downloading

1
Immune Privilege of the Brain

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Introduction

"Immune privilege" is certainly among the most frequently misconstrued terms in the field of neuroimmunology. Numerous papers are introduced by statements such as 'The brain, as an immune-privileged organ does not allow leukocyte entry, but this immune privilege is not complete'. Thus, immune privilege is often defined as the absence of leukocyte recruitment to the brain and is therefore related to the blood–brain barrier (BBB) hindering invasion of immune cells. This ignores not only the origin of the term 'immune privilege', defined as relative tolerance to grafts by Billingham and Boswell (1953), but also the fact that the BBB was originally described as an obstacle for hydrophilic molecules at the level of capillaries, not for leukocytes which, according to current knowledge, leave the bloodstream at the level of postcapillary venules (reviewed in Bechmann *et al.*, 2007).

The original experiment

In his seminal, still-cited paper published in 1948, Medawar reported how immune tolerance to skin grafts, which are tolerated within the brain, can be broken: upon grafting a second piece of skin into the same animal, Medawar noted rapid elimination not only, as anticipated, of this second graft but also of the first one located in the brain. He summarized his observation by stating that "it is concluded that antigens within the brain do not elicit, but can succumb to an immune response". Apparently, the graft within the brain does

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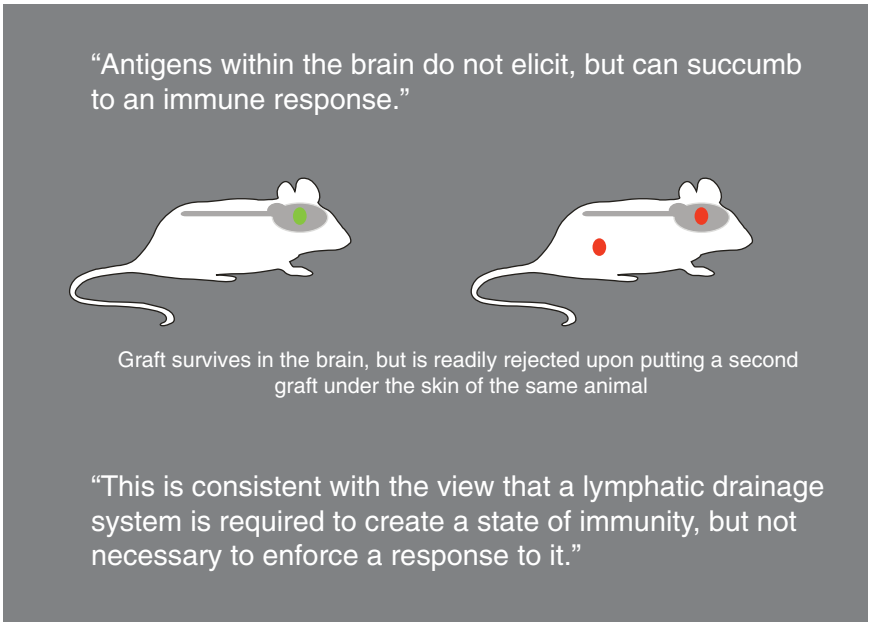

Figure 1.1 Medawar (1948) demonstrated that a skin graft (green) was tolerated when transplanted into the brain. Upon grafting a second piece of skin into the same animal, rapid rejection not only, of this second graft (red) but also of the first one located in the brain took place.

not evoke systemic immunity sufficiently to eliminate the grafted tissue, or it causes a tolerogenic immune response (Figure 1.1). Only if immunity is triggered by the same antigens present in the periphery, is the graft in the brain also attacked. Medawar's interpretation was that "this is consistent with the view that a lymphatic drainage system is required to create a state of immunity, but not necessary to enforce a response to it" (1948). In modern terms, this interpretation suggests that immune ignorance rather than an actively induced state of immune tolerance underlies the observed phenomenon of the maintenance of grafts within the brain.

The late Wayne Streilein (1935–2004), a leading scientist in the field of ocular immune privilege, often stated that "immune privilege comes in two flavours": immune-privileged sites and immune-privileged tissues. While the former are remarkably hostile to grafts, the latter are special in that they can be transplanted elsewhere with a relatively high rate of success (Streilein, 1996). 'Immune-privileged sites' include the brain, eye, pregnant uterus, testis, ovary, adrenal cortex, hair follicles and tumours, while 'immune-privileged tissues' are the cornea, lens, cartilage, placenta and foetus, testis, ovary, liver and also tumours. The threshold for inducing inflammation and immunity in immune-privileged tissue microenvironments is higher than in conventional tissues. This distinction allowed a first estimation of the mechanisms behind privilege: while morphological barriers such as a relative

barrier for leukocytes and the absence of lymphatic vessels may contribute to the state of an immune-privileged site by causing some extent of immune ignorance, immune-privileged tissues seem to provide signals that actively induce immune tolerance. This is probably best studied in tumours which secrete various immunomodulatory cytokines and express death ligands as a prerequisite for maintenance and growth (Crane *et al.*, 2012).

As for the brain, attributing its privileged state to the absence of lymph vessels seems short-sighted: studies in experimental autoimmune encephalomyelitis (EAE) (de Vos *et al.*, 2002), traumatic brain injury (Mutlu *et al.*, 2007) and a transgenic model allowing killing of oligodendrocytes (Locatelli *et al.*, 2012) revealed vivid drainage of myelin epitopes and axonal antigens into cervical lymph nodes (CLNs). Moreover, migration of immune cells along olfactory nerves through the cribriform plate into the nasal mucosa and further into CLNs has been demonstrated (Goldmann *et al.*, 2006; Kaminski *et al.*, 2012). It thus seems that alternate routes allow brain antigens to reach lymphoid tissues, but the mode of antigen presentation therein may favour tolerance induction in the absence of infection or adjuvants (Mutlu *et al.*, 2007; Locatelli *et al.*, 2012). At present, it is unknown whether brain antigens reach CLNs by passive drainage or transportation within brain-derived antigen-presenting cells (APCs).

As for the BBB, an extended view has developed in recent years: the term originally referred to brain capillaries which hold back certain hydrophilic molecules (Lewandowski, 1900), and "belts of tight junctions" have been identified as "morphological correlates" of this barrier (Reese and Karnovski, 1967). Which parts of the vascular tree of parenchymal versus meningeal vessels possess these structures has not yet been studied systematically (reviewed in Dyrna *et al.*, 2013). As for the invasion of leukocytes, however, two differentially regulated steps are required to reach the parenchyma proper (Bechmann *et al.*, 2007; Owens *et al.*, 2008; Engelhardt and Coisne, 2011). Leukocytes passing the vascular wall and the vascular basement membrane are not yet in the parenchyma, but are kept in perivascular spaces, which are separated from the neuropil by an additional basement membrane and the astrocytic and microglial endfeet building up the glia limitans (Lassmann *et al.*, 1991; Bechmann *et al.*, 2001b; Prodinger *et al.*, 2011). Crossing this membrane and the astrocytic endfeet requires induction of matrix metalloproteinases 2 and 9 (MMP2 and MMP9, respectively) which specifically cleave the basement membrane's connection to the endfeet (Sixt *et al.*, 2001; Agrawal *et al.*, 2006; Toft-Hansen *et al.*, 2006).

Physiological turnover of perivascular macrophages has been demonstrated (Bechmann *et al.*, 2001a,b), but further progression of blood-derived monocytes across the glia limitans is dependent on additional pathological signals such as release of the chemoattractant CCL2 (chemokine (C–C motif) ligand-2), which is induced, for example, by axonal injury and irradiation (Babcock *et al.*, 2003; Mildner *et al.*, 2007). There appears to be a physiological immune surveillance of perivascular spaces, but not of the parenchyma. This implies

that the BBB in its original sense as a capillary endothelial barrier for solutes is not a strict barrier for immune cells. In other words, the mechanisms and sites restricting diffusion or regulating transportation of blood solutes into the brain cannot be the same as those that restrict invasion of leukocytes into the neuropil. Therefore, the term 'neurovascular unit' (NVU) (Zlokovic, 2008) has been introduced into the field of immune privilege research: the term 'NVU' acknowledges cells and structural barriers beyond the endothelium, including pericytes, the vascular and parenchymal basement membranes and the glia limitans (Muldoon *et al.*, 2013) (see Chapter 13). Understanding the mechanism and signalling required for cells to pass through individual layers of cells from blood to the brain parenchyma is likely to unravel promising targets to inhibit harmful neuroinflammation (e.g. in multiple sclerosis (MS)).

Mechanisms of the brain's immune privilege

While the absence of lymphatic vessels in the brain seems to be compensated for by alternate routes to lymphoid tissues, most importantly via CLNs, the NVU certainly inhibits a simple immigration of leukocytes from blood to the neuropil proper. However, if staining for class II major histocompatibility complex (MHC-II), another important feature of the brain's parenchyma becomes evident: under normal conditions, there is a sharp border between the parenchyma proper and the perivascular and subarachnoid space demarked by MHC-II expression (Perry, 1998), suggesting that local cues down-modulate APCs within the neuropil. This is in line with the observation that microglia are immature APCs and that a multiple-step activation is required to qualify them for immunogenicity *ex vivo* (Carson *et al.*, 1998; Matyszak *et al.*, 1999). Their lack of CD80, a costimulatory molecule for T cell activation, under normal conditions also supports that microglia may be regarded as a form of tolerogenic dendritic cell (Bechmann *et al.*, 2001). *From an embryological point of view*, this may be due to their origin from yolk sac–liver macrophages (Ginhoux *et al.*, 2010; Kierdorf *et al.*, 2013) or be attributed to local cues down-modulating microglia, *de novo*–recruited and blood-derived monocytes (Bechmann *et al.*, 2005), or both. However, while there are no data supporting the view that yolk sac–derived microglia cannot mature to fully competent APCs, various factors have been detected which potentially down-modulate all mononuclear cells, regardless of whether they are original yolk sac–derived microglia or bone marrow (BM)/blood-derived monocytes which were recruited to the brain later in life. Important molecules include CD200, CX_3CR1 and TREM2 (for a review, see Kierdorf and Prinz, 2013), but also neuropeptides (Reinke and Fabry, 2006). For example, the neuropeptide alpha melanocyte-stimulating hormone (αMSH) is an important mediator of immunoregulation through induction of transforming growth factor beta (TGFβ)-secreting regulatory T cells (Tregs) and immunosuppression. Taylor and Kitaichi (2008) have reported that injection of αMSH at the onset of paralysis in EAE animals diminished both the severity of disease

and its time course. αMSH acts on macrophages via melanocortin-1 and -3 receptors, suppressing the activation of nuclear factor kappa B (NF-κB) and p38 mitogen-activated protein kinase by interleukin-1 (IL1) and tumour necrosis factor alpha. Melanocortin-4 receptor is expressed by microglia and astrocytes, and upon activation it stimulates anti-inflammatory pathways via the release of IL10 and TGFβ from microglia and astrocytes, respectively (Carniglia *et al.*, 2013). CX_3CL1 (fractalkine), CD200 and CD47, which act on microglial CX_3CR1, CD200R and SIRPα receptors, respectively, exert a quiescent effect on the microglia phenotype (Hanisch and Kettenmann, 2007). There are also data supporting the view that the brain can actively inhibit T cell proliferation via induction of the tryptophan-degrading enzyme indolamine 2,3-dioxygenase (IDO) (Kwidzinski *et al.*, 2005) or induce their apoptosis via CD95L (Bechmann *et al.*, 1999). A recent report by Corona *et al.* (2013) on experiments in CX_3CR1 knockout mice suggested that the potential consequence of impaired CX_3CL1–CX_3CR1 signalling in the brain is amplified microglial activation, increased IDO activation in microglia and prolonged depressive symptoms following an inflammatory insult. IDO is activated by pro-inflammatory cytokines.

A potential impact of astrocytes on microglial activation was proposed by Sievers *et al.* (1994) who found that monocytes cultured on monolayers of astrocytes acquired microglia-like ramified morphologies. In similar experiments, Hailer *et al.* (1998) identified TGFβ secreted by astrocytes as an important player in the regulation of microglial activity. However, adoption of monocytes to microglia-like phenotypes does not only occur *in vitro*. Using an entorhinal cortex lesion to induce axonal degeneration within the dentate gyrus in mice grafted with bone marrow cells expressing green fluorescent protein (GFP), the invasion of monocytes into zones of Wallerian degeneration was followed by their subsequent ramification and long-term maintenance (Bechmann *et al.*, 2005). Thus, local cues are driving an immediate adoption of *de novo* invading monocytes, transforming them into microglia-like cells (Priller *et al.*, 2001). Due to their processes in forming the glia limitans, astrocytes are ideally situated to exert their influence on invading monocytes.

Concluding remarks

It has been frequently questioned whether the brain really has a capacity for immunity. Knowing the experimental basis and the original definition, the answer is simple: beyond doubt, the brain is immune privileged in 2013 absolutely to the same extent as was described in 1948. Thus, the phenomenon is still the same, but what has changed is the understanding of the mechanisms underlying the brain's tolerance to non-self-antigens. Coming from a simple picture of the brain being ignored by the immune system due to the BBB and the lack of lymphatic vessels, various molecular mechanisms are now identified. All seem to serve the common purpose of shaping immune responses such that bystander damage is minimized, at least under those circumstances

which provided the strongest evolutionary pressure (i.e. infection). However, evolution – for evident reasons – did not take into account diseases of the elderly, and in that view immune privilege or its underlying mechanisms are now recognized to be not only protective: down-modulating phagocytic activities (via TREM2) may also have their downside and be among the culprits for the pathological accumulation of proteins in Alzheimer's disease (see Chapter 6).

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2
Innate Immunity in the CNS – A Focus on the Myeloid Cell

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Introduction to concepts of innate immunity

The concept of innate immunity is phylogenetically ancient and first appeared in multicellular organisms. Unlike the adaptive immune response, innate immune mechanisms do not rely on 'memory' and reset to baseline following clearance of encountered pathogens. The adaptive immune response is dependent on several aspects of innate immunity that result in the generation of antigen-specific lymphocytes and memory cells that are capable of rapidly responding to recurrent infections, thereby providing long-lasting immunity.

In order for a pathogen to successfully infect its host, several host defences, including physical barriers, must be breached. These innate barriers include epithelial surfaces and respiratory, reproductive tract and gastrointestinal mucosal surfaces. In the context of the central nervous system (CNS), the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB) are considered the most important and relevant barriers; both can be breached during CNS injury. The BBB is both a physical and biochemical barrier, consisting of tight junctions within an endothelial cell barrier that restricts the diffusion of pathogens and large hydrophilic molecules that would otherwise disrupt homeostasis in the CNS. In addition to endothelial cells, astrocytes and pericytes are also integral in forming the BBB and maintaining its integrity by regulating blood flow and providing structural and metabolic

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support. The BCSFB is located at the tight junctions surrounding the epithelial cells on the surface of the choroid plexus, which secrete cerebrospinal fluid (CSF) and express specific transporter proteins that regulate the exchange of molecules between the blood and the CSF. If a given pathogen is successful and infects its host, an inflammatory response in the host is initiated. While the inflammatory process is very complex, in simple terms, it begins with an innate response whereby local vasodilation of vascularized tissue, including the CNS, leads to fluid and leukocyte accumulation. This increase in blood flow is manifested by symptoms such as swelling (oedema) and redness. Within the blood vessels, increased hydrostatic pressure can lead to the contraction of endothelium and the leakage of fluids, such as collectins (i.e. soluble lectin molecules and mannan-binding protein) and complement, which can bind to the surfaces of microbes in a process termed *opsonization*, facilitating their phagocytosis or direct lysis.

In order for immune cells to reach sites of infection or injury, various molecules and chemoattractants (e.g. bacterial products and chemokines) must promote the expression of adhesion molecules (selectins and integrins) on both immune cells and vascular endothelial cells, while chemokines and activated complement components (e.g. C5a) create a gradient that can lead to extravasation of peripheral blood cells into the infected or injured tissue. Upon reaching the site of injury, phagocytic cells (e.g. neutrophils and macrophages) must identify both opsonized and non-opsonized dying cells, pathogens and cellular debris. The phagocyte can then deliver antimicrobial molecules, engulf the elements and, if necessary, kill the microbe through production of reactive oxygen species (ROS). The detailed mechanisms by which innate immune cells recognize microbes and sites of tissue injury will be discussed in subsequent sections of this chapter.

Cells of the innate immune system

All cells of the immune system, in addition to erythrocytes and platelets, are derived from pluripotent haematopoietic stem cells that develop in the bone marrow. From the bone marrow, leukocytes can either migrate to specialized tissues or reside within the circulatory and/or lymphatic systems. Haematopoietic stem cells can further give rise to both common lymphoid and myeloid progenitors. Cells within the lymphoid lineage differentiate into cells of the adaptive immune system (e.g. B and T lymphocytes) in addition to natural killer (NK) cells. NK cells are large granular cells that lack antigen-specific receptors, but can recognize and kill tumour and pathogen-infected cells in a major histocompatibility complex (MHC) unrestricted manner, and they are therefore considered part of the innate immune system. Common myeloid progenitor cells differentiate into cells of the innate immune system, including granulocytes, mast cells, dendritic cells (DCs) and monocytes and macrophages.

Granulocytes are also known as *polymorphonuclear cells* and can be divided into neutrophils, eosinophils and basophils. During infection and inflammation, these cells can migrate into tissues, where they are found in very large numbers but are relatively short-lived. As stated in this chapter, neutrophils are highly phagocytic and are capable of engulfing and degrading microbes by releasing ROS and hydrolytic enzymes. Of the cells comprising the innate immune system, neutrophils are the most numerous and can readily migrate to sites of injury through various chemotactic gradients. While the roles of eosinophils and basophils have been largely described in the context of allergy, eosinophils in particular have been shown to also possess antiparasitic activity (Rosenberg *et al.*, 2012). In neuromyelitis optica (NMO), an inflammatory demyelinating disease of the CNS, active lesions have been characterized by infiltration of macrophages, perivascular granulocytes and eosinophils (Lucchinetti *et al.*, 2002). Both intact and degranulated eosinophils were found within active lesions; however, their exact function is unknown, and it is unclear whether infiltration of eosinophils is a primary or secondary event in NMO. It has been suggested that complement activation in lesions may result in the production of chemotactic factors that recruit eosinophils into the CNS (Lucchinetti *et al.*, 2002). In addition to their capacity to release ROS, cytokines and growth factors, eosinophils have also been shown to possess antiviral activities through the production of RNases.

Mast cells play significant roles in allergy and inflammation, and they are also important in their ability to protect the body from certain pathogens, including parasites (e.g. intestinal worms). While mast cells are capable of secreting a variety of different soluble factors, including histamine, proteases, proteoglycans (heparin), prostaglandins, thromboxanes and leukotrienes, these cells are believed to release several different cytokines that can impact innate immunity, including tumour necrosis factor alpha (TNFα), interleukin-1 (IL1), IL6 and interferon gamma (IFNγ) (Skaper *et al.*, 2012). In the inflamed CNS, mast cells have been found in both multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE) lesions (Brenner *et al.*, 1994; Theoharides, 1990), and can directly cause demyelination and oligodendrocyte death (Johnson *et al.*, 1988; Theoharides *et al.*, 1993; Medic *et al.*, 2010).

Much of the current chapter will focus on innate immune cells that are termed *professional antigen-presenting cells* and include DCs and macrophages. The CNS resident macrophages, referred to as *microglia*, will also be discussed in detail. DCs are highly phagocytic cells that possess long extensions resembling the dendrites of neurons. A high density of DCs can be found in epithelial surfaces and in areas that encounter the external environment. Unlike other phagocytic immune cells, DCs specialize in antigen processing and presentation, and effectively present antigen to lymphocytes and initiate adaptive immune responses. Upon antigen uptake, DCs migrate to lymph nodes where they increase their expression of costimulatory molecules,

present antigen via MHC proteins and can subsequently activate B cells, naïve T cells and antigen-specific memory T cells that will undergo clonal expansion. It is important to note that both macrophages and B cells can similarly activate T cells; however, the spatial distribution of DCs within the lymph nodes makes them superior antigen-presenting cells and the most potent activators of T cells.

In humans, there are two classes of DCs that possess distinct properties that can influence immune responses. These two major cell types are myeloid DCs (mDCs; also termed *classical* or *conventional DCs*) and plasmacytoid DCs (pDCs). mDCs are similar to monocytes and are the conventional dendritic cell responsible for activating naïve T cells. There are several different subsets of these specialized DCs; however, all cells express MHC and costimulatory molecules specialized for activating the adaptive immune response (Johnson *et al.*, 2011). While pDCs are also capable of activating T and B cells, they have been documented to play a significant role in responding to viral infections and express specific Toll-like receptors (TLRs) that are able to sense viral infections (TLR7 and TLR9). In addition, pDCs secrete type I interferons (IFNα and IFNβ), which are specialized cytokines that can further activate the immune system and rid the body of viral infections.

Monocytes, as well as DCs, also arise from specialized myeloid progenitor cells found in the bone marrow. Monocytes circulate within the blood and are capable of extravasation into tissues to become macrophages. Also included in the myeloid cell category are specialized and tissue-specific macrophages, which have been suggested to arise from the yolk sac (Gomez Perdiguero *et al.*, 2013). Depending on their location within the body, examples of tissue-specific macrophages include alveolar macrophages (lung), Kupffer cells (liver), osteoclasts (bone) and microglia (CNS). Previous fate-mapping studies have demonstrated that adult microglia are derived from haematopoietic progenitors in the extra-embryonic yolk sac between embryonic day 7.0 (E7.0) and E9.0 (Ginhoux *et al.*, 2010). Like DCs, these additional myeloid cell types (e.g. monocytes, macrophages and organ-specific myeloid cells) are phagocytic, express MHC class II and costimulatory molecules and promote adaptive immune responses. However, monocytes and macrophages also secrete a wide array of cytokines and chemokines, which function to promote inflammation and recruit additional immune cells to sites of injury. In subsequent sections of this chapter, the phenotypes and specialized functions of these cells will be further discussed in the context of CNS infection, injury and repair.

Innate immune cell receptors

Prior to activating the adaptive immune response, cells of the innate immune system first respond to pathogens, or danger signals, within their environment. DCs, macrophages and monocytes all possess both surface and intracellular receptors capable of recognizing pathogen-associated molecular patterns

(PAMPs), which are small molecular patterns associated with specific classes of pathogens and microorganisms. These specialized patterns are recognized by different families of pattern recognition receptors (PRRs), and they include TLRs, nucleotide-binding oligodimerization domain (NOD)-like receptors (NLRs) and RIG-like receptors (RLGs). The concept of PRRs is important not only in the context of their specialized roles in mediating inflammation, but also in the pathology and identification of specialized and polarized cell types in diseased tissue, as discussed later in this chapter.

There are several types of TLRs capable of recognizing different PAMPs, and these can be found both on the cell surface and within endosomes. Common PAMPs and their receptors include glycolipids and lipoproteins (TLR2), double-stranded RNA (dsRNA) (TLR3), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), single-stranded RNA (ssRNA) (TLR7) and unmethylated CpG DNA (TLR9). A complete table of human TLRs, their respective ligands and their expression within the CNS can be viewed in Table 2.1 (adapted from Hanke and Kielian, 2011). Downstream signalling events of TLR activation are complex, involving several adaptive molecules, kinases and transcription factors. Ultimately, activation of TLRs leads to the transcription of genes that influence inflammatory responses (Athman and Philpott, 2004).

NLRs are cytosolic receptors and activate similar downstream signalling pathways as TLRs. In mammals, NOD proteins recognize bacterial cell wall proteoglycans and possess CARD domains that can readily recruit caspases, thus activating inflammatory cytokines (e.g. IL1). Mutations in several NOD proteins have been associated with certain inflammatory diseases, including

Table 2.1 Toll-like receptor (TLR) expression in the central nervous system (Adapted from Hanke and Kielian, 2011.)

		Cell Type				
TLR	Ligand	Neuron	Astrocyte	Oligodendrocyte	Microglia	Brain Endothelium
TLR1	Triacylated lipoproteins		✓		✓	
TLR2	Glycolipids, lipoproteings		✓	✓	✓	✓
TLR3	dsRNA	✓	✓	✓	✓	✓
TLR4	LPS		✓		✓	✓
TLR5	Flagellin		✓		✓	
TLR6	Diacylated lipoproteins				✓	✓
TLR7	ssRNA	✓			✓	✓
TLR8	ssRNA	✓			✓	✓
TLR9	Unmethylated CpG-DNA	✓	✓		✓	

Crohn's disease (Eckmann and Karin, 2005); however, there are currently no neuroinflammatory diseases identified with such mutations. NLRs are expressed on DCs, macrophages and monocytes, but are also highly expressed in epithelial cells and can activate innate immune responses through downstream signalling. RLGs are also a family of cytoplasmic receptors that contain RNA helicase domains and bind dsRNA, a feature of certain RNA viruses. Downstream signalling of RIG proteins leads to increased expression of type I interferons, important molecules that activate receptors and initiate signalling pathways involved in inhibiting viral replication. PRRs are an important mechanism of infection and injury detection in cells of the innate immune system, and given that expression of these receptors may differ in a pathological context, they may represent novel targets for diagnosis and treatment.

Innate immune responses in the CNS

Within the CNS, inflammation and innate immunity are believed to contribute to the pathogenesis and neurodegeneration that occur in several diseases and conditions, including stroke, Alzheimer's disease and demyelinating diseases such as MS. Traditional views that considered the CNS to be an 'immune-privileged' site are currently being challenged, whereby it is generally accepted that both innate and adaptive immune responses are important and highly relevant in the context of both CNS injury and repair. In the context of neuroinflammation, while there has been a plethora of research that has investigated the roles of innate immune cells (macrophages, DCs and granulocytes) in contributing to CNS disorders, more recent research has focussed on putative roles of CNS resident cells, including astrocytes and microglia. In the subsequent sections of this chapter, we will discuss the roles of the traditional innate immune cell populations in diseases of the CNS, as well as the emerging roles of CNS resident cells.

Roles of dendritic cells

DCs are among the most important cells in processing antigens and initiating an adaptive immune response. Both *in vivo* and *in vitro*, monocytes can give rise to both DCs and macrophages, with each cell lineage being dependent on specific signals that can promote their unique phenotype. Within the non-inflamed or healthy CNS parenchyma, DCs are not present; however, evidence suggests that they can be found in the meninges and the choroid plexus (Anandasabapathy *et al.*, 2011). The highly vascularized nature of these regions suggests that the presence of DCs is likely due to their extravasation from the blood (Prodinger *et al.*, 2011). Following disruption of the BBB, DCs can be found within CNS tissue and readily express surface molecules that are indicative of their activated state (i.e. an increase in costimulatory molecule expression). In EAE, an animal model of MS, studies have indicated that both

antigen-specific (memory) and naïve T cells can be activated directly within the CNS by mDCs. Compared to macrophages, pDCs and microglia, mDCs are superior at inducing pro-inflammatory Th17 responses and disease progression (Miller *et al.*, 2007). In EAE, several subtypes of DCs accumulate in the inflamed CNS and are thought to arise from circulating monocytes (Bailey *et al.*, 2007). Within the CNS, DCs have also been shown to be the most important APCs in terms of promoting epitope spreading, a mechanism by which autoreactive T cells can be activated by *de novo* antigens that are released as a result of ongoing tissue damage (McMahon *et al.*, 2005; Miller *et al.*, 2007).

Within the brains of MS patients, both myelin-containing mDCs (CD209+) and pDCs (CD123+) have been observed in active demyelinating lesions, meninges and normal-appearing grey matter (Plumb *et al.*, 2003; Cudrici *et al.*, 2007; Lande *et al.*, 2008). CD209+ DCs have also been observed in close association with lymphocytes in active MS lesions (Serafini *et al.*, 2006). Both DC subtypes can also be found in the CSF of patients with neurological inflammatory disorders, whereby DC numbers are correlated with several parameters associated with CNS inflammation. Interestingly, cell surface expression levels of several molecules that drive adaptive immune responses (e.g. CD80, CD86 and CD40) were higher in mDCs in the CSF compared to blood-derived DCs (Pashenkov *et al.*, 2001). pDCs express high levels of TLR9 and secrete type I interferons upon ligand binding (e.g. viral DNA). During MS relapses, increased numbers of pDCs have been reported in the CSF (without changes in the blood), consistent with observations that relapses may be driven by systemic viral infection (Longhini *et al.*, 2011).

In MS patients treated with natalizumab, a monoclonal antibody directed at the cell adhesion molecule α4-integrin (VLA4), the numbers of CD209+ DCs and CD4+ T cells were significantly decreased in cerebral perivascular spaces (del Pilar Martin *et al.*, 2008). Patients treated with natalizumab also had decreased VLA4 expression on both pDCs and mDCs, with a resulting decrease in the ability of the DC to stimulate antigen-specific T cell responses (de Andres *et al.*, 2012). Fingolimod (FTY720) is a recently approved oral treatment for MS and has a well-described mechanism of action that leads to sequestering of immune cells in secondary lymphoid tissues. In the context of DCs, FTY720 has been shown to reduce antigen presentation function, decrease pro-inflammatory cytokine production, increase IL10 and block *in vivo* trafficking (Lan *et al.*, 2005; Muller *et al.*, 2005; Durafourt *et al.*, 2011). FTY720 can also promote an anergy-polarized phenotype of both immature and mature DCs (Zeng *et al.*, 2012). Dimethyl fumarate (BG12) is in late clinical trials for the treatment of MS and has also been shown to inhibit DC maturation, decrease MHC class II and costimulatory molecule expression (CD80 and CD86), block IL6 and IL12 secretion and result in fewer activated Th1 and Th17 cells (Peng *et al.*, 2012).

In addition to MS, increased numbers of DCs have also been reported in the CSF of patients with bacterial meningitis and Lyme neuroborreliosis

(Pashenkov *et al.*, 2002). In the CNS of amyotrophic lateral sclerosis (ALS) patients, in addition to its animal models, DCs have also been observed in the ventral horn and corticospinal tracts, supporting a role for an immune response in patients with this neurodegenerative disease (Henkel *et al.*, 2004). In Parkinson's disease (PD), the discovery of anti-melanin antibodies in the sera of PD patients has prompted studies to investigate whether the innate immune system can be activated by this putative autoantigen. *In vitro* studies have shown that neuromelanin can be recognized by DCs and trigger their maturation, thus suggesting a rationale for a potential autoimmune mechanism driving the pathogenesis of PD (Koutsilieri *et al.*, 2012). Both mDCs and pDCs have also been observed in the post-mortem brains of stroke patients (Yilmaz *et al.*, 2010). In an experimental model of stroke, the anti-inflammatory actions of granulocyte colony-stimulating factor (G-CSF) are thought to be at least partly mediated by its effects on DCs. Administration of G-CSF led to a decrease in DC migration and maturation, in addition to reducing infarct size and improving clinical disability (Dietel *et al.*, 2012). While many of these studies provide evidence that antigen presentation and immune system activation can occur within the CNS, animal studies have further demonstrated that DCs from the CSF can also migrate to cervical lymph nodes where they can activate adaptive immune responses, providing evidence that DCs may drive immune responses in both the CNS and periphery. It has been suggested that both CCR7 and CXCR4 (Cravens and Lipsky, 2002) chemokine receptors, which have been shown to be either elevated upon activation or expressed by mature DCs within the CSF (Kivisakk *et al.*, 2004), may mediate migration of DCs into the lymph nodes.

Of interest to CNS pathophysiology is the mechanism by which DCs cross the BBB and promote immune cell activation directly within the CNS. Migration of immune cells across the brain endothelial is complex, requiring cell tethering, rolling, attachment and extravasation. In addition, the expression of cell adhesion molecules, integrins and selectin molecules is important in initiating the attachment of immune cells to endothelial cells. Transmigration of immune cells across the BBB is also mediated by individual chemoattractant molecules called 'chemokines'. CCL2, also known as monocyte chemotactic protein-1 (MCP1), is a chemokine that is important for recruiting monocytes, lymphocytes and DCs to sites of tissue injury (Seguin *et al.*, 2003). In transgenic mouse models, it has been observed that CCL2 is essential for the attraction of mDCs into the CNS (Dogan *et al.*, 2008). Conversely, overproduction of CCL2 in the CNS leads to accumulation of leukocytes across the endothelial basement membrane (Toft-Hansen *et al.*, 2006). CCL2 expression in glial cells is increased in several CNS inflammatory states, including epilepsy (Foresti *et al.*, 2009), Alzheimer's disease (Sokolova *et al.*, 2009), ischaemia (Yamagata *et al.*, 2010) and EAE (Giraud *et al.*, 2010). In addition to CCL2, other chemokines have also been shown to promote DC migration, including CCL20, CXCL10 and CCL3. Many of these chemokines are also expressed by

CNS resident cells during inflammation (Serafini *et al.*, 2000; Williams *et al.*, 2009; Clarkson *et al.*, 2012). Through the use of both transgenic animal studies and *in vitro* BBB modelling, studies have demonstrated the importance of these chemokines in DC recruitment into the CNS (Ambrosini *et al.*, 2005; Zozulya *et al.*, 2007; Wuest & Carr 2008).

In vitro assays using a modified Boyden chamber model of human BBB endothelial cells have identified a subpopulation of monocytes that closely associate with endothelial cells and express DC markers (CD83 and CD209) (Ifergan *et al.*, 2008). These cells have been termed endothelial-associated DCs (eDCs). The presence of these cells has been confirmed *in situ* within active MS lesions. The release of cytokines from brain endothelial cells, such as transforming growth factor beta (TGFβ) and granulocyte–macrophage colony-stimulating factor (GM-CSF), is thought to contribute to the differentiation of eDCs. eDCs are believed to be potent phagocytes; secrete IL12p70, TGFβ and IL6; and promote the proliferation of Th1 and Th17 CD4+ lymphocytes.

Once DCs are in the CNS, there is evidence to support both a detrimental and beneficial role for them in inflammatory responses. As mentioned in this section, DCs can re-stimulate T cells and promote epitope spreading in the CNS. Adoptive transfer studies using the EAE model also demonstrate that MHC class II expression on DCs is sufficient to stimulate T cells and results in disease activity (Greter *et al.*, 2005). DC production of IL12, a potent cytokine that stimulates Th1 responses, also suggests that some DC subtypes are capable of promoting adaptive immune responses in the CNS. pDCs in particular increase OX40L (CD252), which has been previously shown to promote Th2 responses (Kitajima *et al.*, 2011). Studies have been performed *in vivo* whereby intracerebral injection of myelin oligodendrocyte glycoprotein (MOG)-loaded DCs prior to EAE induction significantly increased clinical disability and activation of infiltrating T cells (Zozulya *et al.*, 2009b). In contrast, follow-up experiments using injections of DCs that were pre-exposed to TNFα induced DC tolerance, decreased IL17 and increased IL1 production, thus resulting in a delay or prevention of disease activity (Zozulya *et al.*, 2009a). These findings may indicate that tolerogenic DCs may protect the CNS from immune-mediated injury and have important implications in further study of the roles of DCs in mediating CNS inflammatory injury.

Roles of microglia and macrophages

Microglia are specialized macrophages and glial cells within the CNS, and they have been suggested to originate from the yolk sac during development (Ginhoux *et al.*, 2010). Early in development, microglia populate the CNS parenchyma where they have an extended lifespan with little turnover (Ginhoux *et al.*, 2010, Rock *et al.*, 2004). Fate-mapping studies have demonstrated that postnatal haematopoietic progenitors do not significantly

influence homeostatic mechanisms specific to microglia in the adult brain. Microglia were first described by Pio del Rio-Hortega in 1932, and they have been reported to share many features with other tissue resident macrophages, with the exception of their resting ramified morphology (Rock *et al.*, 2004). This unique morphology allows microglia to sense the local environment, communicate with neighbouring cells (e.g. neurons, oligodendrocytes, astrocytes) and quickly respond to infection and injury. Microglia express several cell surface and intracellular molecules, including CD14, CD11b, CD45, CD68 and EMR1 (F4/80 in rodents) (Prinz *et al.*, 2011). Many of these molecules are also similarly expressed by monocyte and tissue macrophages. In mice lacking the PU.1 transcription factor, a critical transcription factor known to be important during myeloid and B cell development, microglia are notably absent in the neonatal CNS (Beers *et al.*, 2006). Irradiation and bone marrow transplantation (BMT) studies have provided evidence that BMT-derived progenitors can efficiently migrate into the CNS (Eglitis & Mezey 1997, Priller *et al.*, 2001). Animal studies using GFP knock-in CX_3CR1 (fractalkine receptor) transgenic mice, whereby both peripheral macrophages and microglia are fluorescently labelled, have demonstrated that migration of GFP+ cells into the CNS occurs at E10.5, prior to the onset of embryonic haematopoiesis (Ginhoux *et al.*, 2010).

Activation of microglia and infiltrating monocytes and macrophages is observed in most inflammatory CNS disorders, yet the mechanisms and consequences of myeloid cell activation across the various CNS disorders have not been fully elucidated. In addition to the roles of microglia in neuroinflammatory and degenerative disease, as discussed here, these cells also play a role in the developing and normal healthy brain. It has been reported that microglia are essential in the clearance of cellular debris during the development of the foetal brain (Rock *et al.*, 2004). Similar to astrocytes, microglia may also play a significant role in the support of axons and the modelling of synapses in the healthy brain (Nimmerjahn *et al.*, 2005; Tremblay *et al.*, 2010; Paolicelli *et al.*, 2011; Schafer *et al.*, 2013). In postnatal development, microglia have been recently shown to participate in activity-dependent synaptic pruning and can engulf presynaptic inputs, which is dependent on both the complement receptor 3 (CR3)–C3 signalling pathway and neural activity (Schafer *et al.*, 2012). Understanding the basal inherent properties of microglia throughout development may provide important insights into mechanisms that may also be relevant during CNS injury and repair.

Upon activation, microglia quickly adopt a typical amoeboid morphology (see Figure 2.1) and release several cytokines, chemokines, complement proteins, nitric oxide, matrix metalloproteinases (MMPs) and ROS, all of which can influence the innate and adaptive immune responses in the inflamed CNS. While microglia trigger these inflammatory responses in the context of acute pathogenic infection, sustained microglia cell activation, as seen in several chronic neuroinflammatory diseases, can significantly impact the

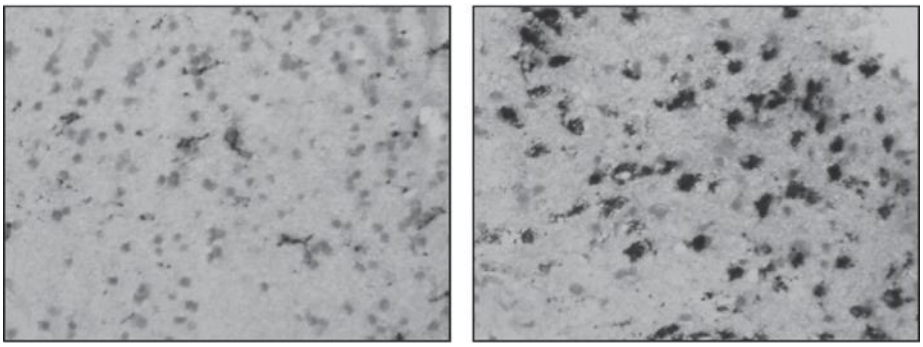

Figure 2.1 Macrophage and microglia activation in the human CNS. (A) In normal healthy tissue, immunohistochemical staining for the lysosomal marker CD68 shows a resting ramified morphology with small cell bodies and cytoplasmic extensions. (B) In contrast, under inflammatory conditions, activated microglia (both *in vitro* and *in vivo*) retract their processes, increase their volume and adopt an amoeboid morphology.

progression of neurodegenerative diseases. Microglia may also possess an anti-inflammatory and/or neurotrophic role as they have been reported to secrete various growth factors and anti-inflammatory cytokines that are associated with dampening Th1 responses and initiating CNS repair, as will be discussed further in this chapter. During CNS inflammation, parabiotic experiments, using genetically similar animals with surgically fused circulatory systems, have shown that monocyte infiltration into the CNS can exacerbate EAE progression but does not affect CNS resident microglia (Ajami *et al.*, 2011). During disease remission, the infiltrated monocytes disappear and do not contribute to the resident microglia population. Results of these experiments suggest that infiltrated monocytes, macrophages and microglia represent separate cell populations with distinct roles during neuroinflammation. In a mouse model of ALS, studies have demonstrated that disease progression is associated with an increase in inflammatory monocytes that are recruited into the CNS and correlate with neuronal loss; numbers of resident microglia decreased in the spinal cord in a mouse model of ALS. Using antibodies to target Ly6C, a murine haematopoietic differentiation marker, monocyte recruitment was reduced and survival was extended, suggesting that inflammatory monocytes play a significant role in disease progression (Butovsky *et al.*, 2012).

In the inflamed CNS, inflammatory monocytes, macrophages and microglia exhibit similar features with respect to phenotype, morphology and functional properties. Currently there are no distinct lineage markers that can distinguish microglia and infiltrating macrophages; however, relative expression levels of the lineage marker CD45 can be used. Several studies have differentiated these myeloid cell types by considering CD11b+/CD45^{low} as the microglia population, and CD11b+/CD45^{high} as infiltrating macrophages

(Babcock *et al.*, 2003; Denker *et al.*, 2007). There remains considerable debate over the use of this paradigm as there is a lack of specificity for either individual cell. As a result, identifying novel genes and molecules that are highly selective for either cell type is being investigated. CCR2, CX_3CR1 and Ly6C have been suggested as possible markers unique to myeloid cell populations; however, under certain pathological circumstances, these markers may also be expressed by other immune cell types (Sato *et al.*, 1996; Prinz and Priller 2010). Similar attempts have also been made to identify unique microglia cell markers, such as GLUT5 and P2Y12 (Horikoshi *et al.*, 2003; Sasaki *et al.*, 2003), and while these molecules have been suggested to be microglia specific, widespread acceptance in the literature does not currently support these claims.

Myeloid cell polarization

Myeloid cell plasticity is essential for an immune response to be adapted as required by the needs of each specific tissue and broadly in response to either infection or injury. Both *in vitro* and *in situ* studies of peripheral macrophages have demonstrated that macrophages can vary with respect to their activation state, allowing for differential responses to contribute to both pro- and anti-inflammatory responses. It has been shown that, similar to the Th1–Th2 T cell polarization axis, myeloid cells may adopt distinct phenotypes in response to the stimuli which they encounter. At the extremes of the activation continuum are the M1, 'pro-inflammatory' or classically activated myeloid cells, and the M2, 'anti-inflammatory' or alternatively activated cells (Gordon 2003); see Figure 2.2. Studies investigating functional and phenotypic properties of human macrophages have traditionally used blood-derived monocytes; murine studies have used both blood-derived and bone marrow–derived monocytes. Monocytes treated with GM-CSF and IL4 adopt a dendritic cell phenotype, whereas macrophage colony-stimulating factor (M-CSF) has traditionally been used to generate macrophages (Lambert *et al.*, 2008). Established polarization paradigms involve differentiation of monocytes into macrophages using GM-CSF or M-CSF for M1 or M2 macrophages, respectively. M1 cells are then activated using IFNγ and LPS, whereas M2 cells are generated using IL4 and IL13 (Leidi *et al.*, 2009). This M2 cell activation generates a subtype of M2 macrophages referred to as M2a; other subtypes of anti-inflammatory macrophages have also been described and are discussed in this section.

M1 phenotype The M1 phenotype occurs in response to pro-inflammatory cytokines such as IFNγ and TNFα, as well as PAMPS such as LPS (Van Ginderachter *et al.*, 2006; Fairweather and Cihakova 2009). This phenotype occurs in response to bacterial and viral infections, leading to promotion of the pro-inflammatory responses required to clear the infection or to promote

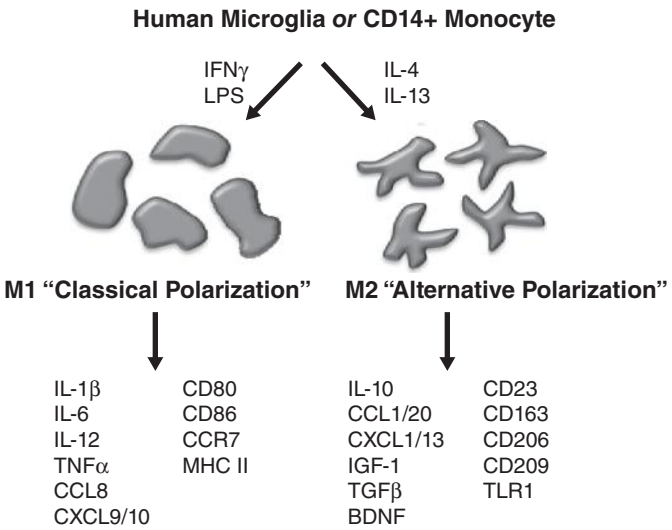

Figure 2.2 Polarization of CD14+ monocytes and human (fetal and adult) microglia *ex vivo*. M1 polarization (aka *classical*) can be achieved in the presence of pro-inflammatory factors, such as IFNγ, TNF and/or LPS, while M2 polarization (aka *alternative*) is achieved through the addition of IL4 and IL13 (IL10 and TGFβ can also be used). Based on cytokine and chemokine profiles and on cell surface expression of 'polarised' cell types, these cells have been associated both *in vitro* and *in situ*, with either a 'pro-inflammatory' (M1) or 'anti-inflammatory' (M2) phenotype and exhibiting unique functional properties.

the adaptive immune response. In the context of immunoregulatory-relevant properties, M1 macrophages express high levels of the costimulatory molecules CD80 and CD86, as well as MHC class II, resulting in efficient antigen presentation capacity (Ishizuka *et al.*, 2012). M1 macrophages also up-regulate levels of TLR2, TLR4 and Fcγ receptors (CD16, CD32 and CD64) (Mantovani *et al.*, 2002). M1 cells also up-regulate expression of the chemokine receptor CCR7, which has been shown to control migration of both innate and adaptive immune cells to secondary lymphoid organs (e.g. lymph nodes) (Randolph *et al.*, 2005). With respect to cytokine production, M1 cells secrete pro-inflammatory and Th1/17-inducing cytokines, including IL1, IL6, IL12, IL23 and TNFα. In addition to cytokines, several chemokines and other effector molecules are produced, including CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL5, CXCL8 as well as inducible nitric oxide synthase (iNOS) and other reactive oxygen and nitrogen species (Mantovani *et al.*, 2002).

M2 phenotype In addition to their role in immunity, macrophages also have an important role in the self-limiting nature of inflammation and in tissue repair. The anti-inflammatory macrophage phenotype is referred to as M2

and has been further subdivided based on the specific activating stimuli. M2a myeloid cells, induced using IL4 and IL13, are capable of differentiating naïve T cells into the Th2 phenotype, and are involved in the elimination of parasites (Sica *et al.*, 2006). M2b cells are generated by activation with immune complexes and TLR ligands, and have been shown to produce inflammatory cytokines, in addition to anti-inflammatory cytokines (Mosser 2003). Myeloid cells treated with IL10 exhibit the M2c, or 'deactivated' phenotype, and such cells are involved in immunoregulation and tissue remodelling (Sica *et al.*, 2006). Microglia, as well as astrocytes, have been shown to act as endogenous sources of IL4 and IL10 (Ponomarev *et al.*, 2005), suggesting that myeloid cells in the CNS can be potentially exposed to these polarizing factors. The role of M2 macrophages in the context of resolving inflammation and promoting repair, particularly within the CNS, will be discussed here.

M2 macrophages produce anti-inflammatory cytokines such as IL10 and TGFβ, and they are associated with the dampening of immune responses and the promotion of tissue remodelling and repair (Schwartz, 2010). IL33, a member of the IL1 family, has also been associated with M2 polarization (Kurowska-Stolarska *et al.*, 2009). The surface marker profile of M2 macrophages includes expression of CD23 (Fcε-RII), the scavenger receptors CD163 and CD204, the mannose receptor CD206, and CD209 (DC-SIGN) (Mantovani *et al.*, 2004; Martinez *et al.*, 2006; Van Ginderachter *et al.*, 2006). CD206 and CD209 are C-type lectins, molecules responsible for the uptake of complex carbohydrates, and are involved in cell-to-cell adhesion and immune responses to pathogens (Van Ginderachter *et al.*, 2006; Saunders *et al.*, 2009). Expression of the scavenger receptor CD163 has been detected in the inflamed CNS, including in Alzheimer's disease, HIV encephalitis and MS (Roberts *et al.*, 2004; Zhang *et al.*, 2011). Murine M2 macrophages up-regulate expression of the secretory and chitinase-like protein YM1 and the enzyme arginase-1, but these have been reported to not be markers of human M2 macrophages (Raes *et al.*, 2005). Human M2 macrophages have been found to be more efficient than M1 cells in phagocytosing opsonized targets (Leidi *et al.*, 2009).

Microglia polarization Similar to macrophages, microglia can also be polarized to both M1 and M2 phenotypes. M1-polarized human microglia are similar to M1 macrophages in their ability to produce pro-inflammatory cytokines and express co-stimulatory molecules (Durafourt *et al.*, 2012). M2 microglia are more restricted in their ability to become M2 polarized cells as defined by expression of M2 surface markers expressed in macrophages, but it is unknown whether novel microglia-specific M2 surface markers are yet to be identified. While both M2 macrophages and microglia have increased phagocytic capacity compared to their M1 counterparts, microglia have been shown to be more efficient at phagocytosis of myelin compared to macrophages (Durafourt *et al.*, 2012). Given that myelin debris can impair remyelination

by restricting the differentiation of oligodendrocyte progenitor cells (OPCs) (Kotter *et al.*, 2006), microglia may play an important role in clearing myelin debris following injury, thus allowing for an efficient repair process. During CNS development, microglia have also been shown to mediate synaptic pruning by engulfing pre-synaptic inputs (Schafer *et al.*, 2012), demonstrating an inherent role of phagocytosis by microglia in the non-inflamed CNS.

Anti-inflammatory and trophic roles of macrophages and microglia

The infiltration of peripheral monocytes and the activation of microglia within the injured or inflamed CNS have been traditionally considered impediments to tissue repair. Evidence supporting this hypothesis has been widely documented in many animal models of CNS injury, directly implicating cytokines, chemokines and other toxic molecules (i.e. oxygen and nitrogen radicals) with disease pathologies. While myeloid cells are indeed a source of many pro-inflammatory factors that can promote tissue injury, a large body of evidence has emerged that supports both an anti-inflammatory and a CNS-regenerative role for monocytes, macrophages and microglia. Because the CNS is equipped with its resident macrophages (microglia) that are activated upon injury, it remains unclear why additional peripheral myeloid cells (i.e. monocytes) are heavily recruited to sites of injury, and it is of interest whether these cells possess distinct properties that can ultimately impact both CNS injury and repair.

Following myelin injury, as occurs in MS and its animal model EAE, macrophages are the predominant cells responsible for phagocytosis of myelin debris, thus clearing the local environment and permitting the migration and differentiation of OPCs at sites of injury. Providing the neurons and axons have remained intact, oligodendrocytes can remyelinate axons and mediate repair. In the lysolecithin model of demyelination, rats depleted of macrophages experienced impaired remyelination, a delayed recruitment of OPCs and altered expression of growth factors such as insulin-like growth factor-1 (IGF1) and TGFβ (Kotter *et al.*, 2005). In a model of spinal cord injury, M2 (alternatively activated) monocyte-derived macrophages directly injected into sites of injury led to partial recovery of motor function (Rapalino *et al.*, 1998). In humans, clinical trials enrolling spinal cord injury patients have been performed using autologous macrophages; however, a phase II clinical trial failed to meet its primary outcomes (Knoller *et al.*, 2005; Kumar *et al.*, 2009; Lammertse *et al.*, 2012). In addition, neurite extension has been reported to be significantly inhibited by treatment with monocyte-conditioned media (Pool *et al.*, 2011). Given the heterogeneity of monocytes, it may be that further investigation into the activation paradigms and selection of monocyte subsets is critical to mediate CNS regeneration. Several groups have

previously reported that M2 macrophages (and M2 microglia) exhibit enhanced ability to phagocytose myelin and secrete anti-inflammatory and neurotrophic factors that can promote repair (Boven *et al.*, 2006; Durafourt *et al.*, 2012; Hu *et al.*, 2012). In contrast, M1-polarized macrophages and microglia appear to be less phagocytic, thereby hindering the remyelinating potential of the CNS (Kigerl *et al.*, 2009; Durafourt *et al.*, 2012; Hu *et al.*, 2012).

While the pro-inflammatory and disease-promoting role of M1-polarized myeloid cells have been largely attributed to their ability to secrete pro-inflammatory cytokines and chemokines and further influence the adaptive immune response, the M2 cell has been viewed by many as an important source of growth factors that can stimulate CNS repair. M2 myeloid cells have been reported to secrete many factors that can directly promote neuronal survival and axon regeneration, including IGF1, nerve growth factor (NGF) and bone marrow–derived neurotrophic factor (BDNF) (Butovsky *et al.*, 2006; Rolls *et al.*, 2008; Kuo *et al.*, 2011). Macrophages have also been recently shown to transfer ferritin to NG2+ OPCs *in vivo*, thereby inducing OPC proliferation and differentiation (Schonberg *et al.*, 2012). In the context of neurogenesis, microglia have been implicated in enhancing neurogenesis *in vitro* (Walton *et al.*, 2006; Choi *et al.*, 2008) and also by secreting molecules such as presenilin-1, which mediates neural progenitor cell proliferation (Choi *et al.*, 2008). In a mouse model of ALS, at disease onset, mSOD1 microglia expressed higher levels of M2 markers and lower levels of M1 markers compared to end-stage disease. Co-culture experiments demonstrated that microglia isolated at disease onset (M2-like) were neuroprotective and enhanced neuronal survival; microglia isolated at end-stage disease (M1-like) were toxic to motor neurons (Liao *et al.*, 2012).

The ability to differentiate between the contributing roles of peripheral-derived macrophages and microglia has been previously described in the context of Alzheimer's disease and the ability of myeloid cells to mediate amyloid clearance. Compared to microglia, infiltrating monocytes were shown to have more highly acidic lysosomes and were therefore superior at clearing amyloid (Majumdar *et al.*, 2008). In line with this observation, mice lacking CCR2, an important chemokine receptor involved in the recruitment of monocytes into the CNS, had exacerbated pathology in a mouse model of Alzheimer's disease (El Khoury *et al.*, 2007; Naert and Rivest, 2011). Furthermore, injection of CCR2 lentiviruses in bone marrow cells, followed by their systemic delivery, prevented cognitive decline and amyloid pathologies in a similar model (Naert and Rivest, 2012).

Astrocytes and their roles in innate immunity

Astrocytes are the most abundant cells within the CNS and are believed to contribute both to promoting inflammatory responses and to remyelination and repair. While their typically defined roles in the CNS include promoting

BBB integrity, providing structural and metabolic support to neurons and modulating synaptic transmission, several studies over the past decade have described novel functions of astrocytes, including influencing immunity. In terms of innate immunity, astrocytes express several receptors capable of recognizing several pathogens, including TLRs and NLRs. Using quantitative real-time polymerase chain reaction, human astrocytes have been shown to express high levels of TLR3 (significantly higher than TLR1, TLR2 and TLR4), both at the cell surface and intracellularly, and can induce potent pro-inflammatory polarizing responses by secreting IL6, IL10, IL12, TNFα and chemokine CXCL10 (Jack *et al.*, 2005).

In order to serve as a functional antigen-presenting cell, a cell must be able to ingest antigen, undergo internal processing of antigen and eventually present antigen via MHC class II molecules. Indeed, astrocytes are also capable of engulfing myelin debris (Lee *et al.*, 1990) and phagocytosing apoptotic encephalitogenic T cells (Magnus *et al.*, 2002). In addition, astrocytes can also express MHC class II and costimulatory molecules (e.g. CD40, CD80 and CD86) and can successfully present antigen. Post-mortem studies of MS tissues have demonstrated that astrocytes stain positively for MHC class II; however, positive staining was observed only in a subset of astrocytes (Zeinstra *et al.*, 2000). It is important to note that astrocytes do not constitutively express MHC class II, yet both IFNγ and TNFα can promote its expression *in vitro* (Vass and Lassmann, 1990; Dong *et al.*, 1999). In addition, studies on mice have shown that astrocytes can present immunodominant myelin epitopes (proteolipid protein (PLP) and myelin basic protein (MBP)) (Soos *et al.*, 1998; Tan *et al.*, 1998), and modulate anti-MOG T cell responses (Kort *et al.*, 2006).

While astrocytes possess similar properties as cells of the innate immune system, albeit not to the same extent as macrophages, microglia and DCs, they may play a significant role in the regulation of innate immune responses. Similar to other cells of the innate immune system, astrocytes can secrete several chemokines (e.g. CCL2, CXCL10 and CXCL12) that can recruit both peripheral and CNS resident immune cells to sites of inflammation within the CNS. Astrocytes also express several cell adhesion molecules, such as intercellular cell adhesion molecule-(ICAM1) and vascular cell adhesion molecule (VCAM1) (Hurwitz *et al.*, 1992; Gimenez *et al.*, 2004), which play critical roles in binding to receptors on lymphocytes; as well as integrins (e.g. LFA1 and VLA4), which mediate lymphocyte entry into the CNS. In the EAE model, astrocytic expression of VCAM1 is required for T cell entry into the CNS parenchyma (Gimenez *et al.*, 2004). Furthermore, astrocytes also produce several cytokines (e.g. IL6, TGFβ and TNFα) that can influence BBB integrity by directly acting on brain endothelial cells (Nair *et al.*, 2008). MMPs and tissue inhibitors of MMPs (TIMPs) are also secreted by activated astrocytes, which can significantly impact the extracellular matrix (ECM) and contribute to disease progression (Yong, 1999, 2005). It is important to note that several

MMPs and TIMPs have been previously associated with promoting remyelination and/or directly influencing oligodendrocyte progenitor cell differentiation (Larsen *et al.*, 2003; Larsen and Yong, 2004; Larsen *et al.*, 2006; Moore *et al.*, 2011).

Summary

The regulation of innate immune responses within the CNS is critical for recognizing and clearing pathogenic infections, activating the adaptive immune system when appropriate and mediating repair. While the CNS was once considered an immune-privileged site, we now recognize that both infiltrating and CNS resident cells actively participate in several aspects of innate immunity and are important for maintaining neural function. Recognizing that both macrophages and microglia adopt polarized phenotypes that can be either detrimental or beneficial in the inflamed brain has provided novel insights and targets that may help to better understand disease pathophysiology and develop therapies that not only regulate inflammatory processes but also mediate repair.

Conflict of interests

CSM and BAD have no disclosures. JPA has sat on advisory boards or data safety monitoring committees and has received compensation from commercial businesses (BiogenIdec, TEVA, emdSerono, Genzyme, Sanofi Aventis and Novartis), from non-profit organizations (the Cleveland Clinic Foundation and Hertie Foundation) and as editor for *Multiple Sclerosis Journal* (Sage Publishing).

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3
Adaptive Immune Responses in the CNS

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Introduction to concepts of adaptive immunity

The adaptive immune response consists of two major lymphocyte populations, T and B lymphocytes. Both cell types have antigen-specific receptors on their cell surface: the T cell receptor (TcR) and the B cell receptor (BcR). Naïve and memory T cells are present within the circulation and respond differently to antigens presented in association with major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs). In addition to interaction between the TcR and MHC peptide on the APC surface, costimulatory molecules (CD80 and CD86) are required to promote T cell activation. T cells can be broadly divided into CD4 T helper cells (Th) which interact with MHC class II (MHC-II) molecules, and cytotoxic T cells expressing CD8, which interact with MHC class I (MHC-I) molecules on APCs. Antigen presentation takes place in six stages:

1. Antigen is internalized by APCs by endocytosis or phagocytosis and degraded.
2. Peptide fragments associate with MHC-II molecules and are transported to the cell surface.
3. The APC binds via adhesion molecules (e.g. ICAM1) to the T cell.
4. Sufficient MHC–antigen complexes are available on the APC to activate the T cell, assuming that it has an appropriate T cell receptor.
5. Costimulatory molecules (e.g. CD80 or CD86) on the APC engage with CD28 on the T cell to induce activation.

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6. Cytokines (e.g. interleukin-1 (IL1)) are released by the APC that induce receptors on the T cell (e.g. IL2 receptor) which allow it to respond to signals for proliferation.

If any of these functions are missing, then the APC will not fully activate the T cell. As a minimum, the APC must complete stages 1–5; lack of costimulation will result in T cell anergy. It is only if the T cell completes all stages that it will then undergo cell division. In practice, lymphocyte division does not occur in the central nervous system (CNS) to any significant extent, either because the threshold for T cell division is not reached, or because there are additional controls limiting lymphocyte proliferation. However if T cell activation occurs, then the release of inflammatory cytokines (interferon gamma (IFNγ), lymphotoxin etc.) can activate immune effector functions with the capacity to produce local damage within the CNS.

The overall effect of these functions is not only to constrain immune reactions in the CNS but also, when they do occur, to polarize them towards Th1-type immune responses or in some cases Th17-type responses (see Figure 3.1). This polarization of response is seen both in the subsets of lymphocytes and phagocytes that accumulate, and in the way that cells become activated once they have entered the CNS.

Leukocyte populations in the CNS

The profile of leukocyte populations seen in neuroinflammation is highly selective. It is worth noting that neutrophils, which are the most abundant leukocytes in the blood (70%), are rarely seen in the CNS, and if so, only in stroke and the most severe cases of acute encephalitis. This is exactly the opposite of what is seen in inflammation in most other tissues, and in bacterial meningitis, where neutrophils predominate. This observation alone indicates that distinctive mechanisms control leukocyte migration into the CNS. The major leukocytes seen in CNS inflammation are lymphocytes and mononuclear phagocytes (derived from blood monocytes), a pattern more typical of sites of chronic inflammation.

T cell populations

Initial observations of multiple sclerosis (MS) post-mortem tissue showed that both CD4+ and CD8+ T cells were present in chronic active inflammatory lesions, with CD4+ cells extending out beyond the lesion and with a relatively high proportion of CD8+ cells at the active edges of the plaques (Traugott *et al.*, 1983). These plaques were often examples of the late stages of a chronic disease, and more active plaques contained higher proportions of CD4+T cells.

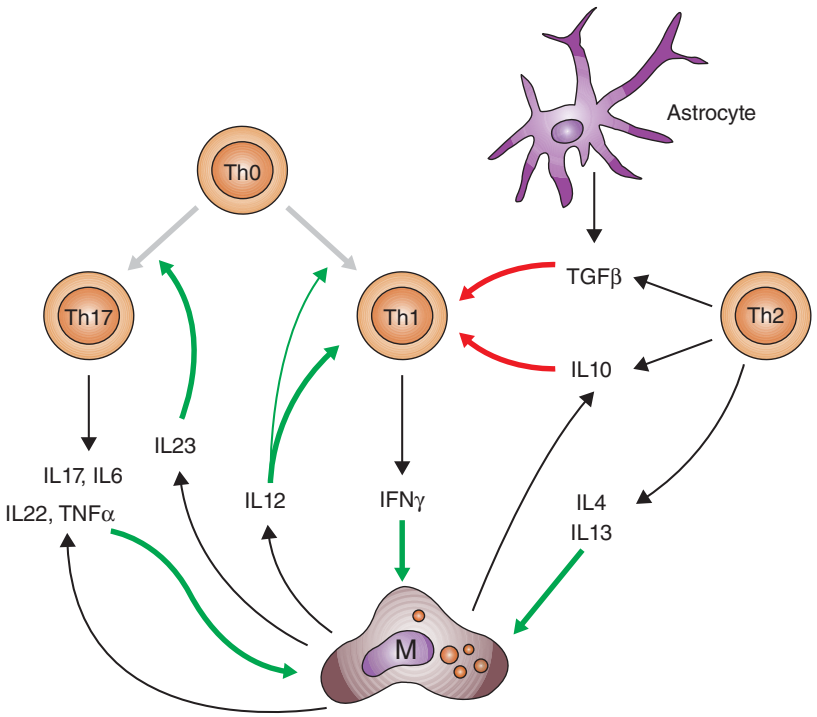

Figure 3.1 Cytokine networks in T cell activation. T helper cells (Th0s) can differentiate into Th17 cells or Th1 cells in response to IL23 or IL12, respectively. Th17 cells release cytokines, including TNFα, which promote macrophage activation. IFNγ released by Th1 cells also activates macrophages and promotes antigen presentation. The action of Th1 cells is counteracted by cytokines released from astrocytes, Th2 cells and in some cases microglia. M = Mononuclear phagocytes, including macrophages and activated microglia; white arrows = differentiation; black arrows = production; red arrows = suppression; green arrows = activation.

Studies of experimental autoimmune encephalomyelitis (EAE) in rodents confirmed the importance of CD4+ T cells in the development of acute neuroinflammation. Not only were CD4+ helper T cells prevalent in the spinal cord during the acute phase of disease, but also the disease could be passively transferred with antigen-activated CD4+ T cells. Moreover, disease development could be blocked with antibodies to CD4 or MHC-II molecules, which interfere with antigen presentation to CD4+ T cells. Although this may show that CD4+ T cells play a dominant role in disease induction, Sun *et al.* (2001) also describe the encephalitogenic potential of CD8+ T cells in mice. Interestingly, however, in passive transfer EAE, the great majority of the T cells found in the affected areas were not donor cells, but host cells and therefore probably not specific for any CNS antigen – although recognition of CNS antigens is required for the development of neuroinflammation, lymphocyte migration

itself is not antigen specific. The process explained in this chapter does, however, favour the accumulation of T cells that have been recently activated by specific antigens in the periphery of the body.

T lymphocyte subsets

Th1, Th2 and Th17 cells

The recognition of subsets of CD4+T cells that secrete different cytokine profiles led to a more thorough investigation of the cell types responsible for neuroinflammation (see Table 3.1). Th1 cells characterized by the secretion of IFNγ respond to antigen presented by mononuclear phagocytes, and are effective at inducing expression of MHC molecules and activating macrophages. Th2 cells secrete IL4, IL10 and IL13 and are more effective at interacting with B cells as APCs, and their cytokines promote B cell development and antibody secretion. The finding of IFNγ in lesions in MS in association with activated macrophages and microglia strongly implied that Th1 cells were primarily responsible for development of lesions. IL12 and IL18 secreted by mononuclear phagocytes also contribute to polarizing the T cell phenotype to Th1 in MS, with IL12 promoting Th1 cell differentiation and IFNγ secretion and IL18 promoting tumour necrosis factor alpha (TNFα) production. This pattern of Th1 cytokine production is found in active MS lesions.

The initial observations in EAE tended to support this view. It is possible to generate Th1-type or Th2-type antigen-specific T cells *in vitro* by treating the cells with different combinations of cytokines and growth factors. In this case, Th1 cells secreting IFNγ, IL2 and TNFα could transfer EAE, whereas Th2 cells that had an identical antigen specificity but secreted IL4, IL5, IL10 and IL13 did not. The theory was further supported by observations that strains of

Table 3.1 T cell phenotypes, the cytokines which induce their polarization and the effector cytokines produced by each subset

T cell phenotypes	Polarizing cytokines	Effector molecules	Function
Th1	IL12, IFNγ	IFNγ	Macrophage activation; induction of MHC II
Th2	IL4	IL4, IL5 and IL13	Interact with B cells as APCs; Th2 cytokines promote B cell development and antibody secretion
Th17	IL6, IL1β, IL23 and TGFβ	IL17, IL22 and GM-CSF	Promotes acute inflammation
Treg		IL10, IL35 and TGFβ	Suppresses/regulates other T cell subsets

GM-CSF = granulocyte–macrophage colony-stimulating factor; TGFβ = transforming growth factor beta.

animals that produce strong antibody responses against CNS antigens often do not develop severe EAE.

A major problem with the hypothesis that Th1 cells are responsible for neuroinflammation arose from the finding that some strains of mice with IFNγ receptor deleted developed *more* serious disease than wild-type animals, and it was possible to induce EAE in TNF knockouts. Emphasis then switched to the investigation of another subclass of CD4+ T cells, Th17 cells, characterized by the production of IL17, IL6, IL22 and TNF. This population of cells is induced and maintained by IL23, produced by inflammatory macrophages and dendritic cells. It was found that mice deficient in IL23 were resistant to the development of EAE, whereas deletion of IL12 did not prevent disease (Cua *et al.*, 2003). Moreover, treatment with anti-IL23 antibody could alleviate EAE in one model, whereas anti-IFNγ did not (Chen *et al.*, 2006). In EAE, Th17 cells promote disease by acting at the choroid plexus, a site which has quite different properties and endothelium compared to the normal CNS parenchyma (Reboldi *et al.*, 2009). Interpreting studies from knockout animals does present some difficulties, because deficiency in one immunological pathway can lead to compensatory activity in others. Nevertheless, the work in EAE lead to a re-examination of the T cell subsets in MS, with the findings that Th17 cells were present and associated with active disease (Tzartos *et al.*, 2008) and that activated microglia secrete IL23 (Li *et al.*, 2007). Zielinski *et al.* (2012) very recently described two types of Th17 cells with different inflammatory properties, producing either IFNγ or IL10, the former representing pathogenic Th17 cells and the latter non-pathogenic cells. IL1β induced Th17 cells producing IFNγ while suppressing IL10 production, and they suggest that IL1β rather than IL23 promotes polarization of highly inflammatory Th1 and Th17 responses in human CNS.

A summary of the interactions between the CD4+ lymphocyte subsets and the role of the different cytokines is shown in Figure 3.1. In viewing schemes of this type, one should be aware that the cytokine production profiles of different cells are variable and may have pleiotropic effects. For example, microglia can secrete IL12 (Th1-type response), IL10 (Th2-type response) and IL23 (Th17-type response).

Cytotoxic T lymphocytes

The role of CD8+ T cells in neuroinflammation has been partly overshadowed by work on CD4+ T cells, even though they sometimes are the predominant cell type. Indeed, some studies have demonstrated the induction of variants of EAE, using CD8+ T cell clones; however, these models are the exception to the general rule that activated CD4+ T cells are required to induce EAE (Mars *et al.*, 2011). In some cases, CD8+ T cell clones are expanded in the cerebrospinal fluid (CSF) of MS patients, and such clones can persist for many years (Skulina *et al.*, 2004). It has been argued that whereas CD4+T cells and

macrophages cause demyelination and damage to oligodendrocytes, CD8+ cells could contribute to the subsequent damage to exposed axons or neurites (McFarland and Martin, 2007).

Regulatory T cells (Tregs)

Tregs, identified by the transcription factor Foxp3 and high levels of the IL2 receptor CD25, are important in controlling tissue-specific autoimmunity and inflammation, particularly in the gut. They are thought to act by the release of anti-inflammatory cytokines including IL10, IL35 and TGFβ (Korn *et al.*, 2007). More recently, a population of CD8+ Tregs producing TGFβ and (surprisingly) IFNγ have been shown to suppress EAE induced by myelin oligodendrocyte glycoprotein (MOG) in SJL mice (Chen *et al.*, 2009).

These observations led to investigations of Tregs in MS. The incidence of Tregs in the blood and the CNS of MS patients appears to be similar to that found in controls, but there is some evidence that the functional activity of Tregs is lower in individuals with relapsing and remitting MS than in those with the progressive disease (Venken *et al.*, 2006).

B cells

Oligoclonal antibodies (i.e. antibodies produced by a small number of B cell clones) are present in the CSF of patients in most infectious diseases of the CNS as well as in MS. Since serum antibodies are effectively excluded by the blood–brain barrier (BBB), this fact alone indicates that some B cells must be present in the brain. The clones represent a small subset of those present in the blood (Owens *et al.*, 2001); however, these clones can be stable over many years, which is longer than the normal lifespan of a plasma cell. This implies that the CNS environment, in some way, allows clones of B lymphocytes to persist. One hypothesis that could explain the persistence of B cells is infection with Epstein–Barr virus (EBV), a recent candidate virus in MS. Although this is an attractive hypothesis, there is still debate regarding the presence of EBV in MS tissues (Lassmann *et al.*, 2011).

B cells and plasma cells were first observed in the Virchow–Robin spaces around larger blood vessels in MS, which explains why they could be detected in the CSF. Although in infection the antibody is usually specific to the pathogen, the antibody targets in MS and other inflammatory conditions were often not identifiable. This led to discussion as to whether these antibodies were merely an epiphenomenon (i.e. random local antibody production, caused by a few persistent B cells that had entered the perivascular space). This has never been a wholly satisfactory explanation, because antigen is normally required to drive the differentiation of B cells to plasma cells and to maintain an antibody response over time. Moreover, antibody-producing cells do occur in early MS lesions (Henderson *et al.*, 2009), and B cell depletion can

alleviate relapses in MS (Hauser *et al.*, 2008). In EAE it appears that small amounts of antibody are important in targeting macrophages against oligodendrocytes, regardless of the immunological target of the T cells. For example, EAE can be induced by an immune response against astrocyte S100B protein or neuronal antigens such as neurofilament light chain (NF-L) (Huizinga *et al.*, 2007). However, myelin damage is clearly due to antibodies to oligodendrocytes, especially MOG (Smith *et al.*, 2005). Consequently, antibody may be required to target the immune reaction even if neuroinflammation is primarily driven by Th1 and Th17 cells.

Mediators of the adaptive immune response

Control of lymphocyte migration into the CNS

The level of leukocyte traffic through the CNS is normally low in the absence of neuroinflammation; hence, there are relatively few lymphocytes present in the normal CNS. Lymphocyte migration into the CNS involves binding to the endothelium following selectin-mediated rolling and subsequent chemokine-mediated integrin activation, leading to firm adhesion and transmigration across the BBB. Within the inflamed CNS, chemokines direct lymphocytes, expressing the appropriate chemokine receptors to sites of inflammation. Lymphocyte migration into the CNS takes place primarily across post-capillary venules, and this is reflected in the location of lymphocytes in the CNS, in perivascular cuffs as seen in MS pathology. The cuff is formed by tightly packed lymphocytes and monocytes in the space between the endothelium and the astrocyte foot-processes, effectively between the double basal lamina produced by the two cell types. Leukocytes are then able to break through the glia limitans barrier following secretion of matrix metalloproteinases (Agrawal *et al.*, 2013) to enter the brain parenchyma.

Lymphocyte migration into the CNS is controlled, as in other tissues, by chemokines and adhesion molecules expressed on the luminal side of the brain endothelium (Male, 2012). These controls are tissue specific, so that the pattern of lymphocyte migration into the CNS, and hence the types of immune reaction that can occur, is constrained by these controls (Engelhardt and Ransohoff, 2005; Man *et al.*, 2007). The BBB prevents the diffusion of chemokines from the CNS to the blood side of the endothelium, via the paracellular route. Additionally, resting brain endothelium has comparatively few caveolae and other transport vesicles, which limits movement of chemokines by transcytosis. This means that chemokines produced by the brain endothelium make a particularly large contribution to the control of lymphocyte migration into the tissue, by comparison with endothelium in other tissues.

Reboldi *et al.* (2009) demonstrated in EAE that the choroid plexus is the first port of entry of inflammatory T cells into the CNS. Specifically,

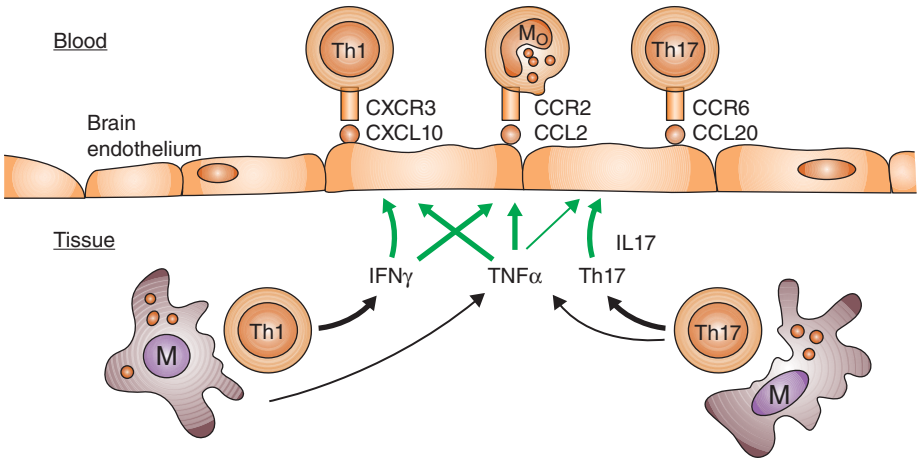

Figure 3.2 Chemokine reinforcement of the T cell response. Th1 cells stimulated to release IFNγ by interaction with antigen-presenting cells (M) induce production of CXCL10 by brain endothelium which promotes further Th1 cell migration. Th17 cells induce CCL20 which promotes further Th17 cell migration. Both types of T cell can promote synthesis of CCL2 which attracts monocytes (Mo) into the CNS.

CCR6 expressing Th17 cells migrate in response to the CCR6 ligand, CCL20, expressed by the epithelial cells of the choroid plexus (Figure 3.2). Sallusto *et al.* (2012) proposed a two-step model for lymphocyte migration, initially entry of CCR6+ Th17 cells via the choroid plexus followed by effector T cells including Th1 cells and inflammatory leukocyte cell migration.

Chemokines

The migration of lymphocytes across brain endothelium requires interaction between integrins on the lymphocyte and cell adhesion molecules (CAMs) on the endothelium. Most important are $\alpha_1\beta_2$ integrins, expressed on most lymphocytes and monocytes, which bind to intercellular CAM-1 and -2 (ICAM1 and ICAM2, respectively), and $\alpha_4\beta_1$ integrin, which binds to vascular CAM-1 (VCAM1). Both the integrins and the CAMs engage with the cytoskeleton inside the cell, which allows the lymphocyte to crawl across the endothelium.

Inflammatory cytokines, including IL1, TNF and IFNγ, all induce ICAM1 and VCAM1 on brain endothelium but reduce ICAM2. For this reason, ICAM1 and VCAM1 are seen as critical in controlling lymphocyte migration in inflammation, whereas ICAM2 is thought to modulate basal cell migration and is used by mononuclear phagocytes during nervous system development.

Blocking the interaction of VCAM1 with VLA4, which is present on activated T cells, is an effective way of limiting cell migration into the CNS in EAE, and treatment of MS patients with anti-VLA4 adhesion-blocking antibodies (natalizumab) also slows progression of the disease. Although adhesion molecules are required to promote lymphocyte migration into the CNS during inflammation, they are not thought to exert the fine level of control mediated by chemokines.

Lymphocyte interaction with APCs

Antigen presentation to T helper cells is a function of a number of cell types of the innate immune system (see Chapter 2), which internalize antigen, process it and present peptide fragments on the cell surface associated with MHC-II molecules. Dendritic cells are the most effective cell at antigen presentation to naïve T cells (i.e. T cells that have not previously encountered their specific antigen). Other leukocytes, including mononuclear phagocytes and B cells, are effective at presenting antigen to previously activated T cells. Mononuclear phagocytes are most effective at antigen presentation to Th1 cells, and B cells to Th2 cells. In some circumstances, particularly at sites of chronic inflammation, resident cells of the tissue can process and present antigen to CD4+ T helper cells.

In some cases, for example following a stroke, CNS antigens may be released into the periphery, inducing immune responses that are usually transient. The normal immunological controls on self-reactivity are re-established within days or weeks. It is only in autoimmune diseases that the initiating antigen is likely to originate from the CNS and provoke an immune response. In these cases, whether a chronic immune reaction develops depends not so much on whether the antigen is presented, as whether the controls on immune reactivity terminate the reaction.

MHC expression on cells of the CNS

Studies *in vitro*

In the normal CNS, APCs consist of dendritic cells and macrophages located in the meninges, choroid plexus and perivascular spaces (Tian *et al.*, 2012). Studies on isolated populations of neurons or glia *in vitro* indicated that expression of MHC-I molecules was low or absent on neurons and oligodendrocytes but was normally detectable on astrocytes and microglia. Single-cell real-time polymerase chain reaction on neurons confirmed that in many cases, it was not possible to detect even a single MHC-I mRNA in the cells. This observation is particularly interesting, because MHC-I expression is an essential element of endogenous antigen presentation, and is essential for

Table 3.2 Expression of MHC molecules on cells of the CNS

	*In vitro**		*In vivo***	
	Class I	Class II	Class I	Class II
Neuron	+/− ↑	−	+/− ↑	−
Oligodendrocyte	+ ↑	−	+ ↑	−
Astrocyte	+ ↑↑	− ↑	+ ↑	−
Microglia	+ ↑↑	+ ↑	+ ↑	+ ↑

*Expression on single cell populations *in vitro*. ↑ indicates that expression is increased or induced by IFNγ.
**Expression on cells in normal CNS. ↑ indicates that expression is increased or induced in neuroinflammation. MHC class-II expression may occur on astrocytes in severe disease (e.g. encephalitis due to infection).

recognition of intracellular antigens by cytotoxic T cells and control of viral infection. In addition, the expression of MHC-I protects a cell from killing by natural killer (NK) cells, which recognize MHC-I using killer inhibitory receptors. The lack of MHC-I on neurons can, therefore, explain why virally infected cells are more readily tolerated in the CNS. However, it begs the question of what mechanism protects the neuron from NK cell–mediated cytotoxicity, as discussed in this chapter.

MHC-II expression on resting CNS cell populations *in vitro* is effectively restricted to microglia. Activation of neurons and glia with IFNγ generally increases the basal level of MHC-I expression on all cell types and induces MHC-II expression on microglia and astrocytes (Table 3.2).

After the initial studies with isolated cell populations, it became clear that interactions between different cell types could cause an even more profound suppression of MHC molecule expression. For example, when neurons were maintained in co-culture with astrocytes, MHC-I was suppressed, both in the resting state and following stimulation with IFNγ. The suppression required cell–cell contact and could not be transferred with culture supernatants (i.e. it was not cytokine dependent). A similar result was found with the IFNγ-mediated induction of MHC-II molecules on astrocytes (Tontsch and Rott, 1993). These findings led to a better understanding of why MHC expression was even lower *in vivo* than on isolated cells *in vitro*.

Studies *in vivo*

Examination of CNS tissue from normal individuals has confirmed that MHC-I expression is low and MHC-II is present at low levels on microglia and perivascular macrophages. The expression of these molecules is kept at a low level through active down-regulation in the immune-quiescent environment within the CNS. In neuroinflammation (e.g. MS), MHC-I expression is increased and MHC-II expression is enhanced on microglia, but is not observed on other cell types. Interestingly, the area of increased expression

extends beyond the MS plaque itself, which may relate to diffusible cytokines (e.g. IFNγ), electrophysiological disturbance extending beyond the plaque and/or an intrinsically higher level of MHC inducibility in MS patients.

In this context, it is worth noting that strains of rat which are susceptible to induction of EAE are more readily induced to express MHC molecules on non-professional APCs, in response to IFNγ, than EAE-resistant strains. However, this may merely reflect an increased sensitivity to IFNγ, affecting many functions, including macrophage activation and induction of endothelial adhesion molecules. Genetic variation in the responses to inflammatory cytokines is an important element in determining susceptibility to immune reactivity generally, not just in the CNS.

Several lines of evidence point towards the importance of normal neuronal function for the suppression of MHC expression both on the neurons themselves and on surrounding glia. For example, electrically active hippocampal neurons have lower expression of MHC-I than inactive neurons, or cells that have been paralysed by tetrodotoxin (Neumann *et al.*, 1995). *In vivo*, it is found that transection of the facial nerve axons activates microglia in the facial nucleus, with increased expression of MHC-II and the opsonic complement receptor CR3. The mechanisms by which neurons inhibit glial activation are outlined further in this chapter.

Costimulatory molecules on microglia

Costimulation by the binding of CD80 or CD86 on APCs to CD28 on the T cell is essential for T cell activation. Activation of Th2 cells is also promoted by the binding of the costimulatory molecule CD40 on the APC with the CD40 ligand on the T cell. Treatment of microglia with IFNγ or granulocyte–macrophage colony-stimulating factor *in vitro* causes an increase in expression of all three costimulatory molecules, and IL4 can suppress expression, although there are minor differences between species. Because microglia are the only CNS resident cell to consistently express MHC-II molecules and costimulatory molecules, it is thought that they are the one endogenous cell type that can effectively present antigen to T helper cells.

Astrocytes and T cell activation

The low level of expression of MHC and costimulatory molecules on astrocytes suggests that these cells may more likely induce Th2 cell responses rather than Th1 cells *in vivo*. Astrocytes, as compared with microglia, produce chemokines which favour Th2 cell recruitment into the CNS as opposed to Th1 cell recruitment by microglial-derived chemokines. Through secretion of TGFβ, astrocytes promote the recruitment and proliferation of Tregs, and other anti-inflammatory cytokines produced by astrocytes (e.g. IL10 and IFNβ) suppress T cell and microglial activation (Tian *et al.*, 2012).

Antigen presentation in the CNS

Immune recognition of CNS antigens

All individuals have some T cells that are capable of recognizing CNS auto-antigens. Central mechanisms of thymic tolerance delete a proportion of the auto-reactive cells during development, but substantial numbers are present in the periphery, and they can potentially mount immune reactions against CNS antigens, if peripheral regulatory mechanisms are bypassed.

The mechanisms which may cause breakdown of tolerance to CNS antigens are:

- Release of the antigen to lymphoid tissue following CNS damage;
- Uptake of antigen by professional APCs, resulting in efficient processing and presentation;
- Presentation of antigen in association with a microbial infection that induces costimulatory molecules; and
- Cross-reaction (molecular similarity) between the CNS antigen and a non-self-antigen, when presented on an MHC molecule.

In EAE models where clearly defined antigens (e.g. myelin basic protein (MBP)) are used in inbred strains of mice, there is restricted usage of the MHC molecules and the T cell receptor repertoire. For example, in the SJL mouse (MHC haplotype I-A^S), the immunodominant peptides of MBP (i.e. the peptides of MBP presented by their MHC-II molecules) are located at positions 18–30 and 85–103. In contrast, the immunodominant peptide in B10-PL mice (I-A^U) is at position 1–12, and in these mice 80% of the encephalitogenic T cells use the T cell receptor gene segment V-beta-8.2.

In humans, the situation is much less clear. The genetic association of specific MHC haplotypes, particularly DRB1∗1501 with MS in Caucasians (Sospedra and Martin, 2005; Compston and Coles, 2008; Sawcer *et al.*, 2011), strongly implies that an antigen-specific immune response is taking place. However, there is little evidence for selective usage of the T cell receptor repertoire even in individuals with DRB1∗1501 (Dyment *et al.*, 2004). The problem in humans is not just that the population is immunologically heterogeneous but also, crucially, that no single antigen has been identified as a target in MS.

Targets of immune responses in CNS

In a number of paraneoplastic conditions, the primary target of the immune response appears to be neurons (see Chapter 10). These conditions are associated with remote neoplasia, particularly small-cell carcinoma of the lung, and they are relatively rare. Demyelinating diseases in which oligodendrocytes are

the primary target are more common, and in these conditions the neurons are often a secondary target of immunopathological damage.

It is understandable how the loss of myelin exposes neurons to secondary damage; however, the susceptibility of oligodendrocytes to immune reactions mediated by Th1 cells requires explanation because they cannot interact directly with CD4+ T helper cells. Observation of demyelinating lesions indicates that the damage is mediated either directly by the action of inflammatory cytokines on the oligodendrocytes, or indirectly by the action of macrophages and activated microglia. However, this raises the question of why oligodendrocytes are particularly susceptible to damage. One explanation is that they are particularly vulnerable to stress due to their high energy demands and may thus be the target of 'autoimmune' responses to stress proteins, although this concept is still under investigation (van Noort *et al.*, 2012).

Several explanations have been proposed:

1. The cells are susceptible because of their physiology – oligodendrocytes maintain large amounts of membrane in the myelin sheath, and the release of inflammatory mediators as well as reactive oxygen and nitrogen species (ROS and RNS) from activated macrophages therefore particularly targets the myelin.
2. Damage to the BBB in response to inflammatory cytokines (TNFα, IL1 and IFNγ) leads to leakage of serum proteins (e.g. complement) into the CNS, and the oligodendrocyte lacks the protective proteins that limit deposition of C3b and membrane attack complexes.
3. The oligodendrocyte is particularly susceptible to cytotoxicity caused by TNFα and lymphotoxin.
4. Small amounts of antibody against myelin proteins enter the CNS, bind to the myelin sheath and allow the macrophage to recognize it, via its high-affinity Fc receptors (CD64).

None of these explanations are completely satisfactory, and because they are not mutually exclusive, it may be that a combination of factors explains why the oligodendrocyte is targeted.

Suppression of immune responses in the CNS

Immune responses in the CNS are normally limited by a combination of direct cell–cell interactions, cytokines and small soluble molecules.

Within the brain parenchyma microglia, in particular, act as sensors of neuronal damage. Microglia express CD200, SIRP-1α and the fractalkine receptor (CX_3CR1) which interact with CD200L, CD47 and fractalkine (CX_3CL1), respectively, on the neuron (Figure 3.3). CX_3CL1 is a membrane-bound chemokine which may be released in a soluble form. Loss of these molecules

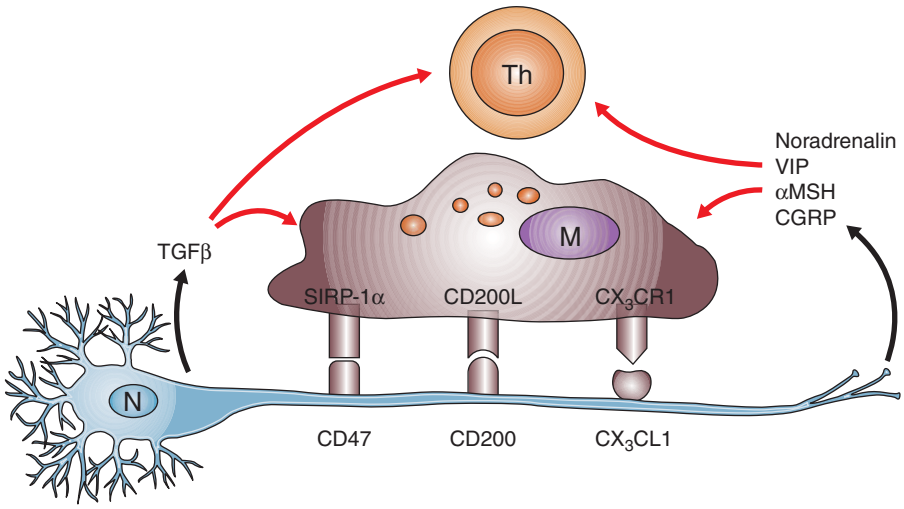

Figure 3.3 Neuronal inhibition of T cell activation. Neurons (N) express CD47, CD200 and CX_3CL1 (fractalkine) which inhibit the activation of microglia (M) and limit antigen presentation to T helper cells (Th). TGFβ, neuropeptides and some neurotransmitters also inhibit T cell activation. These inhibitory actions may be lost if the neuron is damaged.

from the surface of a damaged neuron removes their inhibitory signals to the microglia and increases their ability to present antigen.

Neurons also release soluble factors that suppress antigen presentation and lymphocyte activation, including anti-inflammatory cytokines, chemokines, neuropeptides and neurotransmitters. They also include neurotropins, nerve growth factor (NGF) and brain-derived neurotropic factor (BDNF), which inhibit microglial expression of MHC-II and costimulatory molecules, respectively, and a number of neurotransmitters (noradrenalin, vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP)) which suppress lymphocyte functions (Niederkorn, 2006).

Important anti-inflammatory cytokines, such as TGFβ, are produced by neurons and astrocytes and inhibit the production of proinflammatory cytokines by Th1 cells, thus reducing the activation of brain endothelium and their subsequent chemokine synthesis. IL10 was originally thought to be exclusively a cytokine produced by Th2 cells, although there are reports of microglia secreting IL10 (Chabot *et al.*, 1999). Amongst other effects, it acts on Th1 cells to reduce IFNγ production, hence deviating the immune response to a Th2 type.

Finally, a number of small molecules are also suppressive, for example the eicosanoid prostaglandin E2 (PGE2) released by astrocytes and neurons inhibits lymphocyte proliferation – astrocytes treated with indomethacin to block eicosanoid production are more effective at inducing T cell proliferation *in vitro* than untreated cells which are weak APCs for T helper cells.

Metabolites of tryptophan also inhibit lymphocyte proliferation, and tryptophan itself is required for cell division. Hence, tryptophan catabolism inhibits lymphocyte division. In the CNS, tryptophan catabolism is partly promoted by the enzyme indoleamine 2,3-dioxygenase (IDO) which is present in microglia, induced by IFNγ and enhanced by IL4 and IL13 (Yadav *et al.*, 2007).

Termination of immune responses

Lymphocyte responses are terminated partly by the lack of activation signals (MHC, antigen and costimulation) and partly by signals that prevent further activation and promote cell death. The molecule CTLA-4 (CD154) is an alternative ligand to CD28 for costimulatory signals from CD80 and CD86, and ligation of CD154 inhibits T cell activation. CD154 appears later in immune reactions and is probably partly responsible for the decline in the T cell response.

Another important molecule is PD1 (Programmed Death-1), which is associated with 'exhausted' T cells that have ceased division and cytokine secretion. The ligands PDL1 and PDL2 (CD273 and CD274) are found on APCs, including microglia, and they limit costimulation by CD28. However, they are also present on other cells of the CNS, including astrocytes and retinal pigment epithelium (Ke *et al.*, 2010). In one study, PDL1 was increased on oligodendrocytes in response to a neuronal coronavirus infection, indicating a possible way of controlling cytotoxic reactions against the neurons and oligodendrocytes, but at the expense of tolerating the infection (Phares *et al.*, 2009). Of course, whether the PD1 ligands are functionally effective in curtailing an immune response depends on whether the infiltrating T cells express PD1, and in active disease, this is often not the case.

These mechanisms for terminating immune responses are undoubtedly important, but they are not specific to the CNS.

Relapses and remissions in immune responses

An important feature of chronic immune responses in the CNS is that they often show a pattern of relapses and remissions. For example, the most common clinical course of MS is progressive with relapses and remissions. Recent EAE models show the relapsing-remitting disease pattern, usually with a well-defined periodicity for each of the models, such as chronic relapsing EAE (CREAE) (Figure 3.4). How does this disease pattern arise? The simplest explanation is that the suppressive environment of the CNS is only breached once a sufficiently large number of antigen-activated lymphocytes enter the CNS. The requirement for a threshold number of activated T cells is supported by the observation that the ability to transfer EAE passively depends

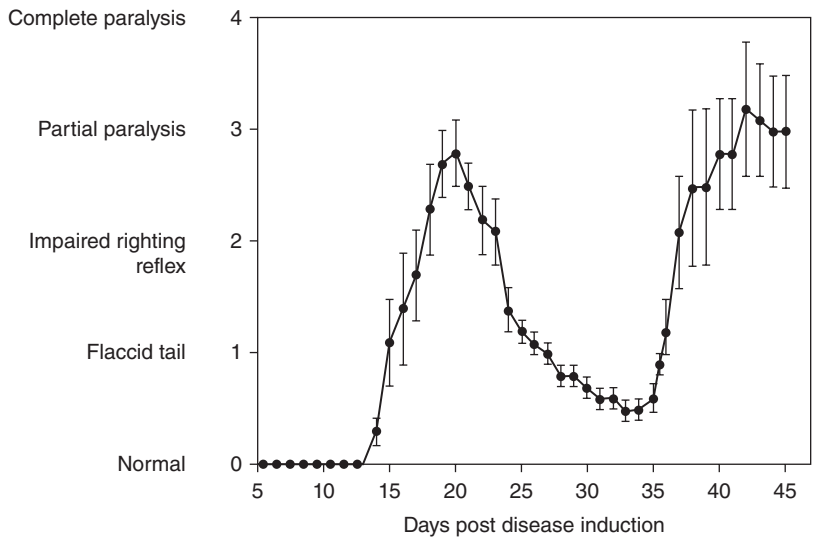

Figure 3.4 Time course of EAE in the Biozzi/AbH mouse. The disease symptoms follow a typical relapsing and remitting time course following disease induction at day 0 by the injection of myelin basic protein in complete Freund's adjuvant.

on the number of antigen-specific T cells that are transferred, and on whether they are in cell cycle. Recall also that antigen-activated cells enter the CNS more effectively than resting cells because they express appropriate adhesion molecules and chemokine receptors. It is also known that antigen-specific T cells are present in the periphery of EAE animals during remissions. These facts imply that the development of the immune response in the CNS does not so much depend on whether antigen-specific cells are present in the animal as on whether they are entering the CNS.

In this theory, an immune reaction in the CNS stimulates the immunosuppressive cytokines described in this chapter (TGFβ and IL10), relapses occur as the suppressive cytokine production declines and sufficient T cells enter the CNS to reactivate the immunopathogenic mechanism with the production of IFNγ, TNF, IL17, CXCL10 and so on. This effect has been described as a switch between a Th1-type response and a Th2-type response; however, although the cytokines controlling the relapses and remissions are characteristic of Th1 and Th2 responses, respectively, there is no evidence for a switch to Th2 responses (i.e. antibody production), although oligoclonal immunoglobulins are present within the CSF in the long term.

Because Th2-type cytokines are associated with protection against or remission of neuroinflammatory disease, a number of attempts have been made to pre-empt the Th1 response by inducing a Th2 response. For example, presenting myelin antigens via the nasal mucosa (aerosol) or gut (ingestion) can prevent the subsequent induction of EAE with the same antigen. This led to

similar attempts to block the immune reaction in MS by feeding myelin antigen (oral tolerance induction). The failure of this approach may well be due to the lack of a clearly defined antigen in MS and the fact that deviating an established immune response is much more difficult than modulating a response in naïve animals. Whatever the explanation, it is very clear that modulating responses by such tolerance regimens is not effective in progressive disease such as secondary progressive EAE.

Allografts and cytotoxic T cell responses

Limited antigen presentation

Acceptance of allografts was one of the first effects that identified the CNS as an immunologically privileged tissue (see Chapter 1). Low expression of MHC-I molecules in the CNS helps limit immune recognition by CD8+ cytotoxic T cells. Indeed, many neurotropic viruses interfere with the presentation of antigenic peptides by the MHC-I pathway. For example, herpes simplex virus produces a protein (ICP47) that prevents transport of peptides from the cytoplasm to the endoplasmic reticulum, and thus prevents the loading and presentation of viral peptides (and cellular peptides) on MHC-I molecules. Another example is cytomegalovirus, which produces two proteins (US2 and US11) that cause removal of MHC-I molecules from the endoplasmic reticulum and promotes their degradation.

Peptides of MHC-I molecules presented by HLA-E molecules are recognized by receptors on NK cells (CD94–NKG2A), and the receptor LILRB recognizes many MHC-I. These receptors recognize low levels of MHC-I and class I–like molecules to inhibit NK cell–mediated cytotoxicity. In theory, the absence of MHC-I on some neurons should render them susceptible to cytotoxicity. In practice, the fact that neurons are not killed indicates that additional mechanisms make them resistant to cytotoxic cells.

Resistance to cytotoxicity

Cytotoxic CD8+ T cells and NK cells kill their targets by engaging the receptor Fas (CD95) with the ligand FasL and by the release of lymphotoxin. They also release perforin which assembles pores in the target cell membrane, allowing granule-associated enzymes, or granzymes, to enter the target. Fas is related to the family of trimeric TNF receptors, and ligation activates the extrinsic pathway of apoptosis with activation of caspases 3, 7, 8 and 10. As they develop, neurons become increasingly resistant to activation of the extrinsic pathway of apoptosis. Moreover, they can themselves express FasL, which inhibits CD8+ T cell degranulation and turns the tables on the cytotoxic T cells to cause their death (Flügel *et al.*, 2000). Trauma in the CNS is associated with increased expression of neuronal FasL, indicating that neurons can

protect themselves against the increased lymphocyte traffic that occurs following CNS injury.

Summary

Protection of the CNS from immune reactions is advantageous to preserve neurons and maintain a physiologically stable environment within the CNS. A variety of mechanisms contribute to this protection, including the blood–brain barrier, limited lymphocyte traffic into the CNS, poor antigen presentation in the brain parenchyma and a variety of immunosuppressive controls maintained by neurons and glia. Infection in the brain may be tolerated, and some neurotropic viruses have evolved to suppress the immune response against them.

Immune reactions can develop in the brain when lymphocytes that have been activated in the periphery enter the CNS in sufficient numbers to respond to CNS antigens and breach the local threshold of immunosuppression.

The pattern of immune responses in the brain is usually typical of Th1- and Th17-type immune responses, with the release of inflammatory cytokines that promote activation of macrophages and microglia. Oligodendrocytes and neurons are sensitive to immunopathological damage mediated by mononuclear phagocytes, although targeting of these cells is not necessarily due to a direct immune response against them.

The termination of an immune response may be due to the actions of regulatory T cells, the local release of Th2 cytokines (from glia as well as lymphocytes) and the induction of cell surface molecules that promote the death of infiltrating T helper cells and the down-regulation of mononuclear phagocytes acting as APCs and effector cells. The characteristic relapsing-remitting pattern of symptoms seen in chronic neuroinflammatory disease can occur when the immunosuppressive actions wane following an immune response, and increased numbers of activated T cells re-enter the brain.

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4

Ageing and the Immune Response in the CNS

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Gene expression in the ageing brain

Gene expression studies have been instrumental in gaining insight in brain ageing. A recent study (Glorioso *et al.*, 2011) highlighted the consistency in results across several molecular studies of human brain ageing using gene expression analysis. Despite different microarray chip platforms and brain areas, a remarkable conservation of age-regulated changes has been demonstrated (Lee *et al.*, 2000; Lu *et al.*, 2004). This suggests the presence of a specific, tightly controlled, age-regulated transcriptional gene expression programme. A recent paper by Colantuoni *et al.* (2011) showed that a wave of gene expression changes during foetal development decreases upon early postnatal life but is resumed upon ageing. Development-related pathways showed a significant overlap with age-regulated pathways, indicating that genes associated with developmental transcriptional programmes may have a dual role in promoting the ageing process in accordance to the 'antagonistic pleiotropy' theory. The authors speculated on how some developmental processes, such as synaptic pruning, mirror ageing phenotypes such as synapse loss and thus could extend from the same transcriptional programmes.

The mechanisms regulating age-related changes in gene expression are largely unknown. Lu *et al.* (2004) found that DNA damage accumulates particularly in the promoters of genes that show low expression with age. Such affected promoter regions were found to be genes that play central

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roles in synaptic plasticity, vesicular transport and mitochondrial function and were thereby proposed to initiate a programme of brain ageing. The idea of a defined transcription programme that underlies ageing also implies that it might be regulated by controlled epigenetic mechanisms making it operate as a clock. Indeed, cytosine methylation used as an epigenetic marker (by genomic mapping for 5-hydroxymethylcytosine) identified loci that are systematically altered during neurodevelopment and ageing. DNA methylation patterns were shown to influence transcriptional states and cellular identity during both development and ageing (Szulwach *et al.*, 2011). A global decrease of genomic DNA methylation with age has been reported (Numata *et al.*, 2012). In addition to global hypomethylation, a number of specific loci such as p16INK4a are known to become hypermethylated during ageing. Epigenetic plasticity in DNA methylation–controlled processes influences activity-dependent gene regulation (Martinowich *et al.*, 2003), learning and memory (Miller and Sweatt, 2007) in the central nervous system (CNS). Alterations in mechanisms of epigenetic programming can thus alter gene expression and induce functional behavioural changes in the ageing brain. The processes of epigenetic coding and orchestration of transcriptional changes during brain ageing are only beginning to be unravelled (Rando and Chang, 2012). Gene expression changes in the ageing brain thus most likely represent underpinnings that orchestrate the behavioural changes and cognitive decline that accompany brain ageing.

Inflammation as a hallmark of the ageing brain

Association of inflammation with ageing is generally acknowledged. It is interesting to note that several longevity polymorphisms associated with human ageing are mediators of inflammation. Various polymorphisms in inflammatory and immune response genes such as interleukin-6 (IL6), tumour necrosis factor alpha (TNFα), IFNγ, IL1β, TGFβ and C-reactive protein (CRP) have been shown to be associated with longevity in humans. A list of longevity candidates related to immune functions and their corresponding studies have been listed in Table 4.1. It would be very interesting to correlate the polymorphism data with functional expression of the protein involved *in vivo* to know the precise role of inflammation mediated by this protein in tissue ageing. Individuals producing low levels of pro-inflammatory cytokines (e.g. IL6 and IFNγ) or high levels of anti-inflammatory cytokines (e.g. IL10) have been associated with longevity, suggesting that an enhanced pro-inflammatory response is a risk factor for shortened lifespan (Jylhava and Hurme, 2010).

Chronic inflammation is also associated with increased risk for age-related cognitive decline and dementia in several species, including rodents (Gemma *et al.*, 2005) and humans (Dik *et al.*, 2005). Cognitive modalities affected by inflammation include learning (Hein *et al.*, 2010) and memory formation and consolidation (Frank *et al.*, 2010). Cytokines are known to act directly on

Table 4.1 Inflammatory genes and human longevity: Polymorphism studies. This table summarizes the studies correlating human lifespan to polymorphisms in inflammation or immune-associated markers (references mention corresponding publications). The studies were carried out in centenarian groups from various populations. Where information is available, the possible function of the polymorphism is also mentioned.

Gene name and symbol	Group studied	Experimental size and age group	Longevity correlation	Possible function of the polymorphism	References
Interleukin-6 (IL6)	Italian	700 individuals; 60 to 110 years of age (323 centenarians)	Homozygotes for the G allele decreases in centenarian males, but not in centenarian females. Negative association between the GG genotype of IL6 single-nucleotide polymorphism (SNP) and longevity in Italian centenarians.[2]	The −174 G/C SNP determines the transcription rate of IL6 mRNA.[3] Among males, homozygotes for the G allele have higher IL6 serum levels in comparison with carriers of the C allele.[1] The IL6 −174 GG genotype has been associated with higher plasma levels.[4]	1. Bonafe *et al.* (2001) 2. Di Bona *et al.* (2009) 3. Terry *et al.* (2000) 4. Olivieri *et al.* (2002)
Interferon gamma (IFNγ)	Italian	174 Italian centenarians > 99 years old and 248 < 60-year-old control subjects	The +874T allele, known to be associated with low IFNγ production, was found less frequently in centenarian women than in centenarian men or in control women, whereas no significant differences were observed in the distribution of the two alleles between male or female controls. Allele frequencies in centenarian men were not found to be significantly different from those of male controls.	The plasma levels of IFNγ bear a genetic regulatory component in which a 12 CA-repeat microsatellite allele in the first exon associates with elevated production *in vitro*.	5. Lio *et al.* (2002) 6. Pravica *et al.* (1999)

(*continued*)

Table 4.1 (*Continued*)

Gene name and symbol	Group studied	Experimental size and age group	Longevity correlation	Possible function of the polymorphism	References
	Finnish	285 people, aged 90 to 95 years	This polymorphism is in absolute correlation with the T allele at the +874 T/A SNP site, and this T allele has been observed to be less frequent in centenarian women than in the control group.[5] The frequency of allele G was higher in the survivors ($n = 114$) than in the non-survivors ($n = 171$).		
Tumour necrosis factor (TNF superfamily, member 2) (TNFα)	Mexican	71 healthy elders aged 80 to 96 years; 99 young people (from 21 to 54 years; mean age 35.2 years)	The TNF2 allele was increased in the elder group when compared to young controls.	Not known	Soto-Vega *et al.* (2005)
Major histocompatibility complex, class II (HLA-DRB1, HLA-DQA1, HLA-DQB1)	Japanese (Okinawa)	Polymorphisms in Okinawan centenarians were analysed.	The DRB1∗1401 allele was significantly increased in the centenarians, while the DRB1∗0101 and DRB1∗1201 alleles were slightly decreased. The DQA10101=0104 and DQA105 alleles were significantly increased in the centenarians. The DQB105 and DQB103 alleles were significantly increased in the centenarians.	Not known	Akisaka *et al.* (1997)

Toll-like receptor 4 (TLR4)	Sicilian		The G allele of this polymorphism has been associated with longevity.	The Asp299Gly (+896 A/G) functional polymorphism alters the receptor structure which leads to attenuation of the TLR4-mediated inflammatory response.	Balistreri *et al.* (2004) Arbour *et al.* (2000)
Heat shock 70 kDa protein 1B (HSPA1B)	Danish	426 participants of various ages	Female carriers of the GG genotype survive better than noncarriers.	Not known	Singh *et al.* (2006)
Complement factor H (CFH)			This allele has been correlated to increased mortality, particularly cardiovascular morbidity (Laine *et al.*, 2007).	The Tyr402His SNP (+1277 T/C) in CFH creates a functional 'proinflammatory' variant (402His) with markedly reduced CRP-binding capacity which predisposes the carriers to unbalanced and excessive inflammatory reactions via insufficient complement downregulation.	Jylhava. (2009) Laine *et al.* (2007)
Interleukin-10 (IL10)	Italian	190 centenarians (159 women and 31 men > 99 years old) and 260 control subjects (99 women and 161 men younger than 60 years)	The −1082G homozygous genotype was increased in centenarian men but not in centenarian women. No difference was found between centenarians and control subjects regarding the other two SNPs.	Associated with high IL10 production	Lio *et al.* (2002)
Interleukin-10 (IL10)	Italian	72 centenarian men, 102 centenarian women and controls (115 men and 112 women, aged 22–60 years)	The number of male centenarians homozygous for the −1082G genotype was significantly increased in comparison with younger control subjects. No significant differences were observed between women and controls.	Suggested to be associated with high IL10 production	Lio *et al.* (2003)

(*continued*)

Table 4.1 (*Continued*)

Gene name and symbol	Group studied	Experimental size and age group	Longevity correlation	Possible function of the polymorphism	References
Interleukin-10 (IL10)	Japanese	500 Japanese persons (mean age: 56.7 years old, range: 19–100)	There was a significant association of −819 T/C with age.	Not known	Okayama *et al.* (2005)
Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (NR3C1)	Dutch	402 men with a mean age of 77.8 years	After a follow-up of 4 years, 73 (19%) of 381 noncarriers died, while none of the 21 ER22/23EK carriers had died. Carriers may have lower C-reactive protein levels.		vanRossum *et al.* (2004)
Transforming growth factor, beta 1 (Camurati–Engelmann disease) (TGFB1)	Italian	419 subjects from northern and central Italy, including 172 centenarians and 247 younger controls	Significant differences were found at the +915 site as far as the C allele and GC genotype were concerned, both of them being lower in centenarians than in young controls, but none of the other tested genetic variants was significantly different between centenarians and controls. Moreover, a particular haplotype combination (G −800, C −509, C 869 and C 915) was notably lower in centenarians than in younger individuals.	Not known	Carrieri *et al.* (2004)

Note: Superscripted footnote numbers refer to numbered sources in the 'References' column.

neurons and affect their functions such as excitability and gene expression (Lisak *et al.*, 2011). Pro-inflammatory cytokines particularly down-regulate the neuronal genes involved in learning and memory (Godbout *et al.*, 2005). Prolonged inflammation and production of IL6 by astrocytes have been shown to suppress proliferation, survival and differentiation of neural progenitors (Vallieres *et al.*, 2002).

To date, the best documented link between neuroinflammatory cytokine production and cognitive impairment has been established for IL1β. Sustained IL1β overexpression in the hippocampus impairs contextual and spatial memory in mice (Hein *et al.*, 2010). IL1β is also demonstrated to be the mediator of lipopolysaccharide (LPS) and chronic stress–induced cognitive dysfunction (Terrando *et al.*, 2010). Also, increased hippocampal expression of IL1β results in age-induced impairment of long-term potentiation (Murray and Lynch, 1998). Caspase-1 is involved in pre-processing of mature IL1β. Transgenic mice lacking the expression of caspase-1 do not suffer loss of contextual memory upon ageing (Gemma *et al.*, 2005). In aged rats, IL1RA, the receptor antagonist for IL1β receptor, prevents *Escherichia coli*–induced suppression of long-term memory (Frank *et al.*, 2010). Other inflammatory mediators that have been most consistently linked to poor performance in individual cognitive capabilities, particularly in memory and executive functions, are CRP and IL6 (Schram *et al.*, 2007). Neuroinflammation-induced cognitive impairment may promote late-life depression disorders as inflammatory markers such as IL6 and CRP are also associated with depression in the elderly (Stewart *et al.*, 2009). Inflammatory markers thus show promising predictive potential for the onset of age-associated dementia (Ravalgia *et al.*, 2007).

Comparison of gene expression profiles from several species and independent studies showed that inflammation is a general hallmark feature of the ageing brain. To estimate the robustness of inflammation in the ageing brain, inflammation-associated genes were analysed in the expression profiles of aged mouse brain tissue. Of the up-regulated genes, a quarter could be assigned to an immune or inflammatory response in the neocortex and cerebellum. Transcriptional alterations varied only between the different brain regions. Up to three-quarters of the gene expression changes were at least partially prevented by calorie restriction. Remarkably, however, the effect of calorie restriction on age-associated alterations in gene expression was highly dependent on transcript class. Calorie restriction was largely shown to reverse changes in immune and stress response genes without affecting alterations in neuronal gene expression in the aged mouse brain (Lee *et al.*, 2000). Such inhibition of brain inflammation and increased production of trophic factors might underlie retardation of the brain ageing process by calorie restriction (Lee *et al.*, 2000). The prominence of inflammatory gene expression was also found in other gene expression studies addressing brain ageing of rats, mice and humans (Lee *et al.*, 2000; Lu *et al.*, 2004; Hickman *et al.*, 2013).

It is also notable that the age-related genes of neuronal origin are predominantly down-regulated whereas genes of glial origin are up-regulated during ageing. In this respect, it remains to be delineated if inflammatory components are glia derived in the ageing brain. It is possible that the process of brain ageing might affect neural and glial cellular compartments in different ways. It is also possible that ageing affects a particular cellular compartment of the tissue depending on the cell type–specific vulnerability, which could in turn cause responsive reactions in glial cells. It, however, remains to be elucidated whether inflammatory changes are intrinsic results of ageing in glial cells or responsive reactions to alterations in the post-mitotic neurons. In any case, it is plausible that a significant portion of the inflammatory gene expression in the ageing brain originates from the local innate immune cells of the brain.

Microglia

Microglia constitute the sentinel immune network of the CNS (Graeber and Streit, 1990). Until the past decade, the role of microglia in an immunologically silent environment was only anticipated. It is now known that microglial responses are diverse and depend on the context and nature of environmental stimuli. Microglia fulfil a variety of functions in the healthy adult CNS: patrolling the brain to detect pathogens, synaptic scanning to check neuronal health, influencing synaptic transmission and promoting adult neurogenesis. It is conceivable that the ageing brain requires considerable homeostatic support. More insight into the basic functions of microglia in the adult brain could aid in unravelling altered functions with ageing.

Surveillance and motility

In 2005, it was reported that the response of microglia to disruption of the blood–brain barrier (BBB) and brain injury could be mimicked by adenosine triphosphate. By using two-photon imaging, it was shown that microglia are highly active in the resting state and extremely vigilant of changes in the brain environment (Nimmerjahn *et al.*, 2005). Upon detecting an abnormality in tissue homeostasis, surrounding microglia are activated and target their branches towards the site of injury. Shielding of damaged sites by microglia may serve a neuroprotective role, as shown in an ischaemic brain model where microglial protrusions form a barrier between healthy and injured tissue (Wake *et al.*, 2009). The vigilant role that microglia play in a healthy adult CNS implies microglial function in continuous monitoring of the brain environment.

Microglial role in synaptic transmission

Microglia can sense synaptic activity through their neurotransmitter receptors, and overt release of neurotransmitters can trigger microglia activation

(Pocock and Kettenmann, 2007). Evidence from a facial nerve transection model suggested that microglia might be able to influence the adult neuronal network by altering synaptic transmission in a peripheral nerve transection model (Blinzinger and Kreutzberg, 1968). Activated microglia were shown to be involved in severing of afferent synaptic boutons from the surface of regenerating motor neurons, and this process was termed 'synaptic stripping'. Evidence for synaptic stripping by cortical microglia was demonstrated in a focal cortical inflammation model system in which activated microglia closely apposed neuronal perikarya and apical dendrites and were found to displace approximately 45% of the axosomatic synapses. Although it has been argued that the role of microglia in synaptic stripping is a case of 'guilt by association', more recent evidence using *in vivo* two-photon imaging indicates functional proof of the interaction of microglia, with neuronal synapses showing that the dynamic nature of microglia is directed in monitoring synapses. These contacts were also shown to be activity dependent, being reduced in frequency by reductions in neuronal activity followed by the disappearance of the presynaptic bouton (Wake *et al.*, 2009). High-resolution electron microscopy has also shown the participation of microglia in synaptic junctions along with astrocytes redefining the interaction as the 'quad-partite junction' (Schafer *et al.*, 2012). Major histocompatibility complex (MHC) class I or Ib antigens are required to regulate synaptic pruning on neuronal bodies that undergo retrograde degeneration after axonal transection (Shatz *et al.*, 2009). Synaptic stripping is now shown to be preceded by a decrease of synaptic activity. Furthermore, C1q, a complement component, has also been shown to mark synaptic boutons that require removal by microglia (Schafer *et al.*, 2012). Several modalities of microglial interactions with synapses were also found to be altered by sensory experience (light deprivation and subsequent exposure) in the visual cortex of juvenile mice. This raises the intriguing possibility that microglia may contribute to fine-tuning the plastic capacities of individual synapses in the healthy brain in accordance to experience and thus being capable of modulating crucial brain functions such as learning and memory (Tremblay *et al.*, 2010).

Microglia in the ageing brain

Upon encountering immune stimuli, microglia perform an orchestrated and controlled activation programme. Such immune stimuli may originate in the brain or come from the periphery. Activation of microglia in the healthy brain leads to an orderly beneficial inflammatory response, unless prolonged, overt or directed to self-antigens as in autoimmunity (Graeber and Streit, 2010). Aged microglia show increased immunoreactivity to CD68, a lysosome-associated phagocytic receptor, Toll-like receptors (TLRs), MHC class II antigen, OX6, the matrix-remodelling enzyme matrix metalloproteinase-12, Cd11b and Cd11c integrins and cytokines in the brain (as reviewed in Luo

et al., 2010). In the ageing brain, microglia have been proposed to acquire an over-reactive phenotype resulting in the exaggerated immune response called microglial 'priming'. Mounting evidence in several models of infection, injury and neurodegeneration indicates that the aged brain maintains a chronically increased level of inflammation. The best characterized and reproduced system for microglial priming is the response to peripheral infection. During a bacterial or viral infection, the neuroimmune axis comprising microglia communicates extensively with the peripheral adaptive and innate immune systems to induce sickness behaviour (Dantzer *et al.*, 2008). The onset of this behaviour is an adaptive response in the animal to restore homeostasis. Mimicking infection by LPS treatment showed higher inflammatory cytokine production in primary glial cultures established from the brain of aged animals compared to young adults (Xie *et al.*, 2003). *In vivo* studies with intraperitoneal injection of LPS or *E. coli* caused a prolonged and exaggerated cytokine response resulting in altered sickness behaviour in aged (22–24 months) Balb/c mice compared to young adults (Godbout *et al.*, 2005). Cytokine mRNA levels in microglia isolated *ex vivo* from aged mice showed increased expression of pro-inflammatory mediators such as IL1β, TNFα and IL6, suggesting that the increased neuroinflammatory gene expression in the ageing brain might originate from microglia (Sierra *et al.*, 2007; Henry *et al.*, 2009). It is for this reason that microglia from aged brain were suggested to be 'primed' or 'sensitized' for activation and may cause excessive bystander injury to the aged brain, preventing functional recovering in the event of injury or insult (Godbout and Johnson, 2006; Perry *et al.*, 2007).

Microglia priming has been demonstrated in relation to several secondary stimuli in an ageing background as compiled in Table 4.2. This has been shown, for example, in studies on 1-methyl- 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse models, used to study Parkinson's disease (PD), where aged animals were used. In an acute MPTP–PD model, old C57BL/6 mice (9–12 months old) were found to be more sensitive to neurotoxicity than young mice (3 months old), with more severe loss of dopaminergic neurons. Neurotoxicity observed in MPTP mouse therefore was shown to be age dependent (Sugama *et al.*, 2003). Intraperitoneal administration of MPTP to old C57BL6 mice (14–15 months old) led to a remarkable loss of dopaminergic neurons with a marked decrease in dopamine levels (Phinney *et al.*, 2006). Kainic acid–induced neurodegeneration and glial reactivity were also found to be more prominent in aged mice (Benkovic *et al.*, 2006). These examples and the other studies summarized in Table 4.2 clearly demonstrate that microglia activation might indeed be more exaggerated in an ageing background. This suggests diminished control of microglia activation during normal ageing.

Priming thus results from a shift of microglia towards the pro-inflammatory state also known as the 'classically activated' or M1 state. In macrophages another variant of activation, called 'alternative activation' or the M2 state, exists which supports wound healing and regeneration. In this context as brain

Table 4.2 Hyperactive inflammatory profile under different stimuli in the ageing brain. This table summarizes studies involving varied stimuli in rodent models to demonstrate immune activation in aged animals. Most models demonstrate immune hyperactivation in the aged brain.

Model system	Priming stimulus	Animal	Findings	References
Peripheral inflammation via infection	Intraperitoneal injection of *Escherichia coli* lipopolysaccharide (LPS) (0.33 mg/kg, serotype 0127:B8)	Rats aged 24 months	Exaggerated and prolonged sickness behaviour and weight loss in aged rats.	Barrientos *et al.* (2009)
	E. coli cultures (ATCC 15746)	Rats aged 24 months	Hippocampal functions such as memory of context, contextual fear, place learning and long-term memory consolidation are impaired in aged rats.	Barrientos *et al.* (2006)
	Intraperitoneal injection of *E. coli* LPS (0.33 mg/kg, serotype 0127:B8)	Male BALB/c mice aged 20–24 months	LPS-induced elevation in the brain inflammatory cytokines and oxidative stress were both exaggerated and prolonged compared with adults.	Godbout *et al.* (2005)
	Intraperitoneal injection of *E. coli* LPS (0.33 mg/kg, serotype 0127:B8)	Male BALB/c mice aged 20–24 months	Increased interleukin-1β (IL1β)-positive microglial cells and increased inflammatory response in the hippocampus of old mice. Cognition was affected in LPS-induced aged mice.	Chen *et al.* (2008)
	Intraperitoneal injection of *E. coli* LPS (0.33 mg/kg, serotype 0127:B8)	Male BALB/c mice aged 20–24 months	Depressive-like behaviour in aged LPS-treated mice was associated with a more pronounced induction of peripheral and brain indoleamine 2,3-dioxygenase and a markedly higher turnover rate of brain serotonin.	Godbout *et al.* (2008)
	Intraperitoneal injection of *E. coli* LPS (0.33 mg/kg, serotype 0127:B8)	Male BALB/c mice aged 18–20 months	Higher induction of inflammatory IL1β and anti-inflammatory IL10, IL1β, IL10, Toll-like receptor-2 (TLR2) and indoleamine 2,3 dioxygenase (IDO) mRNA levels in microglia isolated from aged mice than from adults. Increased MHC class II expression in aged microglia.	Henry *et al.* (2009)

(*continued*)

Table 4.2 (*Continued*)

Model system	Priming stimulus	Animal	Findings	References
	Intraperitoneal LPS (1 mg/kg, 3 h)	Mice aged 18 months	Aging microglia contained lipofuscin granules, decreased processes complexity, altered granularity and increased mRNA expression of both proinflammatory (TNFα, IL1b and IL6) and anti-inflammatory (IL10 and TGFβ1) cytokines.	Sierra *et al.* (2007)
Adjuvant arthritis (AA)	Intradermal injection of complete Freund's adjuvant	Rats	Proinflammatory microglia phenotype expressing ED1 and IL1β and deficits in the formation of LTP in the hippocampus of middle-aged rats.	Liu *et al.* (2012)
Viral infection	Intracranial injection of HIV-1 gp120	Male BALB/c mice aged 20–24 months	Behavioural deficits induced by gp120 were greater in aged mice than in adults.	Abraham *et al.* (2008)
Facial nerve axotomy	The right facial nerve was transected at the stylomastoid foramen.	Male F344/BN F1 rats aged 22–25 months	Age does not affect the glial response to axotomy in the lesioned facial nucleus.	Hurley *et al.* (2003)
Focal cerebral ischaemia	Middle cerebral artery (MCA) occlusion	MaleC57BL/Icrfat mice 26–31 months old	Cerebral oedema after ischaemia induction was seen in aged mice.	Fotheringham *et al.* (2000)
Intracerebral haemorrhage	Microinjection of autologous whole blood (15 mL) into the striatum	Sprague–Dawley rats aged 24 months	OX42-positive microglia with thick processes and swollen cytoplasm were more abundant in the haemorrhagic lesions in aged rats. Also, the expression of IL1b protein after ICH was greater in aged rats.	Lee *et al.* (2009)
Cortical stab injury	Introduce sterile 25-gauge needle through the skull approximately 2 mm lateral to bregma	Fisher 344r brown rats aged 36 months	Increased expression of IL1b, TNFα, IL6, ICAM1, inducible nitric oxide synthase (iNOS), metalloproteinase-9 (MMP9) and complement 3a-chain 1 in aged rats.	Kyrkanides *et al.* (2001)
Traumatic brain injury	Controlled cortical impact to the sensorimotor cortex	Mice aged 21–24 months old	mRNA expression of microglial markers was higher in the aged mice at all time points studied, and the resolution in aged mice was delayed.	Sandhir *et al.* (2008)
Traumatic brain injury	Microprocessor-controlled impact with a 3.5 mm diameter tip	Male C57Bl/6 mice aged 24 months	Aged mice had larger lesions associated with an M1–M2 balance switch and increased numbers of reactive (bushy and hypertrophic) MG–Mϕ in the cortex, hippocampus and thalamus.	Kumar at al. (2012)

Dopaminergic neurotoxicity	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was delivered unilaterally via the internal carotid artery.	8–9 years and 26.5–31 years female rhesus monkeys	Inflammation was evident at 3 months postlesion by severe microglial activation in the ipsilateral midbrain. HLA-DR fluorescence intensity and an abundance of activated microglia (based on morphological criteria) were consistently exacerbated in the SN of both sides of the midbrain.	Kanaan *et al.* (2008)
Rotenone induced Parkinsonism	Pumps infused 2.5 ml/h, and dosing was adjusted for each rat to receive approximately 1.5 mg/kg per day of rotenone for 28 days.	Four-month-old and 15-month-old rats	No effect on young rats, but led to a 20–30% reduction of tyrosine hydroxylase–positive neurons in the substantia nigra of older rats. May be associated with the increase of glial cell activation in older rats.	Phinney *et al.* (2006)
Kainic acid induced neurotoxicity	Single intraperitoneal injection. Adult animals were dosed with 35 mg/kg KA, while aged animals received a dose of 20 mg/kg.	Male C57BL/6J mice aged 20 months	Our data indicate widespread neuronal damage in the brains of KA-treated C57BL/6J mice; that damage is observed following mild behavioural seizure activity, and aged animals appear more sensitive to the effects of this excitotoxicant.	Benkovic *et al.* (2006)
Taupathy model	Tg mouse lines expressing human wild-type tau (line WT16) or P301S tau (lines PS5 and PS19) P301S Tgmice with FK506 attenuated tau pathology	9–12 months of age	Filamentous tau lesions developed in P301S Tg mice at 6 months of age, and progressively accumulated in association with striking neuron loss as well as hippocampal and entorhinal cortical atrophy. Prominent microglial activation also preceded tangle formation. Importantly, immunosuppression of young and increased lifespan, thereby linking neuroinflammation to early progression of tauopathies.	Yoshiyama *et al.* (2007)
PS1xAPP mice model	Double tg mouse overexpressing amyloid beta	2–18 months	Alternative activation state with phagocytic capabilities (at 6 months) to a classic cytotoxic phenotype (expressing TNF and related factors) at 18 months of age.	Jimenez *et al.* (2008)

tissue macrophages, it is interesting that microglia from aged mice were found to resist skewing towards the M2 phenotype in the presence of IL4 (Fenn *et al.*, 2012). Aged microglia also resist 'de-activation' signals like CX_3CL1 and TGFβ (Norden and Godbout, 2012). Furthermore, aged rats showed increased amounts of IFNγ, an inducer of the M1 phenotype, and decreased levels of IL4, an inducer of the M2 phenotype (Maher *et al.*, 2004; Nolan *et al.*, 2005). An age-dependent phenotypic shift from M2 to M1 microglial activation might occur in mouse models of AD, such as the APP–PS1 mouse, owing to accumulation of soluble amyloid beta (Aβ) oligomers and neuronal loss (Jimenez *et al.*, 2008). Additional evidence from the APP mouse model has shown that M2 skewing of microglia facilitated Aβ clearance (He *et al.*, 2001; Yamamoto *et al.*, 2007).

On one hand, while microglia priming is believed to underlie age-related cognitive impairment and predisposition to neurodegenerative conditions, another school of thought suggests age-related microglial dysfunction. This idea is predominantly based on morphological observations in human post-mortem brain samples and suggests that a subpopulation of microglia in aged and diseased brain exhibits signs of de-ramification and cytoplasmic degeneration termed 'microglial dystrophy'. Dystrophic microglia possess morphological characteristics of cytoplasmic spheroid formation containing phagocytic-intake material and have partially or completely broken processes (Streit, 2006) (Figure 4.1). Indeed, the presence of dystrophic microglia has been found to precede age-related neurodegeneration in senescence-accelerated mice models of ageing (Hasegawa-Ishii *et al.*, 2011). Dystrophic microglia were also found to be associated with tau pathology preceding neurodegeneration in AD (Streit *et al.*, 2009). In R6/2, a transgenic mouse model of Huntington's disease, the presence of dystrophic microglia and a consequent decreased microglial density have been observed (Ma *et al.*, 2003). These morphological observations thus suggest that microglia lose their neuronal supportive functions with age, resulting in age-associated neurodegeneration (Streit *et al.*, 2009).

Isolated microglia from aged mice were shown to constitutively secrete greater amounts of IL6 and TNFα relative to microglia from younger mice. This seems a counter-argument for dystrophy. Also, microglia from aged mice were shown to internalize less Aβ peptide and were found to accumulate Aβ rather than degrade it. These studies proposed the idea that microglial cell function changes with ageing and that compromised functionality is a hallmark of microglial 'dystrophy' (Njie *et al.*, 2012). The trophic support by ageing microglia might also be compromised. For instance, in the senescence-accelerated mouse model, SAMP10 was found to secrete unusually low amounts of osteopontin (OPN) after kainic acid treatment compared to senescence-resistant SAMR1 mice. CD44, an OPN receptor, was expressed on neurons and astrocytes, and the OPN–CD44 signalling axis was proposed to have neuroprotective benefits in the aged brain (Hasegawa-Ishii *et al.*, 2011).

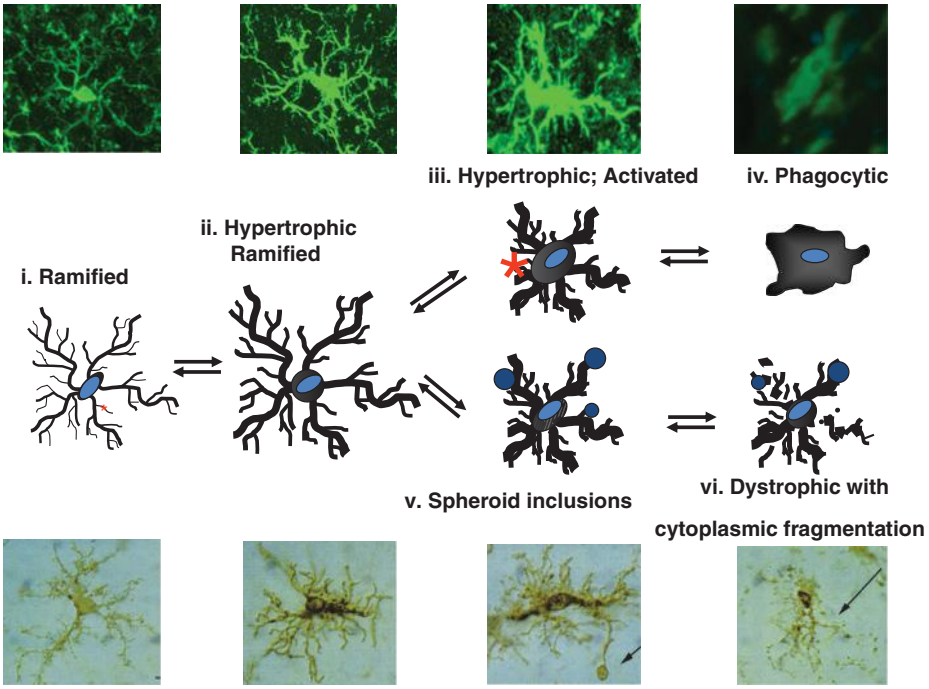

Figure 4.1 Morphological characteristics of primed and dystrophic microglia. Microglia progress through different stages of activation which start with the acquisition of hypertrophic morphology. Hypertrophy is characterized by thickened processes and enlarged cell body (ii). This morphological stage has been shown in both activation and dystrophy. After this stage, microglia might acquire an amoeboid morphology (as in iv) with retrieval of processes (iii) or accumulate spheroid structures in their hypertrophic processes (v), culminating in fragmentation of processes (vi). Stages iv and vi are unique to activation and dystrophy, respectively. *Note the increased cell body size and thickened processes in the intermediary stages of activation and dystrophy. These morphological changes along with functional assays can distinguish activated from dystrophic microglia.

The concepts of 'priming' and 'dystrophy' have entirely different implications for microglial ageing phenotype and function in terms of tissue support and inflammatory damage (Figure 4.2). Furthermore, the two hypotheses offer opposing strategies for therapeutic interventions. Understanding the exact ageing phenotype and functionality of aged microglia and the underlying mechanistic mediators is therefore of utmost importance.

Microglia in the neurogenic niche

Neurogenesis is the process of generating functional neurons that integrate into the neuronal network from neural precursors. It was shown that the process occurs throughout life in two restricted brain regions in the mammalian

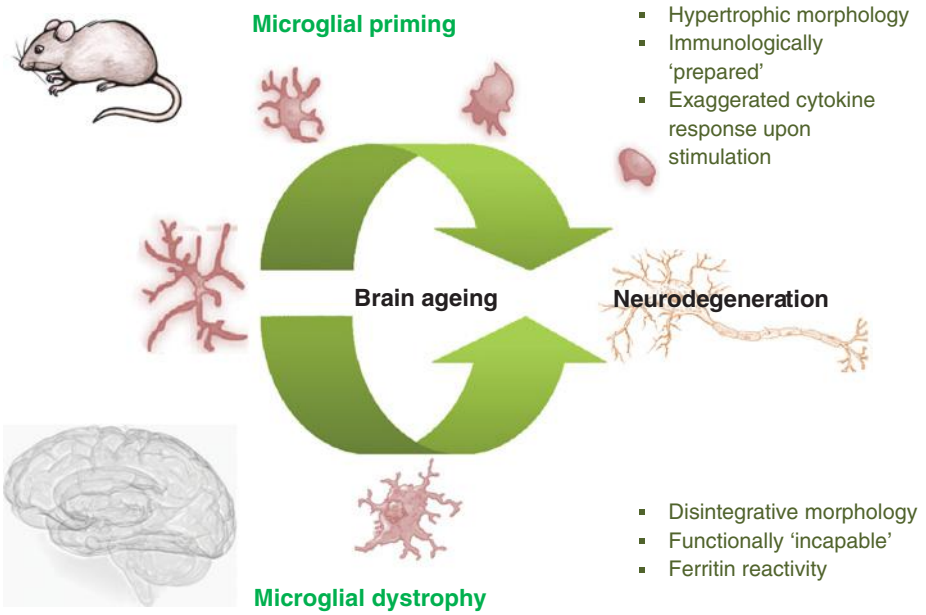

Figure 4.2 Theories of microglia ageing in the brain. Microglia priming has been described in several mouse models and is seen as an intermediate state of alert to microglial activation. Progressive switch of priming to activation might result in neurodegenerative conditions. Predominantly from human studies, microglial dysfunction and dystrophy are also proposed to cause neurodegeneration. Characteristics of microglial priming and dystrophy have been shown.

adult CNS: the subventricular zone (SVZ), which lines the lateral ventricles, and the subgranular zone of the hippocampal dentate gyrus (DG) (Ming and Song, 2005). Depletion of new neurons leads to impairment in hippocampus-dependent cognitive function showing that the newly formed neurons are necessary for the proper cognitive functioning of the brain (Deng *et al.*, 2010).

Ageing affects neurogenesis in a profound manner (Drapeau *et al.*, 2008), although the precise reason for this is not known yet. Two major factors have been proposed to underlie decreased neurogenesis during ageing. Studies show a decrease in proliferation of neural progenitors during ageing in the DG (Kempermann *et al.*, 1998) and SVZ of rodents (Molofsky *et al.*, 2006). The nature of differentiation of aged neural progenitors is biased towards astrocytes rather than neurons (Kempermann *et al.*, 1998). Another major factor that has been suggested to be involved in decreased neurogenesis is the altered 'microenvironment' of ageing neural stem cells. Previously published studies employing infusion of LPS in the brain (Ekdahl *et al.*, 2003) and irradiation of brain (Monje *et al.*, 2003) have highlighted the relationship between inflammation and neurogenesis. LPS-induced inflammation caused

an 85% reduction in the number of new-born neurons with a corresponding increase in the number of ED1+ microglia in the young rat. Treatment with nonsteroidal anti-inflammatory drugs (NSAIDs) could abrogate neuroinflammation and restore neurogenesis to near basal levels (Monje *et al.*, 2003). The mechanism by which inflammation reduces neurogenesis is, however, not by affecting the proliferation of neural stem cells but by reducing the survival of differentiated new-born neurons as observed in long-term BrDU follow-up studies (Ekdahl *et al.*, 2003; Monje *et al.*, 2003).

Microglia have been shown to be modulators of neurogenesis. In the environmental enrichment paradigm, a significant increase in the number of MHC class II–positive hippocampal microglia expressing the neurotrophic factor insulin growth factor-1 (IGF1) was found to have a beneficial role for adult neurogenesis. This increase was attenuated by chronic treatment with minocycline (Ziv *et al.*, 2006). The influence of microglia on neurogenesis was attributed to interferon gamma (IFNγ) and subsequent T cell–microglia interactions (Ziv *et al.*, 2006). Microglia were also shown to play a neurogenesis-supportive role in adrenalectomized rodents via expression of the anti-inflammatory cytokine transforming growth factor beta (TGFβ1).

Considering the relationship between inflammation and neurogenesis and the evidence that microglia modulate neurogenesis, the following studies begin to unravel the possible role of microglia in the aged neurogenic niche. Inhibition of caspase-1, which is an enzyme critically important in the processing of IL1β, abrogates loss of hippocampal neurogenesis associated with ageing and improves hippocampal-dependent memory (Gemma *et al.*, 2005). Adrenalectomy of 10-month-old (middle-aged) rats has been shown to prevent age-related hippocampal neurogenesis and cognitive decline in aged rats (23 months) (Cameron and McKay, 1999).

Fractalkine signalling, which is suppressed upon ageing, leads to an increased microglial activation state and decreased neurogenesis. Fractalkine infusion in aged rat brain was accompanied by a decrease in microglial MHC II expression and restored hippocampal progenitor cell proliferation in aged animals, suggesting that fractalkine modulates at least in part hippocampal neurogenesis in ageing (Vukovic *et al.*, 2011). Other possibilities by which microglia could influence the ageing neurogenic niche include its role in providing trophic support. Indeed, levels of growth factors known to promote neurogenesis, including fibroblast growth factor-2 (FGF2), IGF1, and vascular endothelial growth factor (VEGF), were shown to be significantly reduced in aged rats compared to young rats (Shetty *et al.*, 2005). In summary, the chronic pro-inflammatory state induced by aged microglia in the hippocampal neurogenic environment, lack of trophic support and altered neural progenitor–microglia interactions could cause or contribute to decreased neurogenesis in the aged brain.

Changes in regulation of microglial activation with ageing

It is well known that microglia encounter 'on and off' signals from their environment that regulate their state of activation (Biber *et al.*, 2007). Thus environmental cues, including CD200-CD200R signalling (Hoek *et al.*, 2000) and the fractalkine (CX_3CL1)–fractalkine receptor (CX_3CR1) pathway (Maciejewski-Lenoir *et al.*, 1999), are involved in keeping microglia in a quiescent state. CD200R decreases upon ageing on microglia surface (Lyons *et al.*, 2007). Lowering of CD200R mRNA was found to correlate with increased MHC class II expression (Lyons *et al.*, 2007). While CX_3CL1 expressed on neuronal surface is involved in maintenance of microglia quiescence, as shown by the enhanced activation in $CX_3CR1{-}/{-}$ mice (Cardona *et al.*, 2006), a soluble, cleaved form of fractalkine signals the onset of monocyte infiltration into the brain (Soriano *et al.*, 2002). Fractalkine protein was found to decrease in the brain of aged mice (Wynne *et al.*, 2010). In aged animals, the recovery of fractalkine signalling after encountering an activation signal in microglia seems to be compromised, leading to prolonged activation (Wynne *et al.*, 2010).

Other neuroglia signalling factors include glucocorticoid, cannabinoid and neurotransmitter signalling such as GABA. Recent studies indicate that there is decreased glucocorticoid receptor expression on astrocytes in the hippocampus of older rats (Kasckow *et al.*, 2009). These deficits may be associated with impaired negative feedback of glucocorticoids in the aged brain (Mizoguchi *et al.*, 2009). Microglia also express receptors for neurotransmitters (Pocock and Kettenmann, 2007). GABA, an inhibitory neurotransmitter, attenuated IL1β production by interacting with $GABA_B$ receptors on microglia (Kuhn *et al.*, 2004). Collectively, these data show that impaired immunoregulation in the aged brain by neurons may underlie the enhanced neuroimmune response.

Neural contribution to age-associated brain inflammation

It is becoming increasingly clear that neurons have cell-autonomous mechanisms of sensing danger using an array of immune sensor machinery. The best characterized innate immune receptors that mediate pro-inflammatory signalling are TLRs, which activate nuclear factor kappa B (NF-κB) and/or signal transducer and activator of transcription (Stat) signalling pathways to induce the production of mostly cytokines and chemokines (Iwasaki and Medzhitov, 2004). Neurons and neural progenitor cells express several TLRs, including TLR2, 3, 4, 7/8, and 9, and their functions include regulating neurite outgrowth, adult neurogenesis and cognition (Okun *et al.*, 2011).

Ageing of the brain is associated with changes in the expression of innate immune receptors. The expression of TLR1, 2, 3, 4, 5, 6 and 7 and CD14 was found to be up-regulated during ageing (Letiembre *et al.*, 2007). TLRs are located intracellularly in the endoplasmic reticulum (ER) and in lysosomes (Matsumoto *et al.*, 2003; Leifer *et al.*, 2004), and they are capable of recognizing damage-associated molecular patterns (DAMPs) from within the cell. Other innate immune sensors, nucleotide-binding oligomerization domain–like (NOD-like) receptors (NLRs), can trigger autophagy to rid the cell of accumulating misfolded proteins, aged mitochondria and so on. Hence it is possible that organellar dysfunction such as Lipofuscin-loaded leaky lysosomes or DAMPs generated during neuronal ageing can activate TLR signalling within aged neurons in a cell-autonomous fashion. Other studies have elucidated that well-known immune molecules have specific roles in neuronal ageing and immune activity. For instance, expression of receptor for advanced glycation end products (RAGE) in neurons resulted in abnormalities in learning and memory, synaptic transmission deficits and cognitive impairment in an Aβ-overexpressing background. Overexpression of a dominant negative form of RAGE in neurons, however, led to decreased neurodegeneration and preservation of learning and memory in an Aβ-overexpressing background (Arancio *et al.*, 2004). Another study showed that abrogation of TGFβ signalling by targeted expression of a dominant-negative TGFβ type II receptor in neurons led to increased amyloid accumulation and neurodegeneration in a mutant APP transgenic mice model of AD (Tesseur *et al.*, 2006).

Complement components of the classical and alternative pathway are another class of immune mediators that show altered expression during brain ageing. The mRNA of several complement components (C4, C1qa, C1qb and C1qc) showed increased expression during ageing (Reichwald *et al.*, 2009). However, final mediators of the complement cascade pathway such as C9, although absent in physiological ageing, are produced upon additional pathological injury such as intracerebral haemorrhage in aged rats in an exaggerated manner (Gong *et al.*, 2008). It is however, not completely clear if complement proteins in the ageing brain are produced by ageing neurons or glia.

Role of astrocytes in age-associated neuroinflammation

Astrocytes interact closely with neurons to maintain the ideal ion and metabolic homeostasis, and thereby affect neuronal activity in a significant manner. Astrocytes have also been proposed to have immune functions (Dong and Benveniste, 2001). Surprisingly, changes in astrocytes during ageing have been addressed only recently. Hypertrophic astrocytes with thicker, shorter branches were found in aged rats. They were also found to decrease in number with ageing and found in close association with apoptotic neurons of the aged

brain (Cerbai *et al.*, 2012). Astrocytes were shown to acquire a senescence-associated secretory phenotype (SASP) with age characterized by increased levels of intermediate glial fibrillary acidic protein (GFAP) and vimentin expression, cytokines and accumulation of protein aggregates. Isolated astrocytes from aged brain display the pro-inflammatory phenotype and have been proposed to contribute to neuroinflammation in the ageing brain (Salminen *et al.*, 2011). Loss of trophic support due to decreased availability of astrocyte-derived VEGF and FGF2 factors may contribute to the age-related decline in neurogenesis (Bernal and Peterson, 2011).

Immune cells in the aged brain

The BBB acts as the first wall of defence restricting the entry of leukocytes into the CNS (Bechmann *et al.*, 2007). Alterations of the BBB integrity and permeability have been reported in the normal ageing brain (Lee *et al.*, 2012). Also, tight junction proteins such as occludin and claudin-5 were found to be increased in the aged CNS (Lee *et al.*, 2012). Alterations of a similar nature have been noted in cerebral microvascular pathology in humans (Farrall and Wardlaw, 2007). Entry of leukocytes is also facilitated by active migration making use of adhesion molecules such as chemokine receptors and integrins. Integrins such as ICAM1 and chemokines such as monocyte chemotactic protein (MCP1 and CCL2) and macrophage inflammatory protein-1 (MIP1α) are important mediators of leukocyte migration and are up-regulated in activated glia (Aloisi *et al.*, 2000). During brain ageing, the expression of these chemokines has been shown to be up-regulated (Kumagai *et al.*, 2007)

Dendritic cells are professional antigen presenters and mediators of adaptive immune response. In the normal brain, they are found in the meninges and choroid plexus but not parenchyma of young subjects (Bechmann *et al.*, 2007). Immunostaining using CD11c, however, showed an age-dependent accumulation in the brain (Stichel and Luebbert, 2007). Recent studies also suggest that infiltrating monocytes may originate from a subpopulation of CD11c monocytes when TGFβ signalling is impaired. These cells appear to infiltrate and clear Aβ in a mouse model of AD (Town *et al.*, 2008). Immunostaining with the pan–T cell marker CD3 identified T cells in the brain parenchyma of aged mice (Stichel and Luebbert, 2007). There are no conclusive studies of CD11c-positive cells or T cells in the aged human brain, although a few studies employing aged control samples without neurological disease have shown the presence of T cells (Togo *et al.*, 2002). Brain infiltration of T cells has been shown to increase upon cytokine exposure (Xu *et al.*, 2010). Interestingly, in the facial nerve axotomy model, T cell infiltration was amplified in the axotomized nucleus of aged brain (Dauer *et al.*, 2011). T lymphocyte infiltration in the brain has been discussed in a neuroprotective context previously (Carson *et al.*, 2006). It remains to be determined if age-associated T cell accumulation in the aged brain is beneficial or detrimental.

Implications of altered neuroinflammation for the ageing brain

The ageing brain is characterized by a shift from a homeostatic balance to a pro-inflammatory state. This increase in neuroinflammation is marked by increased expression of immune markers, cytokines and other inflammatory mediators. These conditions have been shown to sensitize the aged brain to produce an exaggerated response in the presence of an immune stimulus in the periphery or following exposure to a stressor. In the brain, pro-inflammatory cytokines exert profound effects on behaviour. Typically after an immune stimulus, aged animals display prolonged sickness behaviour, increased cytokine induction and cognitive impairment. Gene expression profiling shows that a significant fraction of gene expression belongs to glia-derived immune and inflammatory mediators. Microglia are important mediators of the brain's response to trauma, disease and infection. An altered microglial inflammatory profile may therefore underlie the increased neuroinflammation and heightened reactivity of the aged brain. Microglial age-related alterations hence might affect the way the aged brain responds to and recovers from insult. It is therefore of importance to understand ageing-associated changes in microglia and if the functional changes in microglia contribute to altered neuroinflammation in the ageing brain.

It has been proposed that the underlying molecular mechanisms between ageing and neurodegenerative conditions might merely vary in extent, thus making "physiological and pathological brain aging as points on a spectrum varying with age" (Cao *et al.*, 2010). However, this spectrum of changes might be switched to a neurodegenerative state by certain 'precipitating events' during the course of ageing, as proposed by Herrup (2010). One instance of a 'precipitating event' previously proposed to increase the risk of developing AD and worsen the course of ongoing neurodegeneration is the presence of episodes of infection (Perry *et al.*, 2007). The difference in neurotoxicity of Aβ with age gives the idea that it is the nature of the inflammatory response of the ageing brain to the stimulus and not the stimulus itself (in this case, Aβ) that might lead to neurodegeneration. It is therefore likely that neuroinflammatory events in the aged brain initiate or even aid in the progression of AD (Bales *et al.*, 2000; Heneka and O'Banion, 2007). In accordance, several genetic modulation studies of immune and inflammatory pathways render beneficial responses in terms of development of plaque pathology and progression of AD-like disease in mouse Aβ-overexpressing models (reviewed in Wyss-Coray, 2006). Thus the ageing background, precipitating injury and altered immune response to such injury may underlie the initiation and progression of neurodegeneration. Neuroinflammation might thus be more than just an 'epiphenomenon' in the understanding of brain ageing and neurodegeneration.

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5

Brain Repair: The Role of Endogenous and Transplanted Neural Stem Cells

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Introduction

Inflammation and degeneration are typical pathological processes occurring in the central nervous system (CNS) of patients with chronic irreversible neurological conditions. They are only apparently separate processes because, as soon as the pathological process becomes chronic, they have the tendency to become strictly interrelated (Martino *et al.*, 2002, 2011; Martino, 2004). Therefore, primary neurodegeneration triggers secondary inflammatory reactions, while primary inflammatory reactions lead to secondary neurodegeneration.

In the last 50 years, it has become clear that the CNS displays intrinsic 'constitutive' tissue repair ability to prevent the irreversible tissue damage occurring as a consequence of chronic inflammatory and/or degenerative processes. Several molecular and cellular events sustaining innate brain repair mechanisms have been described. On one hand, humoral and cellular inflammatory components shift their function over time, from a tissue-damaging mode to one promoting tissue repair. Not only pro-inflammatory cytokines may, under certain circumstances, act as anti-inflammatory molecules, but also

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pro-inflammatory mononuclear cells may promote neuroprotection by secreting neurotrophic factors (Martino *et al.*, 2002). On the other hand, the recruitment of alternative 'non-damaged' functioning neuronal pathways – occurring also via axonal sprouting and synaptogenesis – takes place as a consequence of brain damage. Whether or not this process is paralleled by central axon regeneration (i.e. an axon growing back along the distal stump of a crushed or transected nerve to re-innervate its normal target) is still a matter of intense debate (Tuszynski and Steward, 2012). Finally, endogenous neural stem and precursor cells – the self-renewing and multipotent cells of the CNS capable of driving neurogenesis and gliogenesis in adult life – may adapt targeted migration into damaged areas, to promote tissue repair and regeneration via cell replacement and/or the so-called bystander (paracrine) effect, which is due to the capacity of such cells to constitutively secrete neurotrophic and anti-inflammatory molecules (Martino *et al.*, 2011). However, 'protective' mechanisms reactive to CNS damage are not strong enough to promote the full recovery of cyto-architecture in most of the chronic inflammatory and degenerative neurological disorders. This evidence supports the ensuing view that irreversible neurodegeneration might be a consequence of the failure of CNS intrinsic repair mechanisms. Thus, it is believed that fostering and/or resetting 'spontaneous' regenerative process may lead to more efficacious, and less toxic, therapies.

Given that stem cells are an integral part of the mechanisms sustaining CNS tissue repair processes, strategies that mobilize neural progenitor cells (NPCs) or *in vivo* transplantation of NPCs might represent promising therapeutic approaches to foster intrinsic repair mechanisms and promote neuroprotection (Martino *et al.*, 2010; Pera and Tam, 2010). In this chapter, we will focus our attention on the self-renewal and differentiation potential and on the recently discovered homeostatic constitutive functions of endogenous NPCs as prerequisites to develop NPC-based transplantation strategies aimed at promoting CNS repair. At the same time, we will also discuss the ability of transplanted NPCs to adapt behaviour and fate in response to the CNS microenvironment and how this behaviour culminates in protection of the CNS from pathogenic signals, the concept of 'therapeutic plasticity'.

The homeostatic regulatory role of endogenous NPCs

In the adult rodent CNS, neurogenesis continues for the rodent's entire life mainly in two regions, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Alvarez-Buylla and Lim, 2004; Zhao *et al.*, 2008). Newly formed adult NPCs of the SGZ migrate short distances and differentiate into dentate granule cells

(DGCs) within the dentate granule cell layer (Kallur *et al.*, 2011). These newly formed DGCs migrate into the granule cell layer to become indistinguishable from pre-existing cells and are necessary for modulating and refining the existing neuronal circuits involved in hippocampus-dependent memory processing and behaviour (Imayoshi *et al.*, 2008; Kitamura *et al.*, 2009; Singer *et al.*, 2011). In the rodent, however, SVZ NPCs migrate along the rostral migratory stream (RMS) to the olfactory bulb where they integrate within the granule and glomerular cell layer in order to maintain and reorganize the olfactory bulb system (Imayoshi *et al.*, 2008).

However, recent evidence challenges the sole and exclusive view that the neurogenic CNS areas mainly act as a source of newly formed neurons, able to replace neuronal cells in the hippocampus and in the olfactory bulb (Imayoshi *et al.*, 2008). As a matter of fact, adult NPCs residing within germinal niches might exert other 'non-neurogenic' homeostatic regulatory functions alternative to cell replacement during either a healthy state or pathology.

Apart from a recent report showing that neurogenesis in the SGZ exerts an antidepressant activity by regulating (via glucocorticoid release buffering) the hypothalamus–pituitary axis (Snyder *et al.*, 2011), evidence collected so far suggests that SVZ represents the more 'strategic' area where NPCs might exert a homeostatic role in addition to neurogenesis. This is because, on one side, these cells are in communication with two different microenvironments, tightly apposing blood vessels and in contact with the cerebrospinal fluid (CSF) through apical processes (Sawamoto *et al.*, 2006; Rolls *et al.*, 2007; Mirzadeh *et al.*, 2008; Tavazoie *et al.*, 2008). On the other side, the SVZ is very close to crucial areas of the midbrain (e.g. the basal ganglia and striatal structures) containing GABAergic neurons capable of efficiently regulating and modulating interconnections between several cortical and sub-cortical brain areas (Koos and Tepper, 1999).

As a matter of fact, SVZ NPCs protect striatal neurons from glutamatergic excitotoxicity, as seen in the early phase of ischaemic stroke and epilepsy, by releasing endogenous endocannabinoids, N-arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2AG) capable of binding to their specific receptors (CB1 and CB2) (Butti *et al.*, 2012). This NPC-mediated operational way of acting is tuned up during CNS-compartmentalized inflammatory insults. It is thus tempting to speculate that endogenous SVZ NPCs act as guardians of the brain, by sensing danger signals coming from the periphery and responding by down-modulating glutamatergic excitotoxic currents, whose deregulation might, in turn, be noxious for proper functioning of brain cells (see Figure 5.1). The above-mentioned strategic positioning of SVZ NPCs within the CNS supports this novel view while questioning the positioning as just representing a developmental relic. In addition, SVZ NPCs have been shown to be capable of exerting a physiological phagocytic activity that requires intracellular engulfment protein, ELMO1, to promote

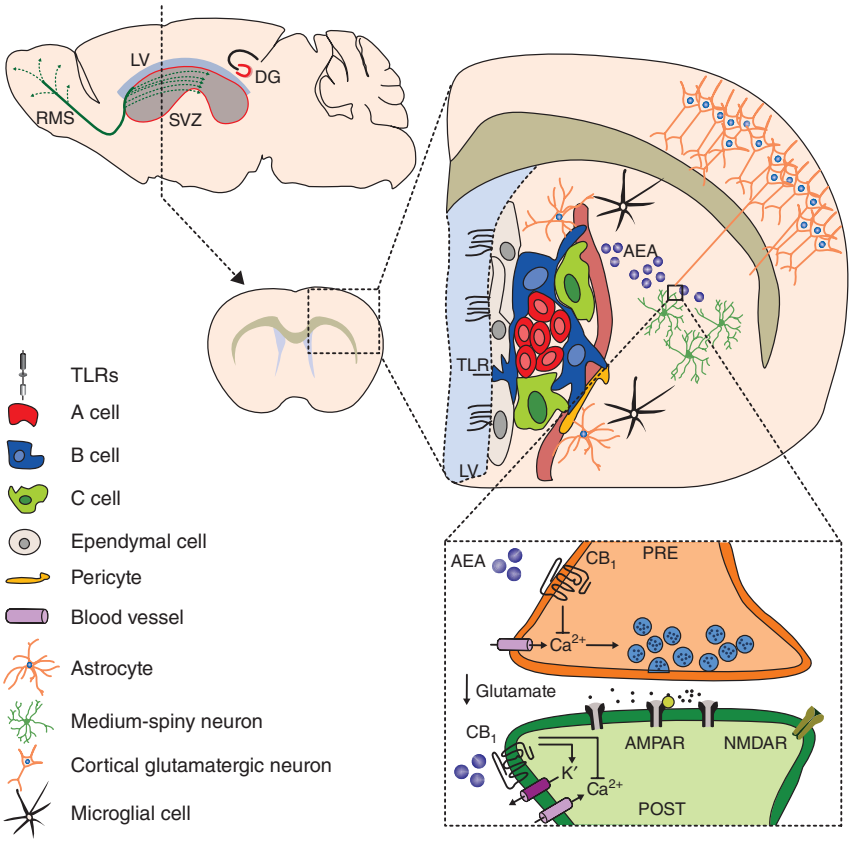

Figure 5.1 Structural and functional characteristics of NPCs in the SVZ. Schematic representation of the functional homeostatic role exerted by NPCs in the SVZ and striatum. SVZ NPCs may sense danger signals, coming from either the CSF or the blood, via Toll-like receptors (TLRs). TLR4 is triggered by several types of endogenous and exogenous danger signals, including those released from neural cells stressed by glutamate-induced excitoxicity (e.g. heat shock protein 70, saturated fatty acids, high-mobility group box 1 proteins (HMGB1) and β-defensins). As a consequence of such triggering, SVZ adult neural precursor cells increase the production of the endogenous cannabinoid AEA (blue dots) which, in turn, binds to pre- and post-synaptic CB1 receptors. CB1 receptor binding decreases Ca^{2+} content within the presynaptic compartment, thus reducing glutamate release within the synaptic space. This regulatory mechanism reduces post-synaptic glutamatergic currents and glutamatergic-mediated excitotoxicity, thus representing an innate protective mechanism.

Rac activation downstream of phagocytic receptors (Lu *et al.*, 2011). Finally, SVZ NPCs may mediate suppression of high-grade astrocytomas (HGA) by releasing endovanilloids that activate the transient receptor potential vanilloid subfamily member-1 (TRPV1) on HGA cells, triggering their death and thus prolonging overall survival time (Stock *et al.*, 2012).

While possibly explaining the role of SVZ NPCs in non-human primates and humans, where the presence of a fully formed RMS is not yet substantiated (Sanai *et al.*, 2004, 2007), the homeostatic role of SVZ NPCs is also supported by recent evidence indicating a long-lasting neurogenic response reactive to injury. This has been observed in both patients (Marti-Fabregas *et al.*, 2010) and animal models (Bengzon *et al.*, 1997; Parent *et al.*, 1997; Jin *et al.*, 2001, 2010; Goings *et al.*, 2004) affected by neurological diseases, but it is not yet confirmed whether such approaches replace damaged and/or dead cells. In stroke, about 80–90% of newly formed SVZ-derived cells die within a few weeks without integrating into the spared neuronal circuitries (Arvidsson *et al.*, 2002), but they seem to protect against tissue injury through the secretion of neurotrophic factors in ischaemic CNS areas (Jin *et al.*, 2010). In the toxin-induced cuprizone model in which demyelination of the corpus callosum is induced, less than 4% of newly formed SVZ-derived cells differentiate into myelinating oligodendrocytes (Menn *et al.*, 2006). However, SVZ-derived newly formed $NG2^+$ cells can still promote remyelination, by forming functional glutamatergic synapses with demyelinated axons (Etxeberria *et al.*, 2010). In experimental epilepsy, SVZ-derived cells migrating towards the hippocampus terminally differentiate into glial but not neuronal cells (Parent *et al.*, 2006). This is likely to exert a protective effect because in temporal lobe epilepsy newly born neurons aberrantly migrate and integrate in the dentate hilus, exacerbating the hippocampal epileptic activity (Hattiangady and Shetty, 2008).

Although the replacement of damaged cells seems not to be the prevailing and sole mechanism of reactive neurogenesis occurring in response to tissue damage (at least in the SVZ), it is very likely that the specific characteristics of the pathological environment influence the behaviour of SVZ-derived newly formed cells. As such, inflammation occurring as a consequence of autoimmunity and/or traumatic and ischaemic injuries has been variably shown to alter NPC proliferation and differentiation characteristics in a non-cell-autonomous fashion. On the other hand, when inflammation fades away and neurodegeneration prevails, endogenous NPCs tend to differentiate into multiple neuronal lineages, depending on the situation, that are partly capable of integrating into damaged neuronal circuits (Kokaia *et al.*, 2012). The increase in the numbers of microglia cells within the SVZ neurogenic area during inflammatory CNS conditions (Pluchino *et al.*, 2008) supports this working hypothesis, as does recent evidence emerging from NPC transplantation studies, which will be discussed in detail in this chapter. Briefly, while remaining undifferentiated, transplanted SVZ-derived NPCs might promote CNS tissue healing via the secretion of immunomodulatory and neuroprotective molecules capable of reducing detrimental tissue responses, the so-called bystander effect (Ourednik *et al.*, 2002; Pluchino *et al.*, 2003; Martino and Pluchino, 2006a; Bacigaluppi *et al.*, 2009a,b).

NPC transplantation as a therapeutic tool to promote brain repair

As already anticipated, CNS pathology results in loss of either neural or glial cells and disrupts the cyto-architecture of the tissue that is defined during development, when neurons and glia precociously acquire specific positional identities (Hochstim *et al.*, 2008; Sauka-Spengler and Bronner-Fraser, 2008) and their integrity is required for correct CNS functioning (Eroglu and Barres, 2010). Therefore, cell replacement strategies would represent an ideal therapeutic approach for CNS disorders, owing to the fact that it not only aims at substituting dead cells with newly differentiated ones but also promotes functional integration into neural circuits of newly formed cells (Ingber and Levin, 2007; Poss, 2010).

With this in mind, several cell replacement–based therapeutic strategies have been established. The results, obtained so far, reveal that this approach does represent a rational and realistic therapeutic strategy for only a restricted category of neurological disorders, and the success of this strategy is very much dependent on the cell type to be substituted and on the CNS region damaged. Cell replacement is efficacious in disorders in which degeneration is caused by either intrinsic cellular defects of, or extrinsic factors that are no longer active in, a specific cell population residing within a discrete CNS area, but where the general architecture of the tissue is maintained. In animal models of Parkinson's disease (PD), dopaminergic neuronal precursors – derived from different animal species (Dunnett *et al.*, 1987; Wictorin *et al.*, 1992; Isacson and Deacon, 1996; Starr *et al.*, 1999) and cell sources (Dunnett *et al.*, 1987; Shim *et al.*, 2007; Wernig *et al.*, 2008) – can survive, re-innervate the striatum and ameliorate clinical outcome, when grafted either in the substantia nigra or in the striatum (Gaillard and Jaber, 2011). In animal models of genetically induced dysmyelination and/or hypomyelination (i.e. shiverer mouse) (Ben-Hur *et al.*, 2005; Duncan *et al.*, 2011) or chemically induced demyelination (Blakemore and Franklin, 2008), intraparenchymal transplantation of many different myelinating cell types extensively remyelinates denuded axons (Windrem *et al.*, 2004; Buchet *et al.*, 2011; Sim *et al.*, 2011).

In contrast, cell replacement has been only partially satisfactory in a persistently unfavourable environment, where different cell sub-populations in different CNS areas are affected and where the tissue architecture is altered, such as in stroke, spinal cord injury (SCI), multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (Chen and Palmer, 2008). In stroke and SCI animal models, NPCs from multiple sources have been demonstrated to functionally integrate into the host neural circuits and differentiate into neurons (Kelly *et al.*, 2004; Cummings *et al.*, 2005; Buhnemann *et al.*, 2006; Yan *et al.*, 2007; Daadi *et al.*, 2009; Braz *et al.*, 2012). Likewise, in animal models of MS and SCI, both NPC and glial-restricted progenitors can remyelinate injured axons (Archer *et al.*, 1997; Pluchino *et al.*, 2003; Keirstead *et al.*, 2005; Lee *et al.*, 2009;

Sharp *et al.*, 2010; Yang *et al.*, 2010). However, it still remains to be demonstrated if this cell replacement mechanism is the sole mechanism functionally accountable for the clinical amelioration (Dubois-Dalcq *et al.*, 2005; Pluchino *et al.*, 2005; Bacigaluppi *et al.*, 2009b; Sahni and Kessler, 2010). Other mechanisms that will be discussed in this chapter (e.g. the bystander effect) may also contribute to the therapeutic effect observed upon NPC transplantation.

Another issue is the degree of complexity of the tissue patterning that transplanted cells need to restore. PD and focal demyelination are diseases in which specific cell types are necessary to re-establish appropriate interactions at the cellular level; in PD dopaminergic circuits should be restored within the striatal region, while in demyelinating disorders a properly functioning saltatory conduction should be achieved via efficient remyelination. However, very few studies have been able to demonstrate that cell transplantation might lead to the generation of long-distance connections in diseases in which degeneration impairs multiple widespread afferent–efferent connections, such as ALS and other motor neuron disorders (Verstraete *et al.*, 2011), Huntington's disease (HD) (Wolf *et al.*, 2008) or spinocerebellar ataxias (Di Giorgio *et al.*, 2011; Yohn *et al.*, 2008; Solodkin *et al.*, 2011; Lepore *et al.*, 2011).

The last issue to be considered when designing therapies aimed at promoting cell replacement is the timing of cell transplantation. In primary neurodegeneration, transplantation should be performed at the beginning of the neurodegenerative process in order to limit the cascade of events leading to the degeneration of other cell populations. Data clearly show that transplanted NPCs acquire an appropriate terminally differentiated fate and integrate functionally in the host tissue (Goldman, 2005; Breysse *et al.*, 2007; Lindvall and Kokaia, 2010), an event particularly evident when there is a partial preservation of the neuronal circuits' cyto-architecture (Shihabuddin *et al.*, 1996). On the other hand, while early transplantation is recommended in degenerative diseases, in primary inflammatory disorders (e.g. stroke, SCI and MS) transplantation aimed at cell replacement would be useful only during the post-acute phase of the disease (Karimi-Abdolrezaee *et al.*, 2006; Bacigaluppi *et al.*, 2009b) when the inflammatory phase is limited. It is now clear that the immune milieu can influence, either detrimentally or protectively, the fate of transplanted cells (Carpentier and Palmer, 2009; Deverman and Patterson, 2009; Ransohoff, 2009; Giannakopoulou *et al.*, 2011; Muja *et al.*, 2011; Kokaia *et al.*, 2012).

The bystander effect of transplanted NPCs

Despite the initial perception that transplantation of NPCs could serve to replace only damaged cells, recent experimental studies have shown that NPCs could exert a plethora of different neuroprotective effects, spanning from neurotrophic support to immunomodulation, when transplanted in chronic inflammatory neurological disorders. (See Figure 5.2.)

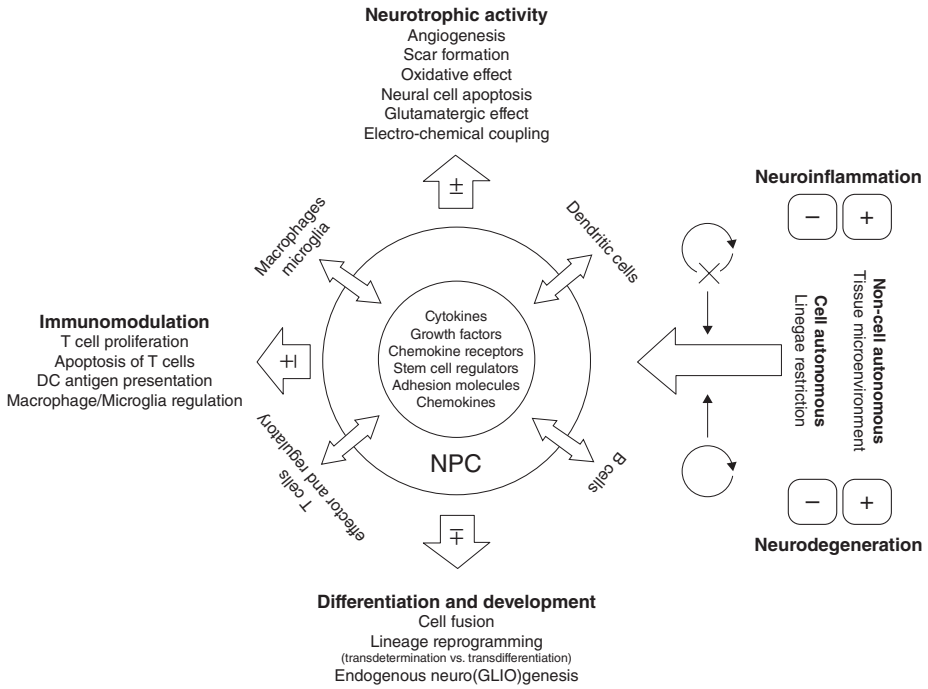

Figure 5.2 Operational mechanism(s) of action of endogenous and transplanted NPCs. The extent of tissue regeneration driven by endogenous as well as transplanted multipotent NPCs is very much dependent on the different operational mechanism(s) of action that such cells adopt while interacting with CNS resident and blood-borne infiltrating mononuclear cells, homing within the microenvironment during both physiological and/or pathological circumstances. Both cell-autonomous and non-cell-autonomous mechanisms may affect the final fate and behaviour of endogenous and transplanted NPCs as well as their interactions with CNS resident and CNS-infiltrating cells. While cell-autonomous mechanisms driving terminal differentiation of exogenous versus transplanted NPCs tend to prevail in pathological conditions mainly characterized by neuronal degeneration and mild reactive inflammation (mainly driven by CNS resident microglia), non-cell-autonomous mechanisms mainly affect NPCs when acute or chronic non-resolving inflammation is ongoing. As a matter of fact, endogenous and transplanted NPCs sense the inflammatory environment and, within such an environment, might promote tissue homeostasis and repair by releasing at the site of tissue damage a milieu of constitutively expressed molecules (chemokines, cytokines, growth factors and stem cell regulators) capable of immunomodulation and trophic support, the so-called bystander effect. The bystander effect is made possible by the fact that inflammation inhibits NPC proliferation, thus maintaining NPCs in an undifferentiated state; this is the best situation possible for NPCs to release constitutively a wide set of neuroprotective molecules. However, cell-autonomous mechanisms, driving terminal differentiation during lineage commitment, limit the amount of molecules constitutively released by NPCs due to epigenetic restriction of transcriptional circuits.

To what extent the interaction between the inflammatory microenvironment and transplanted NPCs results in protective versus detrimental effects is still far from being fully elucidated. Some of these ideas are discussed below.

Firstly, NPCs show a certain degree of pathotropism towards inflammatory foci, which is due to the fact that such cells constitutively express an armamentarium of chemokines and chemokine receptors (e.g. CCR1, CCR5, CXCR3 and CXCR4), cell adhesion molecules (e.g. CD44) (Rampon *et al.*, 2008) and integrins (e.g. VLA4) (Campos *et al.*, 2004, 2006; Leone *et al.*, 2008). Thus, transplanted NPCs are able to follow gradients of chemoattractants and reach multiple inflamed CNS regions when intraparenchymally and/or systemically injected (Ji *et al.*, 2004; Pluchino *et al.*, 2005). This is a very important issue in multifocal CNS inflammatory disorders where multiple intraparenchymal NPC injections are clinically impractical. Once migrated into inflamed CNS areas, transplanted NPCs survive within perivascular inflammatory foci (i.e. the atypical ectopic niche) where they interact with many other cell types such as CNS-infiltrating blood-borne inflammatory cells, endothelial cells and CNS resident astrocytes and microglia. Within these ectopic niches, inflammatory molecules (e.g. interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα)) inhibit NPC differentiation by blocking the cell cycle via upregulated expression of cell cycle–dependent kinase inhibitors (Pluchino *et al.*, 2008). As undifferentiated cells, NPCs can thus produce a wide array of both secreted and transmembrane molecules which, in turn, exert immunomodulatory and neurotrophic functions leading to tissue repair (Irvin *et al.*, 2004; Seifert *et al.*, 2005; Pluchino *et al.*, 2005; Martino and Pluchino, 2006b; Bacigaluppi *et al.*, 2009b; Cusimano *et al.*, 2012), as discussed in the next two sections.

Immunomodulatory effects of transplanted NPCs

In primary inflammatory CNS conditions, bystander immunomodulation exerted by transplanted NPCs can vary depending on the microenvironment. In relapsing-remitting experimental autoimmune encephalomyelitis (EAE), the animal model of MS, intravenously (IV) transplanted NPCs promote the apoptosis of encephalitogenic T cells, via either the expression of death receptor ligands (e.g. FasL, TRAIL and Apo3L) or the production of soluble mediators (i.e. nitric oxide (NO) via inducible NO synthase (iNOS) and IFNγ, involved in mitochondrial-mediated apoptosis) (Einstein *et al.*, 2003, 2006; Pluchino *et al.*, 2005). In the post-acute phase of ischaemic or haemorrhagic stroke, IV transplantation of NPCs inhibits activation of macrophage or microglia cells and CNS recruitment of blood-borne inflammatory cells (Lee *et al.*, 2008; Bacigaluppi *et al.*, 2009b). In the post-acute phase of a contusive model of SCI, intrathecally (IC) as well as intralesionally transplanted NPCs reduce the intensity of the local T cell and microglial response

(Ziv *et al.*, 2006) as well as the recruitment of CNS-infiltrating, classically activated pro-inflammatory macrophages (M1) (Cusimano *et al.*, 2012).

Similar to other stem cell types (i.e. mesenchymal stem cells), NPCs can also exert immunomodulatory effects outside the CNS upon systemic transplantation. It has been variably shown that IV-injected NPCs may limit the initiation and maintenance of neuroinflammation within secondary lymphoid organs (Einstein *et al.*, 2007; Gerdoni *et al.*, 2007; Lee *et al.*, 2008; Pluchino *et al.*, 2009a). As a matter of fact, dendritic cell (DC) antigen presentation (Pluchino *et al.*, 2009a) as well as antigen-specific T cell proliferation (Einstein *et al.*, 2007) within peripheral lymphoid organs are impaired in EAE mice transplanted IV or subcutaneously (SC) with NPCs. The selective inhibition of pathogenic Th17 cell differentiation by the secretion of leukaemia inhibitory factor (LIF) seems to be a crucial event in this peripheral process. NPC-secreted LIF signals through the extracellular signal-regulated kinase (ERK)–suppressor of cytokine signalling 3 (SOCS3) inhibitory pathway that, in turn, antagonizes the IL6-mediated phosphorylation of signal transducer and activator of transcription 3 (STAT3), which are both required for Th17 cell differentiation (Cao *et al.*, 2011).

Finally, immunomodulatory properties of NPCs seem to be a constitutive signature – and possibly an evolutionarily conserved one – of such cells. It has been recently shown also that human foetal NPCs (i) constitutively express a considerable number of immune-related genes (around 18% of the whole transcriptome) (Pluchino *et al.*, 2009b), (ii) inhibit T lymphocyte proliferation as well as DC maturation *in vitro* and (iii) ameliorate EAE severity in non-human primates while persisting in the long term within the host CNS and in the draining lymph nodes (Pluchino *et al.*, 2009a).

Neurotrophic effects of transplanted NPCs

In primary neurodegenerative disorders characterized by reactive inflammation (e.g. PD, HD and epilepsy) as well as in neurodegenerative processes secondary to inflammation (e.g. MS, stroke and SCI), NPCs prevent neuronal programmed cell death and glial scar formation, thus re-establishing functional cell–cell interactions between neural and glial cells. This has been shown to occur mainly via the paracrine secretion of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF) and glial-derived neurotrophic factor (GDNF) (Teng *et al.*, 2002; Lu *et al.*, 2003; Pluchino *et al.*, 2003, 2005; Chu *et al.*, 2004; Ryu *et al.*, 2004; Ziv *et al.*, 2006; Redmond *et al.*, 2007; Bacigaluppi *et al.*, 2009b).

In animal models of MS and SCI, transplanted NPCs enhance endogenous remyelination (Einstein *et al.*, 2003; Pluchino *et al.*, 2005; Ziv *et al.*, 2006; Aharonowiz *et al.*, 2008). Although it has yet to be conclusively established whether or not endogenous remyelination is the result of the anti-inflammatory effect exerted by NPCs once transplanted in an inflammatory

environment (as discussed earlier), data that are so far available suggest that NPCs may *per se* directly interfere with the multiple mechanisms leading to remyelination failure (e.g. depletion–recruitment, failure–differentiation and inhibition of oligodendrocyte precursor cells (OPCs)) (Kuhlmann *et al.*, 2008; Kotter *et al.*, 2011). In EAE, the increased number of endogenous OPCs in demyelinated areas upon NPC transplantation is paralleled by a decrease in the pro-astrogliotic factors TGFβ and fibroblast growth factor-2 (FGF2) (Pluchino *et al.*, 2003). In cuprizone-induced demyelination, intracerebroventricularly transplanted NPCs induced OPC proliferation and enhanced remyelination via the secretion of platelet-derived growth factor-AA (PDGF-AA) and FGF2 (Einstein *et al.*, 2009). Besides PDGF-AA and FGF2, NPCs may also secrete LIF and CNTF: while the former promotes mature oligodendrocyte survival in EAE (Butzkueven *et al.*, 2002; Marriott *et al.*, 2008), the latter is implicated in both OPC survival and differentiation. Indeed, OPCs overexpressing CNTF increased the number of myelinated axons when transplanted in a mouse model of SCI (Cao *et al.*, 2010).

Another bystander effect exerted by transplanted NPCs is to modulate neuronal circuit plasticity (Zhang and Chopp, 2009). In an experimental model of ischaemic stroke, while remaining undifferentiated, human foetal NPCs significantly improved functional outcomes by promoting neuronal dendritic arborization in ipsilesional hemispheres and contralateral corticocortical pathways, and axonal projections within corticostriatal and corticospinal pathways. These effects have been attributed to the capacity of transplanted NPCs to re-express development molecules such as guidance molecules (i.e. Slit and thrombospondin-1 and -2) and also trophic factors such as vascular endothelial growth factor (VEGF) (Andres *et al.*, 2011).

Conclusions and perspectives

In this chapter, we first highlighted the different working modalities that endogenous adult NPCs exert to protect the CNS from damage and to maintain homeostasis. We then underlined how we can take advantage of this constitutive reparative potential of endogenous NPCs to develop new therapeutic approaches for promoting brain repair via cell transplantation. Different mechanisms by which transplanted NPCs might exert their therapeutic effects in neurological disorders were then described to make clear that cellular therapies have different goals depending upon the type of neurological disease they need to confront (see Figure 5.2).

In primary neurodegenerative diseases (e.g. PD, HD and dysmyelinating diseases) in which cell replacement is the most desirable effect, intraparenchymal transplantation of committed precursors with limited differentiating options and high integrating ability is currently considered the most appropriate strategy. To reach this goal, cellular reprogramming of patient-specific cells seems to be the way to go. Induced pluripotent stem cells (iPSCs)

have been proven to produce region-specific neuronal populations (Zeng *et al.*, 2010), including functional dopaminergic neurons (Wernig *et al.*, 2008; Swistowski *et al.*, 2010), as well as myelinating oligodendrocytes and OPCs (Ogawa *et al.*, 2011a, 2011b; Pouya *et al.*, 2011). Nevertheless, the generation of committed precursors via the transition through an iPSC is still hampered by the risk of teratoma formation as well as the risks of incomplete reprogramming, tissue inappropriate differentiation or insertional mutagenesis caused by the reprogramming factors (Wu and Hochedlinger, 2011). To overcome these risks, clonal iPSC cultures should be either screened for integration into 'safe harbour' sites or established and differentiated without any genetic modification (Robinton and Daley, 2012). Another valuable option would be the direct cell reprogramming of fibroblasts into both precursors and terminally differentiated cells of neural lineages (Caiazzo *et al.*, 2011; Kim *et al.*, 2011; Yoo *et al.*, 2011; Lujan *et al.*, 2012). However, a serious disadvantage of direct reprogramming over programming from iPSCs is that somatic cells in general have a limited lifespan and are therefore not expandable, whereas iPSCs have limitless growth and can hence be repeatedly differentiated into the desired cell types.

In diseases in which the NPC-mediated bystander effect is desirable, mainly those characterized by primary inflammation (e.g. MS, SCI and stroke), we still need to understand how to optimize the treatment in order to avoid unwanted side effects. As recently suggested, treatment optimization can be possibly obtained by guiding the transplantation timing and route of cell administration. An early time window for NPC transplantation seems to be the most appropriate approach to prevent tissue damage but not to reconstruct neuronal circuits. A systemic (intrathecal, intravenous or intra-arterial) approach may be effective in multifocal inflammatory disorders but can cause serious side effects as recent data indicated that various sources of stem cells do form tumours, in response to microenvironment-mediated signals, when heterotopically implanted (Fazel *et al.*, 2008; Amariglio *et al.*, 2009; Melzi *et al.*, 2010; Thirabanjasak *et al.*, 2010; Jeong *et al.*, 2011).

While there are still more questions than answers, it is currently clear that NPCs might represent valuable therapeutic options in still incurable neurological disorders. While the possibility of having large amounts of autologous committed precursors – using both progenitors of foetal origin and reprogrammed cells – seems to be the way forward for cell replacement strategies, the discovery of factors governing the NPC-mediated neuroprotective effect is mandatory to optimize the treatment and avoid unwanted side effects. This is likely to play a relevant role not only in directing and manipulating the migration, homing and differentiation of endogenous versus transplanted NPCs and, therefore, their therapeutic effect but also in the refinement of the optimal timing and route of cell transplantation. However, the identification of factors involved in the bystander effect is still in its infancy, thus representing the most problematic issue to be resolved. Bench-to-bedside translation

cannot be foreseen without contemplating further experimental studies aimed at addressing the unexpected and challenging findings that NPC-based therapies have revealed in recent years.

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6

Neuroinflammation in Alzheimer's, Parkinson's and Huntington's Diseases

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General introduction

Parkinson's and Alzheimer's disease are common neurodegenerative diseases which occur in individuals with advancing age (>65 years). Alzheimer's disease (AD) initially presents with short-term memory loss, followed by widespread cognitive and motor impairment caused by a widespread neuronal loss in the hippocampus and selected cortical and subcortical regions. In Parkinson's disease (PD), there is loss of motor co-ordination which is characterized by resting tremor, muscle rigidity, bradykinesia and postural instability. This is principally due to the loss of dopaminergic neurons in the substantia nigra (SN) which forms part of the basal ganglia which controls movement. Both AD and PD are polygenic diseases, meaning that a wide number of risk genes have been implicated in the development of these two neurodegenerative diseases of ageing. In contrast, Huntington's disease (HD) occurs during midlife (at approximately 40–50 years of age), affects 1 in 10,000 individuals

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worldwide and is characterized by movement disorders, cognitive deterioration and psychiatric disturbances. The most sensitive region affected is the striatum, where there is loss of GABAergic medium-sized spiny striatal (caudate nucleus and putamen) neurons. HD is caused by an expansion of CAG repeats coding for a poly-Q tract in the huntingtin protein. In individuals with less than 35 CAG repeats, the likelihood of the disease developing is negligible. However, as the number of CAG repeats increases (i.e. to over 40 CAG repeats), the disease will develop. An autosomal dominant pattern of inheritance of this huntingtin gene has been demonstrated.

The common feature of these three diseases is the presence of intracellular aggregates, or inclusion bodies containing misfolded proteins (i.e. amyloid-beta (Aβ), alpha-synuclein (contained within Lewy bodies) and huntingtin for AD, PD and HD, respectively). The exact roles of these aggregated proteins remain unclear; whether they are protective or are involved in the neuroinflammation of these diseases remains to be elucidated. Other proteins affected which may play a role include chaperones and elements of the cytosolic protein degradation pathway, such as ubiquitin and components of the proteosomal apparatus.

Alzheimer's disease

The main pathological hallmarks of AD include the presence of neuritic plaques, neurofibrillary tangles, synaptic loss and, ultimately, neuronal death. Neuroinflammation in AD is characterized by an inflammatory response to Aβ, inducing the activation of microglia and the recruitment of astrocytes to the sites where Aβ deposits occur (Sastre *et al.*, 2006b) (Figure 6.1). At present, it is still unclear whether inflammation is the cause or consequence of disease progression. However, there is evidence that suggests that it increases Aβ generation, tau phosphorylation and cognitive impairment. In this

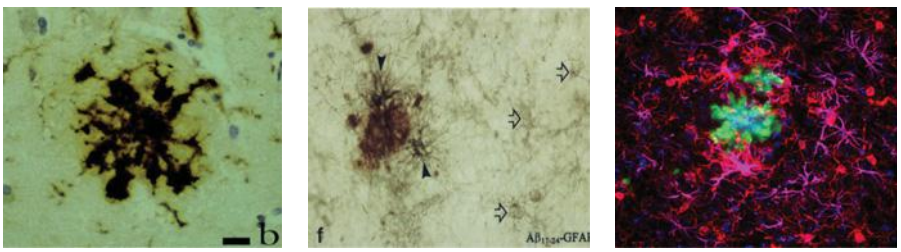

Figure 6.1 Glial cells surround amyloid-β deposits in human and transgenic mouse brain. (A) MHC class II antigen positive microglial cells display a circular distribution around the centre of the plaque in the brain of a 63-year-old human (Thal *et al.*, 1997); and (B) fleecy amyloid is associated with a number of small GFAP-positive astrocytes in the human brain (Thal *et al.*, 2000). Immunohistochemical detection for GFAP (magenta), IBA1 (red) and thioflavin-S (green) in the brain of an APP23 transgenic mouse.

chapter, we will discuss all of these issues, including the cell types and molecular mediators involved.

Microglia in AD

Aβ is able to bind and activate microglia. The mechanism of action for this is through interaction with pattern recognition receptors (PRRs). Microglia express many PRRs (Farina *et al.*, 2007; Falsig *et al.*, 2008) which recognize and bind PRR ligands, that is, pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), such as Aβ (Salminen *et al.*, 2009). Microglia interact with fibrillar Aβ through an ensemble of surface receptors composed of the alpha(6)beta(1) integrin, CD36, CD47 and the class A scavenger receptor (Reed-Geaghan *et al.*, 2009). Interaction of microglia with Aβ via PRRs provokes their inflammatory actions (Solito and Sastre, 2012) (see Chapter 2). However, microglia internalize soluble Aβ from the extracellular milieu through a nonsaturable, fluid-phase macropinocytic mechanism that is distinct from phagocytosis and receptor-mediated endocytosis both *in vitro* and *in vivo* (Mandrekar *et al.*, 2009).

Clinical reports and studies in animal models suggest that microglial activation precedes amyloid plaque and tangle formation (Griffin *et al.*, 1989; Heneka *et al.*, 2005), while positron emission tomography (PET) studies have reported inflammatory changes in one-third of amnestic mildly cognitively impaired subjects (Cagnin *et al.*, 2001; Okello *et al.*, 2009). Two-photon microscopy has clarified the anatomic relationships of microglia to Aβ, showing that microglia sample and react to Aβ deposits in transgenic mouse models. In this report, recruitment of microglia to newly formed plaques occurred within a few days (Meyer-Luehmann *et al.*, 2008), while other investigations revealed the establishment of a dynamic interface between microglial processes and Aβ deposits (Bolmont *et al.*, 2008; Koenigsknecht-Talboo *et al.*, 2008). These studies do not fully agree with previous reports (Heneka *et al.*, 2005), a finding that may depend on the animal model used.

It was proposed that early microglial activation in AD delays disease progression by promoting clearance of Aβ before the formation of senile plaques, suggesting that glial activation is protective early in the disease (Wyss-Coray *et al.*, 2003; Maragakis and Rothstein, 2006; Wyss-Coray, 2006). Studies have shown that bone marrow–derived macrophages (BMDMs) are able to efficiently eliminate amyloid and confer neuroprotection by secretion of growth factors such as the glia-derived neurotrophic factor (GDNF) which are potentially beneficial to the survival of neurons (Liu and Hong, 2003). Activated microglia in early stages of AD can reduce Aβ accumulation by increasing its phagocytosis, clearance and degradation (Frautschy *et al.*, 1998; Qiu *et al.*, 1998). The mechanism by which Aβ is phagocytosed depends on the physical properties of Aβ and whether it is soluble or fibrillar. Secreted Aβ1–40 and Aβ1–42 peptides are constitutively degraded by neprilysin and the

insulin-degrading enzyme (IDE), a metalloprotease released by microglia and other neural cells, whose enzymatic activity is enhanced by inflammatory events, such as lipopolysaccharide (LPS) stimulation (Qiu *et al.*, 1997).

In later stages, with persistent production of pro-inflammatory cytokines, microglia lose their protective effect (Hickman *et al.*, 2008; Jimenez *et al.*, 2008) and may become detrimental through the release of cytokines and chemokines (Hickman *et al.*, 2008). These inflammatory mediators modulate immune and inflammatory function and may also alter neuronal function. In addition, microglia from aged transgenic APP–PS1 mice have a decrease in the expression of the Aβ-binding scavenger receptors A (SRA), CD36 and RAGE and the Aβ-degrading enzymes IDE, neprilysin and matrix metalloproteinase-9 (MMP9), compared with wild-type controls (Hickman *et al.*, 2008). Therefore, evidence supports the idea that over-activated microglia could cause uncontrolled inflammation that may drive the chronic progression of AD by exacerbating Aβ deposition and stimulating neuronal death (Mrak and Griffin, 2005; Gao and Hong, 2008). This concept constitutes the 'neuroinflammatory hypothesis'.

By comparison, the 'microglial dysfunction hypothesis' stipulates that rather than an increase of inflammatory function, there is a loss of the microglial neuroprotective function in AD (Polazzi *et al.*, 2010). Research has shown that the phagocytic abilities of microglia are altered in aging and impaired in neurodegenerative diseases. Therefore, this 'senescent' or dystrophic microglia can also contribute to the onset of sporadic AD (Streit *et al.*, 2004, 2009).

Astrocytes in AD

The actual role of astrocytes in AD still remains elusive, because astrocytes seem to adopt different functions depending on disease progression and the extent of the accompanying parenchymal inflammation.

When healthy astrocytes are exposed to high levels of certain pro-inflammatory cytokines, reactive oxygen species or even Aβ, they undergo a process called astrogliosis whereby they become activated. Astrogliosis is a defensive transformation in response to inflammation during which astrocytes undergo a complex morphological and functional remodelling involving hypertrophy, altered gene expression and up-regulation of inflammatory mediators (Sofroniew, 2009). If the inflammatory response is severe or particularly persistent, as it is in AD, reactive astrocytes will also proliferate to form a glial scar in an effort to isolate damaged tissue (Rodríguez *et al.*, 2009; Medeiros and LaFerla, 2013).

While there is no significant correlation between plaque load and cognitive deterioration, studies on brain tissues obtained from aged AD patients show a correlation between astrogliosis and cognitive decline, which suggests that astrogliosis may be partially responsible for the synaptic dysfunction that

is undoubtedly implicated in the deterioration of brain function during AD (Brenner, 2005; Simpson *et al.*, 2010; Verkhratsky *et al.*, 2010). Although the exact processes underlying this dysfunction remain elusive, it is well established that astrogliosis leads to elevated expression and secretion of cytokines and chemokines, whose accumulation results in neurotoxicity (Li *et al.*, 2011). Reactive astrocytes also up-regulate inducible nitric oxide synthase (iNOS), stimulating not only high concentrations of astrocytic neurotoxic nitric oxide (NO) but also nitration of the Aβ tyrosine at position 10, which can potentiate the propensity of this amyloidogenic protein to self-aggregate (Kummer *et al.*, 2011). In addition, astrocytes also contribute to neuronal death through release of glutamate.

However, it has also been suggested that reactive astrocytes play a beneficial role during AD, for example by being directly involved in the clearance of Aβ by both phagocytosis and secretion of Aβ-degrading proteases such as neprilysin, as well as apolipoprotein E (ApoE) (Apelt *et al.*, 2003; Wyss-Coray *et al.*, 2003; Koistinaho *et al.*, 2004; Pihlaja *et al.*, 2007, 2011; Terwel *et al.*, 2011). Reactive astrocytes are essential in limiting inflammation and neuronal damage in models of acute central nervous system (CNS) injury (Faulkner, 2004; Myer, 2006). Therefore, it is plausible that in chronic CNS diseases, such as AD, reactive astrocytes may initially play a similar protective role while prolonged activation gradually reduces their capacity to act in a neuroprotective manner. Interestingly, preventing astrocyte activation at an early stage of pathology (through gene deletion of GFAP and vimentin in an AD mouse model) results in an increased plaque load accompanied by neuronal dystrophy. These studies underscore the idea that reactive astrocytes could indeed play a neuroprotective role during early disease stages, which may gradually be outweighed by increased cytokine production, oxidative stress and excessive Aβ production exhibited by reactive astrocytes with disease progression.

Several studies have attempted to determine at what stage of AD astrogliosis occurs and how it could impact clinical disease. Reactive astrocytes become plaque associated at early stages in both transgenic mice and AD patients (Kato *et al.*, 1998; Heneka *et al.*, 2005; Van Dam *et al.*, 2005; Carter *et al.*, 2012). To track the extent of astrogliosis with the progression of amyloid pathology, an animal model of AD was used where transgenic APP23 mice were crossed with glial fibrillary acidic protein (GFAP)-luc mice, which express luciferase under the control of the GFAP promoter (Watts *et al.*, 2011). This study showed that *in vivo* bioluminescence imaging levels increased at 14 months of age, which correlates to a late activation of astrocytes when AD pathology had already progressed considerably.

Together the studies presented here suggest that astrocytes have evolved to deal with acute localized inflammation, rather than chronic disseminated inflammation, and that astrogliosis represents a common mechanism by which the brain attempts to deal with early disruptions caused by trauma or neurodegenerative disease.

Molecular mediators in AD

Cytokines Aβ can increase gene expression and protein production of a number of cytokines by human microglia *in vitro*, including interleukin-1 beta (IL1β), IL8, IL10, IL12, tumour necrosis factor alpha (TNFα), macrophage inflammatory protein-1 alpha (MIP1α, aka CCL3), MIP1β (CCL4) and monocyte chemoattractant protein-1 (MCP1, CCL2) (Lee *et al.*, 2002). It is no surprise, therefore, that their expression is reported to be altered in the brain, blood and cerebrospinal fluid (CSF) of AD patients (Akiyama *et al.*, 2000). Animal models of AD, such as the APP transgenic line Tg2567 carrying the Swedish mutation, also show enhanced levels of TNFα, IL1β, IL1α, chemoattractant protein-1, COX2 and complement component 1q (Sastre *et al.*, 2008).

Despite the strong links between inflammation and AD, there are a surprisingly small number of studies assessing the expression of many inflammatory cytokines. Evidence of an increased risk of AD is observed when the IL10–1082 GA gene polymorphism is present; however, the association is only marginally significant (Di Bona *et al.*, 2012). Adeno-associated virus (AAV)-mediated gene delivery of this anti-inflammatory cytokine reduced glial activation, enhanced Aβ clearance into the plasma, upregulated neurogenesis and improved spatial memory in APP–PS1 mice (Kiyota *et al.*, 2012); however, it had no effect on Aβ plaque load. Increased concentrations of p40 (a common subunit of the pro-inflammatory cytokines IL12 and IL23) are found in the CSF of AD patients, and this correlates with cognitive performance (vom Berg *et al.*, 2012). In addition, ablation of IL12 and IL23 signalling in APP–PS1 mice reduces amyloid burden (vom Berg *et al.*, 2012). IL6 is elevated in the serum of patients with AD (Helmy *et al.*, 2012), and there is an interaction between IL6 and IL10 gene polymorphisms associated with increased risk for AD (Arosio *et al.*, 2004; Combarros *et al.*, 2009). These studies strongly suggest that cytokine dysregulation is a significant candidate for initiating the development of AD. The most commonly studied cytokines are IL1β and TNFα; up-regulation of these pro-inflammatory cytokines has been heavily implicated as common cellular response markers to Aβ release and deposition (Hickman *et al.*, 2008).

IL1 is a primary mediator of the pro-inflammatory response in the body and is capable of inducing the production of many other cytokines. A number of studies have analysed the possibility that polymorphisms in IL1 could lead to increased risk for the development of AD. A specific IL1α gene polymorphism in allele 2 triples the risk of developing AD (Du *et al.*, 2000; Grimaldi *et al.*, 2000; Rebeck, 2000), and an even greater risk is seen with patients carrying both IL1α and IL1β allele 2 gene polymorphisms (Griffin *et al.*, 2000). In addition, this IL1α gene polymorphism is associated with increased microglial cell numbers in post-mortem AD brains, and the increase was even higher in patients carrying the APOE ε4 allele (Hayes *et al.*, 2004). These polymorphisms increase expression of IL1 and may therefore drive IL1-mediated

mechanisms that lead to an overall enhancement of neuroinflammation in the brain and consequently increased risk for AD. Overexpression of IL1β has been reported in microglia and astrocytes of AD brains since the late 1980s (Griffin *et al.*, 1989), in particular in plaque-associated microglia (Mrak and Griffin, 2005). This association is transient; high IL1β expression is associated with early amyloid deposits and the conversion of diffuse amyloid plaques into compact forms (Griffin *et al.*, 1995). IL1α and IL1β increase translation of APP in astrocytes *in vitro*, with the latter enhancing synthesis to a greater extent (Rogers *et al.*, 1999). In addition, IL1β drives astrocyte activation and increases β-secretase-cleaved carboxyl-terminal fragment (βCTF) production *in vivo*, suggesting that this cytokine may have a significant role to play in the progression of AD (Sheng *et al.*, 1996). Indeed, many argue that early and sustained overexpression of IL1 could be an initiator of disease pathology (Griffin and Mrak, 2002). IL1 may also contribute directly to neuronal dysfunction in AD; chronic release of exogenous IL1β *in vivo* induced overexpression and phosphorylation of neurofilament protein and tau, which are components of neurofibrillary tangles of AD (Sheng *et al.*, 2000). This overexpression was specifically associated with swollen, dystrophic neurites. Interestingly, a recent paper by Ghosh and colleagues reports that sustained IL1β overexpression in a triple transgenic (3xTg) mouse model of AD increases tau hyperphosphorylation but leads to a corresponding decrease in amyloid burden (Ghosh *et al.*, 2013). They also found a four- to sixfold increase in plaque-associated microglia, suggesting that IL1β may directly affect tau phosphorylation, possibly via p38 mitogen-activated kinase and glycogen synthase kinase-3β activity, but regulates amyloid indirectly via glial activation. This builds on similar findings from their group showing that overexpression of IL1β specific to the hippocampus in amyloid precursor protein–presenilin-1 (APP–PS1) mice induces significant astrocyte and glial activation with a corresponding decrease in amyloid plaque deposition (Shaftel *et al.*, 2007). The differential effect of IL1 on tau and amyloid makes it an unlikely candidate for therapeutic manipulation; however, it does lead to intriguing questions about targeting certain microglial or astrocytic processes to reduce amyloid burden.

TNFα is increased in the serum of AD patients (Fillit *et al.*, 1991; Bruunsgaard *et al.*, 1999; Tarkowski *et al.*, 1999), and increased expression is found in activated microglia surrounding amyloid plaques in brains of AD patients (Dickson *et al.*, 1993). Microglia produce TNFα that is neurotoxic in response to Aβ exposure (Meda *et al.*, 1995; Combs *et al.*, 2001), and Aβ itself can induce neuronal apoptosis via TNF receptor type I (TNF-RI) (Li *et al.*, 2004). These studies indicate that there may be TNFα-mediated apoptosis in AD. Increased TNFα is also found in 3xTg-AD and APPswe mouse models, expressed by both neurons and microglia (Mehlhorn *et al.*, 2000; Janelsins *et al.*, 2005). Overexpression of TNFα in 3xTg-AD mice induces significant neurodegeneration (Janelsins *et al.*, 2008), providing further evidence for its role in the cell death seen in AD. However, chronic down-regulation of TNF-RII in

3xTg-AD mice exacerbates amyloid and tau pathology (Montgomery *et al.*, 2013), although interestingly this effect was evident only in mice whose TNF-RII receptors were knocked down from 12 to 21 months of age. Young and aged mice in which TNF-RI or both TNF-RI and TNF-RII receptors were knocked down showed no change in amyloid burden or tau hyperphosphorylation. TNF-RI and TNF-RII show divergent signalling pathways, with TNF-RI being heavily implicated in neuronal apoptosis (Yang *et al.*, 2002), whereas TNF-RII is reported to activate alternative nuclear factor kappa B (NF-κB) signalling associated with the down-regulation of pro-inflammatory cytokines (Rauert *et al.*, 2010). This once again highlights the complex nature of cytokine involvement in AD, and any therapeutic intervention targeted at TNFα must take into account disease state and receptor specificity.

Pro-inflammatory cytokines are known to affect Aβ generation, suggesting that a positive feedback loop may develop following an Aβ-triggered inflammatory response in which cytokines contribute to further Aβ production (Sastre *et al.*, 2006a,b). The potential mechanisms include increasing the susceptibility for Aβ deposition or aggregation (Games *et al.*, 1995; Guo *et al.*, 2002), up-regulating β-secretase (BACE1) mRNA (Sastre *et al.*, 2003, 2006b) and elevating APP transcription (Amara *et al.*, 1999; Rogers *et al.*, 1999).

Reactive oxygen species (ROS) in AD ROS are chemically reactive molecules that are cytotoxic by-products of oxygen metabolism. These include hydroxyl radicals (−OH), peroxide (H_2O_2) and superoxide radicals (O_2^-). Endogenous ROS are mainly produced by mitochondria, peroxisomes, endoplasmic reticulum and the NADPH oxidative complex (NOX) in the cell membranes (Vitale *et al.*, 2013). The main mechanisms for eliminating ROS are superoxide dismutases (SODs), catalase, heme-oxygenase and glutathione peroxidase, enzymes that are increased in the vicinity of Aβ plaques (Furuta *et al.*, 1995; Pappolla *et al.*, 1998). During ageing or inflammation, there is accumulation of ROS, which causes oxidation of lipids, sugars, proteins and DNA, thus leading to cell dysfunction and consequently cell death (Chami and Checler, 2012). The neurons are highly susceptible to oxidative stress because of their high metabolic demands and poor anti-oxidant capabilities. ROS can also contribute to further inflammation through induction of cyclooxygenase-2 (COX2), inflammatory cytokines and pro-inflammatory transcription factors such as NF-κβ (Yan *et al.*, 1995). In AD brains, several oxidative markers are increased (Guglielmotto *et al.*, 2010), including protein oxidation (Smith *et al.*, 1997), lipid peroxidation (Mark *et al.*, 1997; Sayre *et al.*, 1997), advanced glycation end products (Smith *et al.*, 1994) and oxidation of nucleic acids (Nunomura *et al.*, 1999).

Oxidative stress is greatest early in the disease (Nunomura, 2001). Increased levels of isoprostanes, a marker for lipid peroxidation, were detected in the urine, blood and CSF of both mild cognitive impairment (MCI) and AD patients (Praticò *et al.*, 2000, 2002; Mecocci, 2004), suggesting that oxidative markers could be potentially used as biomarkers for the early

detection of AD. Increased ROS and oxidative stress were also confirmed in mouse models of AD (Pappolla *et al.*, 1998; Smith *et al.*, 1998; Matsuoka *et al.*, 2001; Praticò *et al.*, 2001).

It is well established that Aβ causes oxidative stress which in turn causes more Aβ, resulting in a positive feedback loop that further contributes to the progress of the disease (Tamagno *et al.*, 2012; Butterfield *et al.*, 2013). This is because ROS affect the rate-limiting enzyme of AD, beta-site APP cleaving enzyme-1 (BACE1), by altering its distribution in non-apoptotic conditions (Tan *et al.*, 2013) as well as its activity and expression (Tamagno *et al.*, 2002). Oxidative stress-mediated BACE activity is γ-secretase-dependent as absence of PS1 abolished the effect (Tamagno *et al.*, 2008). Further studies strengthened the correlation of BACE1, γ-secretase and oxidative stress, showing that treatment with dehydroepiandrosterone, an antioxidant, reduced BACE1 activity in NT2 neurons exposed to oxidative stress (Tamagno *et al.*, 2003) and that mild impairment of oxidative metabolism by thiamine deficiency increased Aβ, carboxyl-terminal fragments and plaque burden in an AD mouse model (Karuppagounder *et al.*, 2009).

Dysfunctional homeostasis of transition metals, such as copper and particularly iron (Bush, 2013), may play an important role in the pathogenesis of AD by enhancing oxidative stress. (Crichton *et al.* 2011). Increased concentrations of iron are present in the basal ganglia (Bartzokis and Tishler, 2000), hippocampus (Ding *et al.*, 2009) and neocortex (Cornett *et al.*, 1998) and in or around the plaques and tangles (Morris *et al.*, 1994). In addition, iron modulates the ability of α-secretase to cleave APP (Rivera-Mancía *et al.*, 2010), promotes Aβ toxicity (Bodovitz *et al.*, 1995) and aggregation, and directly regulates the expression and the synthesis of APP via the iron responsive element at the 5′ untranslated region of *APP* mRNA (Rogers *et al.*, 2002; Rivera-Mancía *et al.*, 2010). Levels of furin, an enzyme responsible for the activation of β-secretases involved in the amyloidogenic pathway, are also modulated by iron (Crichton *et al.*, 2011), which will play an important role in AD pathogenesis (Silvestri and Camaschella, 2008).

Complement in AD Complement receptors are one of the categories of cell surface molecules on microglia that are up-regulated in response to the activation of these cells (Liu and Hong, 2003). Aβ-induced complement activation leads to generation of C1q, C4 and C3 activation fragments around the plaques. Here microglia express complement proteins C1q and C3 and receptors C1qR, CR3, CR4 and C5aR, which support phagocytic uptake (Keene *et al.*, 2011). Inhibition of the complement system results in an increase of Aβ plaque formation and neurodegeneration in AD transgenic mice (Shen and Meri, 2003).

In contrast, lack of C1q in mouse models of AD results in decreased pathology (Hafer-Macko *et al.*, 2000). This indicates that one mechanism by which microglia could recruit further reactive cells to the site of a plaque and cause

neurotoxic damage is by activating the classical complement pathway and the inflammatory machinery associated with it (i.e. pro-inflammatory cytokines and oxidative products) through production of C1q (McGeer and McGeer, 1998; Bonifati and Kishore, 2007).

Adaptive immunity in AD There have been a number of reports demonstrating the presence of T cells in the brain of AD patients since the original observation 25 years ago (Rogers *et al.*, 1988). Furthermore, inflammatory IFNγ-secreting Th1 cells and IL17–secreting Th17 cells have been shown to infiltrate the brain of older APP–PS1 mice (Browne *et al.*, 2013).

Therapeutic vaccination with Aβ antibodies in mice evidenced the Fc-mediated uptake and clearance of Aβ antibody complexes by local activated microglia (Bard *et al.*, 2000). However, because a human trial of Aβ immunization led to meningoencephalitis in some patients, this treatment has been discontinued. Recently, it was found that nasal vaccination in mice was able to decrease Aβ, and the extent of this reduction correlated with microglial activation, suggesting that this may be a promising approach for human Aβ immunization (Frenkel *et al.*, 2005).

Anti-inflammatory therapy in AD

Non-steroidal anti-inflammatory drugs (NSAIDs) The finding that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) is associated with a reduced risk and age of onset of AD reinforced the hypothesis that modulating inflammation could have therapeutic efficacy. The beneficial effects of NSAIDs have also been associated with reductions in Aβ generation, because experiments *in vitro* and in AD animal models indicate that certain NSAIDs are able to decrease Aβ levels, plaque size and tau phosphorylation (Yoshiyama *et al.*, 2007; El Khoury and Luster, 2008; Sastre and Gentleman, 2010).

However, clinical trials have failed to reproduce the beneficial effects of NSAIDs in AD patients. This has led to further analysis of the previously published epidemiological data, which has revealed that the use of NSAIDs prevents cognitive decline in older adults if started in midlife (prior to age 65) rather than late in life (Hayden *et al.*, 2007). In addition, it was recently shown that while NSAIDs may indeed protect those with healthier brains, they can accelerate AD pathogenesis in patients with advanced stages of the disease (Breitner *et al.*, 2009). This is supported by studies in transgenic mice, in which NSAIDs can prevent the appearance of cell cycle protein markers in neurons in young mice, but not after cell cycle entry has been initiated (Varvel *et al.*, 2009). Therefore, it seems that the protective effects of NSAIDs depend very much on the stage of the disease at which the medication is started as well as the duration of the treatment (Sastre and Gentleman, 2010).

Peroxisome Proliferator-activated Receptor Gamma (PPARγ) agonists PPARγ inhibition regulates the transcription of pro-inflammatory genes, such as IL1β; therefore, activation of PPARγ consequently inhibits the inflammatory response. In addition, our group found that PPARγ activators are able to decrease total Aβ levels under inflammatory conditions by affecting BACE1 transcription (Sastre *et al.*, 2003, 2006b). Recently it was shown that ibuprofen is able to suppress RhoA activity in neuronal cells through PPARγ activation, promoting neurite elongation (Dill *et al.*, 2010). Therefore, PPARγ activation could be beneficial in AD at several levels.

However, other groups have suggested that PPARγ may affect Aβ clearance and degradation. Landreth demonstrated that PPARγ activation induces lxrα, apoe, and abca1 expression, promoting Aβ clearance by both microglia and astrocytes (Mandrekar-Colucci *et al.*, 2012). Furthermore, PPARγ stimulates an increase of Aβ phagocytosis, which was mediated by the up-regulation of scavenger receptor CD36 expression. In addition, combined treatment with agonists for the heterodimeric binding partners of PPARγ, the retinoid X receptors (RXRs), showed additive enhancement of the Aβ uptake that was mediated by RXRα activation (Yamanaka *et al.*, 2012).

A recent prospective randomized, open-controlled study with pioglitazone (a typical PPARγ agonist) showed that at 6 months, the Wechsler Memory Scale-Revised logical memory-I scores significantly increased in the pioglitazone group, but not in the control group (Hanyu *et al.*, 2009). Another PPARγ agonist, rosiglitazone, has been trialled with inconsistent results. In contrast to pioglitazone, rosiglitazone cannot cross the blood–brain barrier (BBB) (Festuccia *et al.*, 2008), and it was suggested that the protective effects are mediated through its effects on insulin and glucocorticoids that are able to penetrate the brain.

Minocycline Minocycline, a tetracycline derivative, has potent anti-inflammatory, anti-apoptotic and neuroprotective properties. Minocycline is able to cross the BBB and effectively delays disease progression and reduces neuronal death in mouse models of amyotrophic lateral sclerosis (Zhu *et al.*, 2002), Huntington's disease (Chen *et al.*, 2000) and Parkinson's disease (Wu *et al.*, 2002). In many cases, the neuroprotective properties of minocycline have been attributed to inhibition of caspases. In primary cortical neurons, minocycline was shown to reduce caspase-3 activation and lowered the generation of caspase-3-cleaved tau fragments (Noble *et al.*, 2009). Recently, minocycline was shown to protect against Aβ-induced cell death and prevent fibrillization of Aβ *in vitro* (Familian *et al.*, 2006), reduce iNOS levels (Ferretti *et al.*, 2012), prevent Aβ deposition and cognitive decline in APP transgenic mice (Seabrook *et al.*, 2006; Ferretti *et al.*, 2012) by reducing BACE1 levels (Ferretti *et al.*, 2012) and inhibit neuronal death and attenuate learning and memory deficits following administration of Aβ to rats (Ryu *et al.*, 2004; Choi *et al.*, 2007). In addition, treatment of tangle-forming transgenic mice (htau

line) with minocycline resulted in reduced levels of tau phosphorylation and insoluble tau aggregates (Noble *et al.*, 2009).

Another potential mechanism of action of minocycline has been related to the inhibition of microglial activation. Administration of minocycline in animal models of ALS attenuated the induction of the expression of M1 microglia markers during the progressive phase, whereas it did not affect the transient enhancement of expression of M2 microglia markers during the early pathogenesis phase (Kobayashi *et al.*, 2013). This study suggests that minocycline may selectively inhibit the microglia polarization to a pro-inflammatory state.

Anti-TNFα Interestingly, anti-TNFα treatment reduced Aβ and tau phosphorylation in transgenic mice. Treatment with the antibody against TNFα, infliximab, increased the number of CD11c-positive dendritic-like cells and the expression of CD11c. These data suggested that the CD11c-positive dendritic-like cells might contribute to the infliximab-induced reduction of AD-like pathology (Shi *et al.*, 2011).

Neuroinflammation in Parkinson's disease

Although the disease mechanisms involved in PD are not fully understood, the progressive neurodegeneration of dopaminergic neurons in the SN that occurs in PD over a number of years is associated with chronic inflammation. This is driven by the inflammatory response of microglia cells, reactive microgliosis.

Microglia in PD

Early evidence for the involvement of neuroinflammation in PD was reported by McGeer and colleagues (1988b), who identified reactive microglia in the SN of post-mortem brains of PD patients +/− dementia, by immunohistochemical techniques, using HLA-DR-specific antibodies. Further immunohistochemical studies by Imamura *et al.* (2003) similarly identified major histocompatibility complex class II (MHC-II)-positive microglia, not only in the SN but also in the hippocampus, transentorhinal cortex, cingulate cortex and temporal cortex in PD brains with a range of 4–14 years of disease duration. Furthermore, the numbers of activated microglia increased in the SN and putamen as the neuronal degeneration of the SN proceeded. In contrast, Mirza *et al.* (2000) showed activation of microglia only in the SN of PD autopsies verified by immunohistochemical detection of MHC-II and ferritin. Overall clinical and animal studies have supported the role for activated microglia in PD (reviewed by Collins, 2012). In *in vivo* studies, where PET was utilized in PD patients, activated microglia, which were identified with the translocator protein (TSPO) ligand PK11195, [1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide], the 'peripheral benzodiazepine-binding site' on activated microglia, showed increased binding potential,

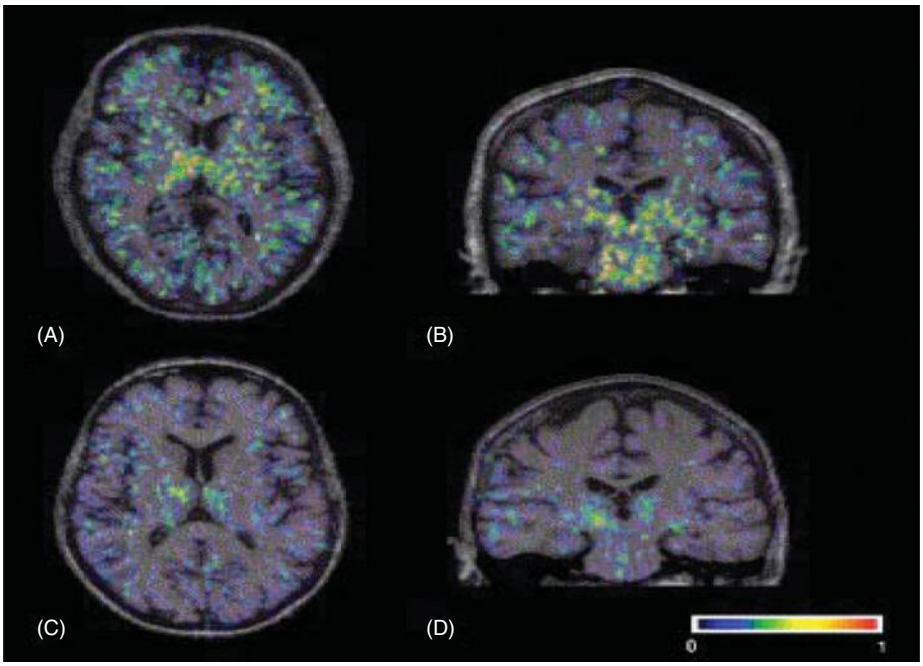

Figure 6.2 Transverse and coronal sections of binding potential maps co-registered to the individual MRI. In the PD patient (A and B), binding is increased in the basal ganglia, pons and frontal regions, while the healthy control person (C and D) only shows constitutive [11C](*R*)-PK11195 binding in the thalamus and pons. (From Gerhard, A., Pavese, N., Hotton G., *et al.*, 2006. In vivo imaging of microglial activation with [11C](*R*)-PK11195 PET in idiopathic Parkinson's disease. Neurobiology of Disease 21, 404–412, Elsevier.)

values only evident in the midbrain of PD patients in early disease stages (Ouchi *et al.*, 2005) (see Figure 6.2). In PD patients with more advanced disease, there were significant increases in mean [^{11}C](*R*)-PK11195 binding in the subcortical region, which included the striatum, pallidum, thalamus and pons (Gerhard *et al.*,2006). Such PET results indicated that there might be different patterns of microglia activation between early- and late-stage PD patients. In addition, these results indicated that microglia become activated very early in the disease process and remained activated for a long duration.

Only a certain neuronal cell population (i.e. the dopaminergic neurons in the SN) are affected in PD, which is caused by the pro-oxidative state in which these neurons function relative to other brain regions; that is, they have an increased metabolic rate and a high content of (i) oxidizable products, such as dopamine; (ii) iron; and (iii) polyunsaturated fatty acid, but also, most importantly, a low content of antioxidants such as reduced glutathione (GSH) (reviewed by Niranjan, 2013). It seems that once the dopaminergic neuron destruction has begun, a self-renewing cycle of microglia activation

(i.e. microgliosis) ensues. The vulnerability of these dopaminergic neurons was demonstrated with *in vitro* studies of mixed mesencephalic neuron–glia cultures where the soluble neuron factor, mu-Calpain, was released from damaged dopaminergic neurons, which activated microglia via activation of NADPH oxidase (Levesque *et al.*, 2010).

Such activated microglia in the nigrostriatal dopamine region will release prostaglandins, chemokines, enzymes associated with inflammation (e.g. iNOS and COX2) as well as pro-inflammatory cytokines (e.g. TNFα, IL1β, IL2, IL4, IL6, transforming growth factor alpha (TGFα), TGFβ1 and TGFβ2) (Dexter, 2013). Decreased secretions of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) by the microglia will further compromise the neurons. In addition, the death-signalling receptor TNFR1 is expressed on dopaminergic neurons in human SN, which will contribute to their demise; blockade of this receptor in animal studies attenuated the death of dopaminergic neurons (reviewed in Collins, 2012).

The phagocytosis of extracellular aggregated α-synuclein will further activate microglia and propel dopaminergic degeneration (Zhang *et al.*, 2005). *In vivo* and *in vitro* studies where extracellular α-synuclein was either incubated with BV2 microglia cells or intra-nigrally injected into mouse brains, respectively, resulted in a positive NF-κB response with the production of pro-inflammatory cytokines (Couch *et al.*, 2011). Furthermore, it has been suggested that the α-synuclein had a 'priming effect' on the microglia which will make them more susceptible to subsequent challenges from direct environmental pro-inflammatory challenge or via peripheral systemic inflammation (Morgan and Liu, 2011). In addition to microglial activators, there may also be changes in several anti-inflammatory systems in PD that play a role in regulating microglia activation. These include the CD200–CD200R receptor, the vitamin D receptor, PPARs and the soluble receptor for advanced glycation end products (reviewed by Ward *et al.*, 2011).

Astrocytes in PD

Astrocytes, the most numerous of the glial cells, are a major class of cells in the mammalian brain, outnumbering neurons by several-fold (Sherwood *et al.*, 2006). Astrocytes are present in all brain regions and in close contact with neuronal structures, playing a critical and integral role in mediating the physiologic and pathologic states of neurons and the integrity of the BBB. Astrocytes can release and supply various neurotrophic factors (e.g. NGF, neurotrophin-3), metabolic substrates (e.g. lactate) and reduced GSH to ensure the survival and correct functioning of the neurons. It is of note that the concentration of GSH in astrocytes (approximately 3.8 mM) is estimated to be higher than that of neurons (approximately 2.5 mM) (Rice and Russo-Menna, 1998), probably as a result of higher specific activity of the γ-glutamylcysteine synthetase in astrocytes (Gegg *et al.*, 2003). Uniquely astrocytes can release this antioxidant

into the extracellular space to adjacent neurons via the transporter multidrug resistance protein, where it is cleaved by γ-glutamyltranspeptidase on the astrocytic plasma membrane, to generate precursors for neuronal GSH synthesis. The GSH content in the SN of PD patients has been observed to be significantly reduced (40%) (Sian *et al.*, 1994), although the explanation for this loss remains unclear (reviewed by Rappold and Tieu, 2010). In addition, astrocytes are positioned close to synaptic clefts to remove excess glutamate, as well as potassium and calcium. However, if activated in response to neuronal damage or a toxic insult, cytokines and chemokines will be released which can have an adverse effect on neurons (reviewed by Rappold and Tieu, 2010). Although Mirza and colleagues (2000) could not identify reactive astrocytosis in SN and putamen of post-mortem tissues from PD patients, later studies revealed an active astrogliosis (reviewed by Niranjan, 2013) that could maintain the degeneration of the dopaminergic neurons. In support of this, astrocytic α-synuclein-immunoreactive inclusions have recently been noted to develop in sporadic PD. α-synuclein-labelled astrocytes occur preferentially in prosencephalic regions (the amygdala, thalamus, septum, striatum, claustrum and cerebral cortex). The topographical distribution pattern of these astrocytes closely parallels that of the cortical intraneuronal Lewy neurites and Lewy bodies (Braak *et al.*, 2007). Mice injected with the toxin (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (MPTP) and then killed at different time points showed a direct correlation between reactive astrocyte formation and dopaminergic structure destruction in both striatum and SN. Interestingly, although the neuronal death ultimately declined, GFAP) expression remained up-regulated (reviewed by Niranjan, 2013). Other brain regions (e.g. the frontal cortex and caudate nucleus) also showed an increase in expression of GFAP in PD brains, although the authors concluded that these astrocytes protected non-SN brain regions from oxidative and mitochondrial damage (Mythri *et al.*, 2011). That astrocytes play an important regulatory role in PD is indicated by the finding that astroglial-derived GDNF is a potent inhibitor of microglial activation, and that there are two different types of reactive astrogliosis which may be important in the pathogenesis of PD (reviewed by Niranjan, 2013). Therefore, astrocyte proliferation may protect or exacerbate SN neuronal loss, although further studies are needed for clarification. The 30% increase in astrocytes in the SN of PD brains might indicate an attempt at neuroprotection in response to oxidative damage (Mythri *et al.*, 2011).

Oligodendrocytes (OLs) in PD

OLs are mature glial cells that myelinate axons in the brain and spinal cord and are essential for efficient neuronal signalling. These cells, together with their precursor cells, are particularly vulnerable to inflammation and oxidative stress, and elevated glutamate levels leading to excitotoxicity (McTigue and

Tripathi, 2008), such that these cells may become dysfunctional in PD, especially in late stages of the disease. Increasing levels of iron and/or α-synuclein within these cells could contribute to neurodegeneration.

Molecular mediators in microglia activation

Cytokines in PD The pro-inflammatory cytokine IL1β is present in the periphery, brain and CSF of PD patients. IL1β gene polymorphisms have been associated with an earlier age of onset in sporadic PD in some but not all studies. The toxic action of IL1β on dopaminergic cell loss within the SN will be dependent on a number of variables, which include its concentration and the duration of expression. Indeed, experimental models, where sustained IL1β expression in the SN was achieved using adenoviral vectors, showed induction of neurodegeneration and motor symptoms after 21 days, although IL1β expression was detectable at 7 days (reviewed by Leal *et al.*, 2013). Although IL1β mRNA levels are increased after 6-hydroxydopmaine (6-OHDA) nigrostriatal administration, this is not reflected at the protein levels. However, this transcriptional block can be removed by the administration of LPS, thereby confirming the hypothesis of Perry, of an involvement of 'primed' microglia in the aetiology of PD (reviewed by Leal *et al.*, 2013).

High levels of TNFα are present in the CSF and post-mortem brains of PD patients as well as in the various animal models of PD (i.e. 6-OHDA and MPTP) (reviewed by Leal *et al.*, 2013). Although overexpression of TNFα via adenoviral vectors in the SN or acute TNFα administration did not adversely affect dopaminergic neurons, sustained long-term expression of TNFα levels did evoke a toxic effect in the SN (reviewed by Leal *et al.*, 2013).

Cytokines may alter dopaminergic neuronal functions; for example, IL1β increases intracellular calcium in C6 rat astrocytes, IL1α and IL6 promote growth of neuronal terminals in a mouse model, while IL6 and its receptor are involved in the regulation of VCAM1 gene expression in astrocytes (reviewed by Niranjan, 2013). Interestingly, there is normally a high density of microglia in the midbrain relative to other brain regions which, together with the expression of MHC-I and β2-microglobulin in the SN, may be responsible for the robust microglia activation in this region. Animal studies where toxins have been used to induce PD-type lesions showed that microglia were activated before a loss of tyrosine hydroxylase was evident (Marinova-Mutafchieva *et al.*, 2009) and that these were activated earlier than astrocytes and reached a peak before dopaminergic neurodegeneration (reviewed by Niranjan, 2013).

Complement in PD Complement proteins, in addition to all of the other components of the membrane attack proteins, have been identified within Lewy bodies and oligodendrocytes in the SN of PD patients. Complement could promote the secretion of pro-inflammatory cytokines from glial cells (Whitton, 2007).

Matrix metalloproteinases A variety of mediators will be released from damaged dopaminergic neurons. These include MMPs such as MMP3 and MMP9 (Nolan *et al.*, 2013) which will exert anti-inflammatory effects by transrepression of NF-κB as well as regulate oxidative stress pathways.

Adaptive immunity in PD

Many studies have associated changes in the adaptive immune system with PD progression. There is a high glia–neuron ratio of 3:1 in the brain, with the density of microglia within the SN being the highest of any region in the brain. Therefore, any increase in the inflammatory status of the patient may additively, if not synergistically, amplify neuroinflammation. In early studies, McGeer *et al.* (1998a) identified cytotoxic T cells in the SN of one PD patient, and found high expression levels of polymorphic MHC-II molecules, HLA-DR and HLA-DQ expressed by monocytes in the CSF and blood of PD patients compared to controls (McGeer *et al.*, 1998b). Hunot *et al.* (1999) showed a dramatic increase of IFNγ-positive cells in the brains of PD patients which were indicative of the involvement of T cell mobilization in the nigrostriatal injury in PD. In one further study, Brochard *et al.* (2009), identified higher densities of $CD8^+$ and $CD4^+$ T cells post-mortem in PD brains. This could indicate that there are changes in the function of the BBB (the CD4–CD8 ratio in PD was 1:5 compared to the 2:1 ratio for peripheral T cells in controls) which would allow peripheral cells to enter the brain parenchyma. However, the exact mechanisms by which these T cells gain access to the SN remain to be answered.

A variety of antibodies directed against globally expressed tissue antigens such as heat shock protein 65 (HSP65) and HSP70 have been identified in PD patients as well as brain-associated autoantibodies, including those directed against GM1, S100B, glial fibrillar acidic protein (GFAP), NGF, neurofilament, myelin basic protein, tau, Aβ and neuronal calcium channels, together with α-syn and its modified and fibrillary forms (reviewed by Lee Mosley *et al.*, 2012). Extraneuronal nitrated α-synuclein is able to cross the BBB to the CSF, where it will activate antigen-presenting cells (i.e. naïve T cells) (reviewed by Kosloski *et al.*, 2010). With appropriate costimulatory signals, these cells will differentiate into Teffs that will expand into different effector cell subtypes (e.g. Th1 and Th17 cells) (reviewed by Kosloski *et al.*, 2010). Such cells will drive the disease processes towards a pro-inflammatory situation. Th1 cells that express IL2, IFNγ and TNFα will be pro-inflammatory and activate microglia. Th2 effector cells release IL4, IL5 and IL13 and support anti-inflammatory responses. Th17 will also elicit a pro-inflammatory effect (Kosloski *et al.*, 2010) and secrete granzyme B, a cytolytic enzyme (Kebir *et al.*, 2007). In addition Th1, Th2 and Th17 help in the production of antibodies which specifically target modified proteins for their removal by microglia. Th1 and Th17 Teffs are synthesized in the periphery, traverse the BBB to

the inflammatory foci of the nigrostriatum and identify the N-α-synuclein and MHC-II which are presented by the antigen-presenting microglia. The induction of Teffs will drive the microglia and the innate immune responses.

T cell infiltration occurs in the CNS tissue of PD. Nitrated α-synuclein may activate peripheral leucocytes and mediate the adaptive immune system to potentiate microglial activation. Several changes in cellular and humoral immune responses are reported to occur in the peripheral immune system of PD patients, although no clear demonstration of leucocyte involvement at the site of the neuronal damage has been reported (Brochard *et al.*, 2009). In a recent study, Castellani *et al.* (2011) identified a subunit of CD3, part of the T receptor complex (TRC) in mature T cells, in Lewy bodies in PD. This subunit of CD3 has also been shown to be involved in dendritic outgrowth and synaptic formation, thus raising the possibility that CD3 dysregulation is a pathogenic factor in PD.

Role of oxidative stress in PD Considerable evidence supports a role for oxidative stress in the initiation of the nigral dopamine-producing neuronal loss, as either a primary or secondary event of the disease (see Figure 6.3). A considerable number of markers of oxidative stress have been identified within the SN, which include lipid peroxidation products (e.g. malondialdehyde, 4-hydroxynonenal and advanced glycation end products), protein and DNA oxidation products (carbonylated proteins and 8-hydroxyguanosine), altered activities of cytoprotective enzymes, mitochondrial SOD and GSH peroxidase as well as antioxidants such as GSH (Dexter and Jenner, 2013). Ageing is associated with increased oxidative stress such that nigral dopaminergic neurons will be particularly vulnerable, even in normal circumstances, because of the decreased levels of GSH and increased iron content (Crichton and Ward, 2013). Impaired mitochondrial function of complex I in the SN has been identified (Schapira *et al.*, 1990), which will increase the release of O_2^- as well as other free radical products by the impaired electron flow from NADH to ubiquinone. Clinically untreated PD patients show decreased mitochondrial activity of complexes I and III (reviewed by Niranjan, 2013). Such deficiency of complex I was not observed in other non-SN dopaminergic regions in PD brains (Mythri *et al.*, 2011). The vulnerability of SN neurons to mitochondrial complex I deficiency and neurodegeneration has been related to significant GSH depletion. GSH depletion in the SN is possibly the first sign of oxidative stress during PD, with parallel increases in ROS and perhaps an early event in the development of PD.

In addition to mitochondrial dysfunction, NADPH oxidase, a multi-protein electron transport system that produces large amounts of O_2^- via the reduction of molecular oxygen, has also been implicated. NOX2, one of the NOX isoforms involved in the formation of the NADPH oxidase complex, may play an important role in the loss of dopaminergic neurons as evidenced by animal

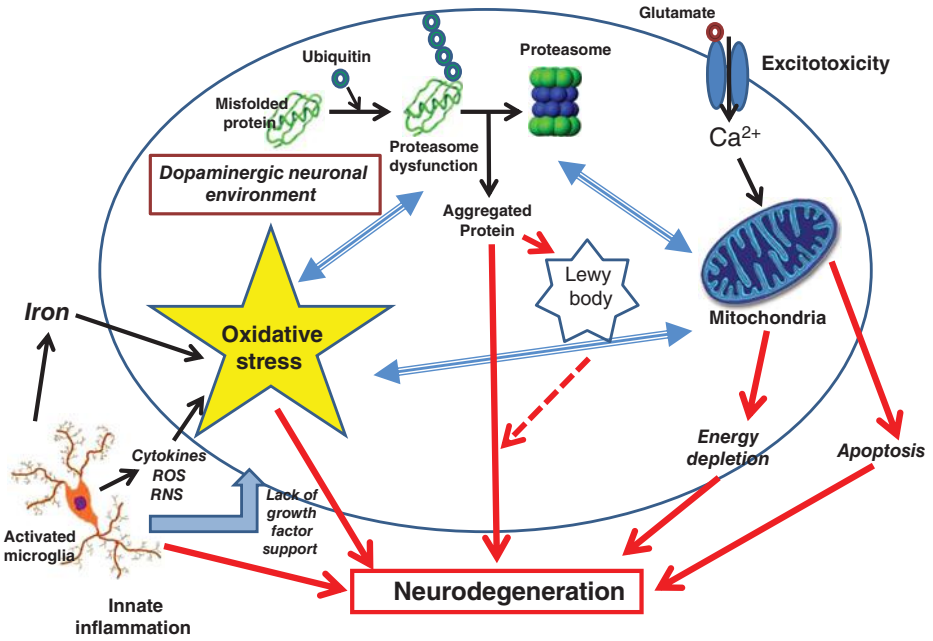

Figure 6.3 Mechanisms of oxidative stress and inflammation in Parkinson's disease. Inflammation-induced pathways are linked to oxidative stress. Excessive production of alpha synuclein will lead to misfolding of the protein and proteasomal dysfunction with alpha synuclein dysfunction and Lewy body formation, which may contribute to excessive oxidative stress and neurodegeneration. Microglia, activated by the dopaminergic neuronal destruction, will release pro-inflammatory cytokines as well as reactive oxygen species (ROS) and reactive nitrogen species which will contribute to the oxidative milieu. The increase in iron will exacerbate ROS production. An increase in glutamate will induce changes in calcium homeostasis leading to mitochondrial dysfunction.

models of PD, such as the MPTP and 6-OHDA models (reviewed by Hernandes and Britto, 2012).

Neuroinflammation can induce oxidative stress via production of high levels of ROS and reactive nitrogen species (RNS) by activated glial cells and via arachidonic acid signalling via the activation of cyclooxygenase and lipoxygenase pathways. COX1 is up-regulated in activated microglia while COX2, regulated by pro-inflammatory mediators, can catalyse the oxidation of cytosolic dopamine. Prostaglandins (J2 series) can induce oxidative stress by inducing decreases in GSH and GSH peroxidase, decreasing mitochondrial membrane potential and increasing lipid peroxidation (e.g. increased levels of 4-hydroxy-2-nonenal) (reviewed by Niranjan, 2013).

Dopamine is normally stored in vesicles to protect it from breakdown, as any cytosolic dopamine can be readily oxidized both spontaneously and enzymatically to produce DA quinone. Such oxidation will contribute to the

vulnerability of dopaminergic neurons, because this may cause inactivation of the DA transporter and tyrosine hydroxylase, and also induce mitochondrial dysfunction (reviewed by Hwang, 2013). In addition, dopamine may be metabolized by monoamine oxidase to form hydrogen peroxide and dihydroxyphenylacetic acid (DOPAC). Alternatively, dopamine could be autooxidized in the oxygen-rich environment to produce O_2^-, which could react with NO to produce peroxynitrite or be dismutased by SOD to hydrogen peroxide (reviewed by Taylor *et al.*, 2013).

A twofold increase of iron in the SN (bound within either H-ferritin or neuromelanin) may enhance intracellular aggregation of α-synuclein, leading to the formation of advanced glycosylation end products as well as contributing to the oxidative stress (Crichton & Ward, 2006). Neuromelanin, a granular dark brown pigment, is produced in catecholaminergic neurons of the SN pars compacta (SNc) and locus coeruleus (Götz *et al.*, 2004), and it is of unknown function, although it may play a protective role via attenuation of free radical damage by binding transition metals, particularly iron. The neuromelanin–iron complex increases linearly with age in the SNc (aging being a predisposing factor for PD), which, if released from damaged dopaminergic neurons, will trigger microgliosis, microglial chemotaxis and activation with the subsequent release of neurotoxic mediators (reviewed in Crichton and Ward, 2006). Recent immunohistochemical data indicated that the influenza A virus is associated within neuromelanin granules in the SN brain sections from post-mortem PD brain (Rohn and Catlin, 2011). This is of interest because infectious agents have emerged as possible candidates for serving as external triggers for initiating the pathology in underlying idiopathic PD.

Over the last decade, there has been continuing debate as to whether ROS can interact with the pathway which activates NF-κB translocation to the nucleus to release a number of pro-inflammatory cytokines. Recently, it has been suggested that ROS have various inhibitory and stimulatory roles with NF-κB signalling pathways (Morgan and Liu, 2011). If such ROS were stimulatory in NF-κB activation, this would enhance the production of a variety of pro-inflammatory mediators, thereby enhancing the perpetual cycle of neuroinflammation.

The contribution of the peripheral immune response to the exacerbation and promotion of the chronic dopaminergic degeneration has recently gained some credence (Collins, 2012). Some immune alterations in patients with PD have been reported, such as impaired ability of PD peripheral blood phagocytic cells to engulf latex particles (Salman *et al.*, 1999), increased levels of $CD4^+$ T lymphocytes and elevated levels of certain pro-inflammatory cytokines, TNFα, IL1β and IL6, in both mononuclear cells (Bessler *et al.*, 1999) and the plasma (Ward and Dexter, unpublished data). Interestingly, *in vitro* administration of DOPA to mononuclear cells modified the release of IL6 and TNFα (Bessler *et al.*, 1999). Increased levels of RNS are also present in the

SN of PD patients, which are possibly reflected by increases in nitrite in the CSF (Qureshi *et al.*, 1995). Reaction products with NO, such as 3-nitrotyrosine adducts, have also been identified in SN of PD as well as extensive and widespread accumulations of nitrated α-synuclein (Giasson *et al.*, 2000).

Neuroinflammation in Huntington's disease

In HD the mutant Huntingtin protein (Htt), aggregates are associated with neostriatal atrophy and massive neurodegeneration in the putamen and caudate (Vonsattel *et al.*, 1985). The pathogenic mutant Htt aggregates may be recognized as foreign bodies by microglial cells. It is also possible that mutant Htt aggregates cause neuronal death through apoptosis, and that the apoptotic bodies can then activate microglia and the CNS innate immune system. However, in pre-manifest HD, where the carriers of the gene do not exhibit the classical signs and symptoms of the typical HD patient, the presence of activated microglia in the striatum as a result of mHtt aggregation induces early neuronal dysfunction, which includes elevated pathogenic extrasynaptic NMDA receptor signalling, reduced synaptic connectivity and loss of BDNF (Milnerwood and Raymond, 2010).

Microglia in HD

Inflammatory processes have been clearly demonstrated in HD pathophysiology, although the role of chronic neuroinflammation in HD pathogenesis is not fully understood (Moller, 2010). Post-mortem studies have shown high levels of activated microglia close to degenerating neurons (McGeer *et al.*, 1988; Messmer and Reynolds, 1998; Singhrao *et al.*, 1999; Sapp *et al.*, 2001). Increased levels of IL6 are present in the serum and CNS approximately 16 years before the onset of symptoms in HD patients (Björkqvist *et al.*, 2008), and its level correlates with disease development. Other cytokines, including IL1β, IL8 and TNFα, are also up-regulated in striatum and plasma of HD patients as well as in animal models of HD (Björkqvist *et al.*, 2008; Silvestroni *et al.*, 2009), which is indicative of an inflammatory component in HD (Dalrymple *et al.*, 2007; Björkqvist *et al.*, 2008; Wild *et al.*, 2011). Activated microglia, a likely source of elevated cytokines in the CNS, are detected in preclinical HD brains, and their accumulation coincides with striatal neuronal dysfunction (Tai *et al.*, 2007).

A major inducer of these inflammatory mediators is the I kappa B kinase (IKK)–NF-κB pathway, which is dysregulated in HD (Khoshnan *et al.*, 2004; Hacker and Karin, 2006). Elevated IKKβ activity is widespread in the CNS of R6/2 mice, a genetic mouse model of HD which expresses a toxic N-terminal fragment of mutant Htt (Khoshnan *et al.*, 2004; Zuccato and Cattaneo, 2010). High levels of pro-inflammatory cytokines, including IL6, IL1β and TNFα, are found in the serum and CNS of these animals, consistent with a

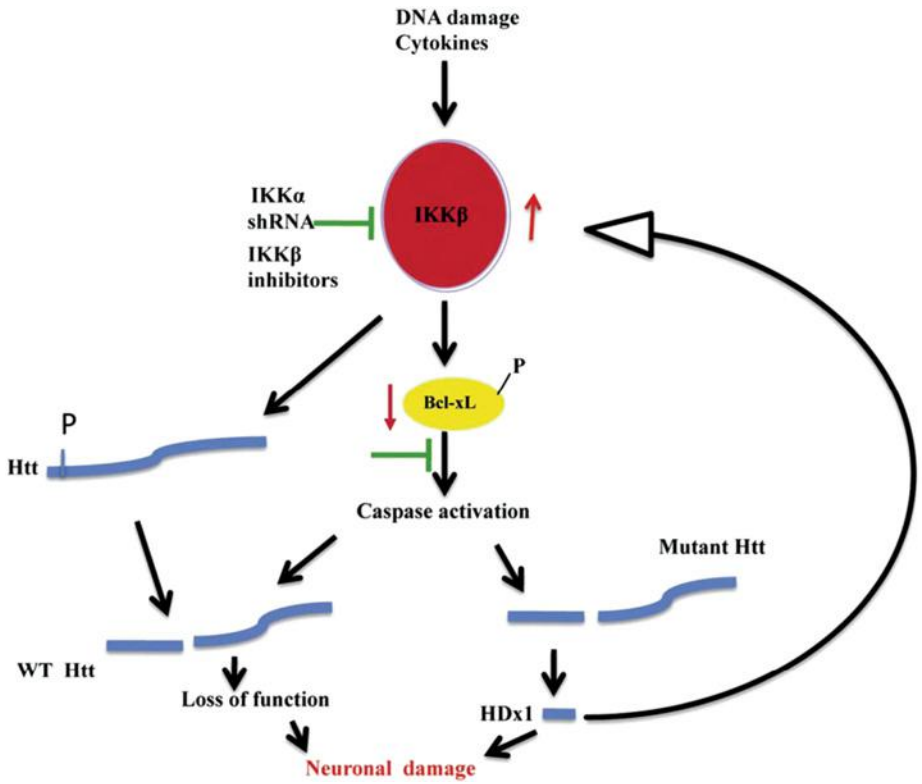

Figure 6.4 A schematic diagram illustrates the signalling pathway for IKKβ-mediated Htt proteolysis. DNA damage or cytokines activate IKKβ, which can phosphorylate Bcl-xL and reduce its level. Reduction of Bcl-xL levels triggers the activation of caspases, which cleave Htt. Inhibition of IKKβ or elevation of IKKα and Bcl-xL prevent Htt proteolysis. N-terminal fragments of mutant HDx1 could further stimulate IKKβ by direct binding to the IKK complex (Khoshnan *et al.*, 2004). These interactions could form a persistent cycle of IKK activation and Htt cleavage. (From Khoshnan, A., Patterson, P.H., 2011. The role of IκB kinase complex in the neurobiology of Huntington's disease. Neurobiology of Disease 43, 305–311. Copyright 2011 with permission from Elsevier.)

deregulated IKKβ–NF-κB pathway (Björkqvist *et al.*, 2008). The core IKK complex has two kinases, IKKα and IKKβ, and a regulatory subunit, IKKγ. IKK is induced by various stimuli, including cytokines, oxidative stress and DNA damage, and it phosphorylates the inhibitors of κB (IκB), a family of proteins that sequesters NF-κB in the cytoplasm. IKKβ is the predominant kinase responsible for inflammatory responses, and it has been proposed that IKKβ may also directly regulate the neurotoxicity of Htt (see Figure 6.4). Activation of IKKβ by DNA damage triggers caspase-dependent cleavage of WT and mutant Htt and enhances the accumulation of oligomeric fragments.

Moreover, the N-terminal fragments of mutant Htt (HDx1) directly bind to and activate IKKβ. Thus, the IKKβ-dependent cleavage of full-length mutant Htt and the build-up of HDx1 could form a deleterious feed-forward loop (Khoshnan and Patterson, 2011). Activated microglia can be distinguished by a number of morphological and immunophenotypic changes (Dheen *et al.*, 2007; Ransohoff and Perry, 2009) which include up-regulation of the expression of the 18 kDa TSPO (Chen and Guilarte, 2008; Cosenza-Nashat *et al.*, 2009; Scarf *et al.*, 2009), which can be detected *in vivo* using PET imaging with the selective TSPO radioligand ^{11}C-PK11195 (Benavides *et al.*, 1988; Pike *et al.*, 1993; Banati *et al.*, 1999). The neuropathological signs of HD, visible in many structures of the CNS, include a prominent loss of neurons associated with protein aggregates composed of the mutated form of the Htt protein, accompanied by significant activation of microglia (Sapp *et al.*, 2001). Increased microglial activation is found both in HD patients and in pre-manifest HD gene carriers (Pavese *et al.*, 2006; Tai *et al.*, 2007; Politis *et al.*, 2008, 2011), and there appears to be a correlation between the level of microglial activation and disease severity (Pavese *et al.*, 2006).

Astrocytes and oligodendrocytes in HD

There is an important astrocytic response in HD, and in the early descriptions of the pathology of HD, the original grading system of striatal atrophy and disease severity by Vonsattel and DiFiglia (1998) used this response. The astrocytic response follows neuronal loss such that the striatal atrophy is mirrored by the intensity of the astrocytosis.

It has been hypothesized that the pathogenesis of HD begins with a deleterious effect of the mutant Htt on myelinated neurons, particularly in the striatum (Bartzokis *et al.*, 2006, 2007). Myelin breakdown leads to a failure of afferent transmission causing the underlying neurons to be overstimulated by its efferent feedback, and the resulting excitotoxicity eventually destroys the neurons. Throughout life, oligodendrocytes continue developing and increase their numbers (Peters and Sethares, 2004), and in HD, in their effort to repair and remyelinate, they become markedly elevated in numbers, which is often observed years before the appearance of symptoms (Myers *et al.*, 1991; Sotrel *et al.*, 1991; Gomez-Tortosa *et al.*, 2001). Iron is required for myelination, and because oligodendrocytes have the highest iron content of all brain cells (reviewed in Bartzokis, 2004; Zecca *et al.*, 2004), there is an increase in iron levels in early myelinating regions like the basal ganglia (Dexter *et al.*, 1992; Bartzokis *et al.*, 1999). These increased iron levels may add to the neuronal excitotoxicity by promoting free radical generation (Crichton and Ward, 2006). Elevated brain iron in HD has been well documented (Bartzokis *et al.*, 1999; Dumas *et al.*, 2012), notably in dystrophic microglia in the form of ferritin (Simmons *et al.*, 2007).

Molecular mediators

Cytokines Pro-inflammatory cytokines, such as IL6, IL8 and TNFα, seem to be clearly involved in HD, because they are detected in the peripheral nervous system of HD patients, irrespective of their disease state (Dalrymple *et al.*, 2007; Bjorquist *et al.*, 2008). Up-regulation of other innate immune proteins, such as α_2-macroglobulin and clusterin, has also been detected (Dalrymple *et al.*, 2007).

ROS in Huntington's disease Excitotoxicity is a phenomenon driven by excessive synaptic accumulation of glutamate and associated with dysregulation of intraneuronal Ca^{2+} ($[Ca^{2+}]_i$) homeostasis (Choi, 2005). A major feature of excitotoxicity is the N-methyl-D-aspartate receptor (NMDAR)/Ca^{2+}-dependent enhanced generation of NO and ROS (Forder and Tymianski, 2009; Szydlowska and Tymianski, 2010). Evidence for oxidative damage has been found in the brain and peripheral blood of HD patients (Ma and Nicholson, 2004; Stoy *et al.*, 2005; del Hoyo *et al.*, 2006; Chen *et al.*, 2007; Klepac *et al.*, 2007) as well as in tissues from animal models of HD (Maksimović *et al.*, 2001; De Luca *et al.*, 2008). In the R6/2 transgenic mouse model of HD, increases in striatal lipid peroxidation parallel the progression of neurological phenotypes (Pérez-Severiano *et al.*, 2000), and DNA damage has been found (Bogdanov *et al.*, 2001). Antioxidant treatments in these HD transgenic mice reduce protein aggregates and improve motor performance (Schilling *et al.*, 2001: Stack *et al.*, 2006), although treatment of oxidative stress in HD patients was ineffective (Stack *et al.*, 2008). Brain levels of NOX, which generates ROS, were elevated in human HD post-mortem cortex and striatum and were highest in striatum of presymptomatic individuals compared to controls. Elevated NOX activity was also found in synaptosomes from cortex and striatum of $HD^{140Q/140Q}$ mice at 3, 6 and 12 months compared to age-matched controls (Valencia *et al.*, 2013). Mutant Htt co-localized at plasma membrane lipid rafts with gp91-phox, a catalytic subunit of NOX2, in the $HD^{140Q/140Q}$ mouse model. ROS overproduction from mitochodrial dysfunction in neurons is found in advanced HD, but is not observed in early stages (Guidetti *et al.*, 2001; Valencia *et al.*, 2010), and it was therefore suggested that NOX activity in lipid rafts may be an early and major source of oxidative stress and cell death in $HD^{140Q/140Q}$ neurons (Valencia *et al.*, 2013).

Complement in HD

Early studies found activated microglia throughout brain areas affected in HD, and the intensity of their accumulation coincided with the degree of disease progression (Sapp *et al.*, 2001); the precise mechanism involved thereafter in ultimate neuronal loss remained unclear. However, several studies have suggested a role for components of the immune system in the initiation

of gliosis and neurodegeneration (Haque *et al.*, 1997; Singrao *et al.*, 1999; Björqvist *et al.*, 2008). The first of these mechanisms involved in immune surveillance is the complement system, which is activated by mutant Htt, which is established as a key factor in several neurodegenerative diseases (Rus *et al.*, 2006). The expression of a number of components of complement, and of regulators of complement in HD brain samples with severe atrophy, does indeed suggest that the complement system is recruited in HD pathogenesis (Singrao *et al.*, 1999).

Conclusions

Clearly, neuroinflammation plays an important role in the toxicity and the progression of the disease process in AD, PD and HD, and these similarities in the inflammatory responses could be utilized to develop new therapeutic approaches for their amelioration. However, the underlying cause of the enhanced neuroinflammation in each of these diseases still remains unresolved (i.e. the misfolded proteins), such that these need to be reduced to remove the stimuli associated with the inflammatory responses.

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7
CNS Infections

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Introduction

Numerous microorganisms infect the human central nervous system (CNS). These encompass viral, bacterial, fungal and protozoan infections. In this chapter we focus primarily on neurological diseases caused by viruses and the virus-like agents, prions. Types of virus infection which occur in humans differ with both the geographical location and the immune status of the host. In Europe and North America, encephalitis due to viral infections is the predominant cause of CNS infection. Herpes viruses are the most common cause of CNS infection in temperate climates, but new and emerging infections which can result in CNS infection are spreading due to sociological factors, climate change and opportunity in immunosuppressed individuals.

While poliovirus has been mainly eradicated from developed countries due to an effective vaccination policy (Minor, 2012), other CNS infections are becoming more frequent. Enterovirus 71 (EV71), in the same picornavirus family as polio, has emerged as an important human pathogen which may cause severe neurological complications and has caused death in children in outbreaks in the Asia–Pacific region during the past two decades (Solomon *et al.*, 2010; Wilson, 2013). The World Health Organization has set regional elimination goals for measles virus (MV) eradication to be achieved by 2020 or earlier. However, there are concerns that zoonotic infection from the closely related and more neurovirulent veterinary morbilliviruses, particularly canine

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distemper virus, could occur (Cosby, 2012). Infections spread as a result of climate change by allowing the insect vectors to, for example, move northwards are exemplified by West Nile virus (WNV), Japanese encephalitis virus (JEV), the newly emerging Toscana virus and enterovirus-71 (EV71) (Charrel *et al.*, 2005; Tyler, 2009; Wang *et al.*, 2012). Transplant-associated cases of viral CNS infection caused by WNV, rabies virus, lymphocytic choriomeningitis–like viruses and human herpes virus-6 (HHV6) are becoming more commonplace (Wilson, 2013). JC virus (JCV) is associated with the occurrence of progressive multifocal leukoencephalopathy (PML) and is also associated with immunomodulatory therapies but has a high occurrence in HIV/AIDS patients.

The aim of this chapter is to give an overview of the major virus infections of the human CNS, focussing on the related neuroimmunology. A summary table is included (Table 7.1) to guide the reader through the virus groups and summarize the related diseases. For particular virus groups, extensive studies have elucidated underlying mechanisms of brain infection and the associated immune response, while information for other viruses is lacking. The information in this chapter reflects this balance.

Herpes viruses

Human herpes viruses (HHVs; Table 7.1) cause a range of acute, subacute and long-term neurological diseases in both immunocompetent and immunocompromised individuals. This is manifested most commonly as encephalitis, but myelitis is also associated with varicella zoster virus (VZV), human cytomegalovirus (HCMV), Epstein–Barr virus (EBV) and human herpes simplex virus-2 (HSV2). EBV can also cause CNS lymphomas and VZV leukoencephalitis in immunocompromised patients (Kleinschmidt-DeMasters and Gilden, 2001).

Herpes simplex viruses-1 and -2

Herpes simplex virus-1 (HSV1) is the most common cause of cold sores (herpes labialis) and herpes simplex-2 (HSV2) of genital herpes. Herpes simplex encephalitis (HSE) is the commonest fatal sporadic encephalitis in humans in Western countries (Rozenberg, 2013), with HSV1 being the primary cause of HSE with an estimated 2–4 cases per one million individuals in Western countries (Conrady *et al.*, 2010). However, there is evidence from cerebrospinal fluid (CSF) testing that HSV2 infection in the CNS is becoming more common, particularly in younger age groups (Peter and Sevall, 2001). Other less common presentations such as a symmetrical brainstem inflammation with lesions associated with vasogenic oedema also occur (Miura *et al.*, 2009). In neonates and young children, disease presentation is more frequently diffuse and/or multifocal (Kleinschmidt-DeMasters and Gilden, 2001).

Table 7.1 Viruses, prions and neurological diseases in humans

Genome	Family	Genus or subgroup	Viruses	Neurological diseases
DNA, double stand	Herpesviridae	Alpha	HSV1/2, VZV	Sporadic encephalitis, myelitis latency in sensory ganglia, acute meningitis
		Beta	HHV6, HCMV	Encephalitis in immunocompromised, neurological complication in newborn
		Gamma	EBV	meningoencephalitis and myelitis
	Papovaviridae	Polyoma virus	JC	PML
RNA, single strand, negative sense	Paramyxoviridae	*Morbillivirus*	Measles	Post infection encephalitis, SSPE, MIBE
		Rubulavirus	Mumps	Meningitis
		Henipaviruses	Hendra, Nipah	Encephalitis with vasculitis
	Bunyaviridae	*Bunyavirus*	150 viruses transmitted by mosquitoes, including California group (e.g. La Crosse virus)	Encephalitis, meningitis
		Phlebovirus	50 viruses transmitted by mosquitoes or phlebotomine flies (e.g. RVFV and Toscana)	Encephalitis

(*continued*)

Table 7.1 (*Continued*)

Genome	Family	Genus or subgroup	Viruses	Neurological diseases
		Nairovirus	CCHV	Encephalitis
		Hantavirus	Transmitted by rodents (e.g. Hantaan and Puumala)	Encephalitis, meningitis, mononeuritis and polyneuritis, including Guillain–Barré syndrome
	Rhaboviridae	*Lyssavirus*	Rabies and bat lyssaviruses transmitted by animal bite	Encephalitis (furious and dumb forms)
RNA, single strand, positive sense	Togaviridae	*Alphavirus*	WEEV, VEEV, EEEV, WEV	Encephalitis
		Rubivirus	Rubella	Congenital defects, rare post-infection encephalitis
	Flaviviridae	*Flavivirus*	E.g. WNV, TBEV, JEV	Encephalitis
	Picornaviridae	*Enterovirus*	Poliovirus	Myelitis
		Enterovirus	CV, EV71	Encephalitis, meningitis, associated delayed neuropathologies
RNA with DNA intermediate	Retroviridae		HIV	Dementia, neuroAIDS, CNS infection due to numerous opportunistic infections
			HTLV1	Myelopathy, tropical spastic paraparesis
Infectious protein	Prions	Human diseases		CJD, vCJD, GSS, FFI, kuru

Genetic factors involved in HSE have been examined in susceptible DA and resistant PVG rats to infection. The calcitonin receptor protein was identified as potentially critical for infection and viral spread to the CNS and subsequent HSE development (Abdelmagid *et al.*, 2012). In addition, HSV1 CNS infection in the context of an Apo E4 phenotype has been linked to Alzheimer's disease (Dobson and Itzhaki, 1999; Wozniak *et al.*, 2009).

Following acute infection, HSV establishes latency in sensory ganglion cells (Bertke *et al.*, 2009) where it is thought to remain dormant until reactivation. However, it has been demonstrated that both HSV1 and HSV2 are shed intermittently in asymptomatic as well as symptomatic individuals in saliva (Scott *et al.*, 1997; Tronstein *et al.*, 2011). Furthermore, it has been found experimentally that HSV1 causes localized incomplete or low-level lytic infection causing a persistent immune response during latency (Halford *et al.*, 1996; Feldman *et al.*, 2002). Daily treatment with the antiviral drug acyclovir significantly reduces the localized immune response within the ganglion during latency (Halford *et al.*, 1997). Therefore, the virus continuously undergoes incomplete or low-level reactivation until such circumstances allow it to fully reactivate. Furthermore, Woszniak *et al.* (2009) have data (*in situ* PCR; Figure 7.1) which has led them to propose that the virus can also be reactivated in the CNS, in a similar manner to the peripheral nervous system, by events such as stress and general infections, and that repeated reactivation causes cumulative damage, which in individuals with a genetic predisposition can lead to Alzheimer's disease (AD). They also suggest that acyclovir should be used in clinical trials in the early stages of AD (Itzhaki and Wozniak, 2012).

Varicella zoster virus

VZV is usually acquired in childhood when it causes varicella (chickenpox), following which the virus establishes a latent state in trigeminal and dorsal root ganglia which lasts throughout life. VZV subsequently reactivates, spontaneously or after specific triggering factors, including immunosuppression, to cause herpes zoster (shingles), which may be complicated by post-herpetic neuralgia and several other neurological complications including vasculopathy (Gershon *et al.*, 2010; Kennedy and Cohrs, 2010).

Cytomegalovirus

Congenital cytomegalovirus (CMV) infection is the leading infectious cause of mental retardation and hearing loss due to infection *in utero* in the developed world; the virus also causes CNS complications in immunocompromised individuals (Tsutsui *et al.*, 2005; Cheeran *et al.*, 2009). CMV is also the commonest infectious agent to affect allograft recipients. The virus can be reactivated in immunosuppressed individuals such as patients with AIDS,

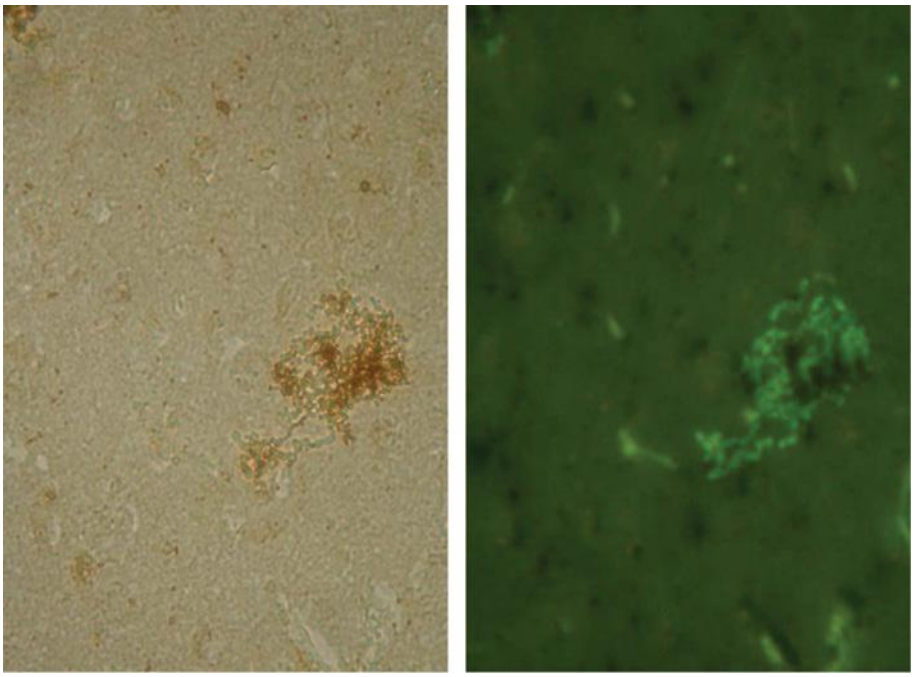

Figure 7.1 Herpes simplex virus type-1 (HSV1) DNA is localized in senile plaques of Alzheimer's disease brain. *In situ* polymerase chain reaction (PCR) was used to locate the HSV1 DNA in sections of human brain. In this method, which is far more sensitive than *in situ* hybridization, a digoxigenin-labelled probe is hybridized to the target sequence in the tissue section after PCR amplification; it thus allows simultaneous amplification and localization. To locate plaques, thioflavin S staining was used. Sections were viewed under fluorescent light to locate the plaques (green staining) and then under white light to reveal the HSV1 DNA (brown staining). (Wosniak and Itzhaki, unpublished data.)

transplant patients, elderly people and individuals admitted to intensive-care units (Griffiths, 2004).

In mouse models, it has been shown that neural stem and progenitor cells are the most susceptible to CMV infection in the developing brain. During brain development, lytic infection tends to occur in immature glial cells. In longer term infection, the virus preferentially infects neuronal cells and in developing brains it may become latent in neural immature cells. It has also been shown that brain disorders may occur long after infection by reactivation of the latent infection (Tsutsui *et al.*, 2005).

Epstein–Barr virus

EBV infects approximately 95% of the human population and persists latently in the memory B cell pool throughout life. EBV is the primary cause of

infectious mononucleosis. The virus is also associated with tumours, such as Burkitt's lymphoma, nasopharyngeal carcinoma and gastric cancers (Kaneda *et al.*, 2012). Neurological complications include meningoencephalitis and myelitis (Kleinschmidt-DeMasters and Gilden, 2001). It has been reported that a history of infectious mononucleosis approximately doubles an individual's risk of developing multiple sclerosis (MS) (Ascherio *et al.*, 2001; Lünemann and Münz, 2009) (see Chapter 9). However, whether EBV or other viruses are causal agents of MS is a long-standing but inconclusive area of research. In common with other viruses which have been implicated, disease mechanisms for EBV are hypothetical. These include (i) autoreactive T cells that could be activated by EBV through molecular mimicry, (ii) enhanced breakdown and presentation of self-antigens, (iii) expression of viral superantigens or (iv) bystander activation. Most contentious is a direct role for EBV infection in CNS inflammation as a consequence of potential selective enrichment of EBV-infected B cells in ectopic meningeal follicles and perivascular CNS inflammatory infiltrates in MS brain (Serafini *et al.*, 2007; Peferoen *et al.*, 2010; Owens *et al.*, 2011; Owens and Bennett, 2012).

Human herpes virus-6 (HHV6)

HHV6 infection is usually asymptomatic and commonly occurs during childhood. Clinically the virus causes exanthema subitum, also known as roseala infantum or the '6th disease' (Ward, 2005). However, HHV6 reactivation (as for CMV) can lead to serious systemic diseases, especially encephalopathy, that may be fatal in immune-compromised or grafted patients (Michael and Solomon, 2012). HHV6 transplacental infection can also lead to neurological complications in the newborn (Hall *et al.*, 2004). HHV6 DNA was detected in a total of 52 out of 126 (41.3%) nasal mucous samples from autopsy specimens from MS and cancer patients, showing that the nasal cavity is a reservoir for HHV6. Furthermore, specialized olfactory ensheathing glial cells were demonstrated to support HHV6 replication *in vitro*. These results indicate HHV6 utilization of the olfactory pathway as a route of entry into the CNS (Harberts *et al.*, 2011).

Immune response in herpes virus infections Evidence for the role of different aspects of the immune response in the CNS in herpes virus infections comes from clinical studies and *in vivo* or *in vitro* experimental models. Natural killer (NK) cell knockout mice or NK cell–depleted mice are susceptible to HSV-induced lesions in the CNS (Nandakumar *et al.*, 2008). Chemokines regulate leukocyte trafficking to inflamed tissues and play a crucial role in orchestrating the immune response to HSV1 infection. Control of viral infections is associated with production of CXCR3 and CCR5 agonists and recruitment of leukocytes bearing these receptors. However, single knockouts of CXCR3 and CCR5 exhibit a mild phenotype when compared to ablation of leukocyte

subsets associated with these receptors, suggesting functional redundancy between these two and most likely other chemokine receptors (Wuest and Carr, 2008). Intercellular adhesion molecule-5 (ICAM5), an immune modulator in the CNS, interacts with neurovirulence factor UOL as higher numbers of lymphocytes, but unaltered soluble ICAM5 and chemokine levels, were detected in ΔUOL HSV1-infected mouse brains. This suggests that ICAM5 plays a critical role in modulating chemokine production in the CNS (Tse *et al.*, 2009).

Humans and mice lacking signal transducer and activator of transcription-1 (STAT1) display increased susceptibility to HSV CNS infection (Pasieka *et al.*, 2011), while STAT4 has been shown to regulate antiviral interferon gamma (IFNγ) responses and disease severity during chronic HSV2 infections in humans (Svensson *et al.*, 2012). IFN lambda (IFNλ), a newly identified member of the IFN family (Kotenko *et al.*, 2003), has also been shown to have the ability to inhibit HSV1 infection of primary human astrocytes and neurons. Furthermore, IFNλ treatment of astrocytes and neurons inhibits or reduces suppressor of cytokine signalling-1 (SOCS1), a key negative regulator of the IFN pathway (Li *et al.*, 2011), which leads to increased IFN production.

Immune evasion in herpes virus infections Viral immune evasion has also been extensively studied with HSV. HSV1 glycoprotein E (gE) mediates cell-to-cell spread and functions as an immunoglobulin (IgG) Fc receptor (FcγR) that blocks the Fc domain of antibody targeting the virus or infected cell thus blocking both IgG Fc-mediated complement activation and antibody-dependent cellular cytotoxicity against the virus (Lubinski *et al.*, 2011) Therefore, gE promotes immune evasion from IgG Fc-mediated activities and probably contributes to virulence when antiviral antibody is present, such as during recurrent infections. HSV1 has been found to decrease immune cell types by causing apoptosis. The virus induces *de novo* expression of Fas L on the cell surface and causes the death of interacting human $CD4^+$ T cells, $CD8^+$ T cells and NK cells (Iannello *et al.*, 2011).

Immune suppression functions of specific virus proteins have also been identified. The HSV1-encoded glycoprotein B (gB) manipulates the MHC class II processing pathway by perturbing endosomal sorting and trafficking of HLA-DR molecules (Temme *et al.*, 2010). Furthermore, the viral proteins gC and gE have been shown to provide a shield against neutralizing antibodies that interfere with the interaction of the virus glycoproteins gB, gD and gH–gL. Fusion of the virus envelope with the cell plasma membrane, necessary for virus entry, is dependent on interaction of these four viral glycoproteins. The HSV1 latency-associated transcript (LAT) also appears to play a role in immune evasion. Using LAT(+) and LAT(−) infected mice, results were obtained which suggest a novel immune evasion mechanism whereby the HSV1 LAT increases the number of HSV1 antigen–positive dendritic cells

(DCs) in latently infected trigeminal ganglia, and interferes with DC phenotypic and functional maturation (Chentoufi *et al.*, 2012).

HCMV may persist by evasion of immune reactions which promote virus clearance. The viral glycoprotein UL141 targets the tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) death receptors preventing apoptosis of infected cells (Smith *et al.*, 2013). Viral immune evasion of antigen presentation by both MHC class I and II molecules occurs; by restricting the presentation of viral antigens during both productive and latent infection, HCMV limits elimination by the human immune system (Noriega *et al.*, 2012; Hesse *et al.*, 2013). HCMV encodes homologues of cellular cytokines and chemokines and their receptors, which facilitates viral evasion of immune clearance (McSharry *et al.*, 2012).

The EBV genes, BCRF1 and BNLF2a, encode the viral homologue of interleukin-10 (IL10) (vIL10) and an inhibitor of the transporter associated with antigen processing (TAP), respectively. Both of these factors are known immune-evasins in the lytic phase of EBV infection. vIL10 impairs NK cell–mediated killing of infected B cells, interferes with $CD4^+$ T cell activity and modulates cytokine responses. BNLF2a reduces antigen presentation and recognition of newly infected cells by EBV-specific $CD8^+$ T cells. Together, both factors significantly diminish the immunogenicity of EBV-infected cells during the initial, early phase of infection before latency is established. This may also reduce recognition of infected B cells in the CNS (Jochum *et al.*, 2012).

Mononegavirales

The mononegavirales are an order of viruses with single-strand negative-sense non-segmented RNA genomes (Table 7.1). Genera predominately associated with CNS infection are the morbilliviruses and the more newly recognized henipaviruses, within the family Paramyxoviridae. Mumps virus in the genus *Rubulavirus* and also within the Paramyxoviridae is primarily associated with meningitis rather than encephalitis. Lyssaviruses within the family Rhabdoviridae including rabies viruses are still a major cause of CNS infection in many countries. Bunyaviridae have negative-strand segmented genomes and include the newly recognized Toscana virus within the genus *Phlebovirus*, and are vector borne. The exception to this mode of infection are the hantaviruses which are spread by rodent bite but can also cause CNS involvement. One member of the hantaviruses, Puumala virus, is known to be associated with meningitis, encephalitis, mononeuritis and polyneuritis, including Guillain–Barré syndrome (Clement *et al.*, 2000).

Measles virus

Measles virus (MV), the only human pathogen within the morbilliviruses and one of the most infectious human viruses, remains an important cause of child

morbidity and mortality worldwide, with the greatest burden in the youngest children. Most acute measles deaths are due to secondary infections that result from measles-induced suppression of immune responses (Schneider-Schaulies and ter Meulen, 2002).Neurological complications with MV are much less frequent than with particular veterinary members of the genus, namely, canine distemper virus, phocine distemper virus and cetacean morbillivirus. However, MV causes an acute post-infection encephalitis in 1 in 1000 cases following primary infection. This is suggested to be due to an autoimmune response, as virus entry to the brain is not thought to occur. In addition, MV causes two fatal infections of the CNS, the long-term MV complication subacute sclerosing panencephalitis (SSPE) and measles inclusion body encephalitis (MIBE) (Cosby *et al.*, 2002). The latter occurs in immunocompromised individuals, including children who are HIV-positive (McQuaid *et al.*, 1998). Approximately 1 in 10,000 children (with a higher incidence in males) develop SSPE (Takasu *et al.*, 2003; Bellini *et al.*, 2005). This long-term persistent infection is most common in individuals who contract their initial measles infection before 2 years of age (Norrby and Oxman, 1990).

Neuropathology MV antigen and RNA is present in neurons, in oligodendrocytes and in a much lower percentage of astrocytes in SSPE brain (Figure 7.2). Viral RNA and antigens are also present in inflammatory cells in

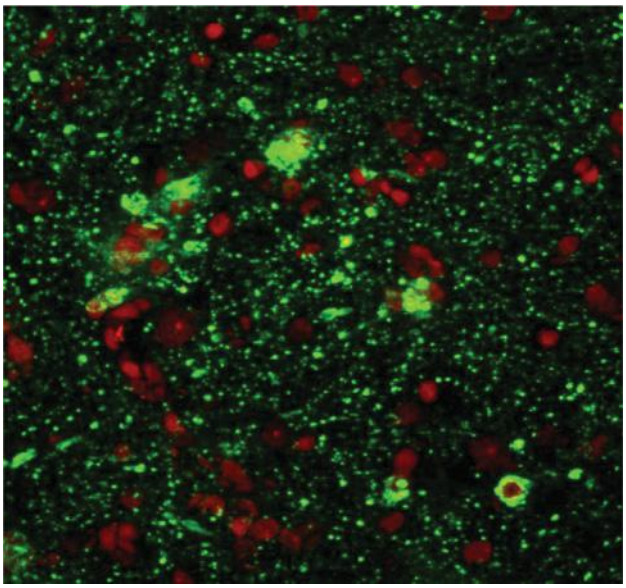

Figure 7.2 MV antigen visualized by confocal microscopy in the frontal lobe grey matter in SSPE brain. Virus is present in neurons, oligodendrocytes and extensive cellular processes. Inflammatory cells are seen surrounding virus infected cells. Virus (green); nuclei (red). (McQuaid and Cosby, unpublished data.)

perivascular cuffs in the infected brain (Cosby *et al.*, 2002). In the establishment of both SSPE and MIBE, the infection of brain endothelial cells (BECs) at the blood–brain barrier (BBB) is likely to play a major role in virus spread into surrounding brain tissue. A small number of BECs in some blood vessels in SSPE brain were shown to be infected (Kirk *et al.*, 1991) and it is also possible that the virus infects these cells in cases of measles but rarely enters the CNS unless individuals are immunocompromised. If the virus does cross the BBB and enters the neuronal network, spread has been shown to be transsynaptic. Exit from the cell by the budding and production of mature virus is not necessary, and the envelope proteins are not required (McQuaid *et al.*, 1998). This lack of selective pressure allows the accumulation of mutations in genes encoding these proteins, which further contributes to the persistent state (Baczko *et al.*, 1993).

Cytokines and chemokines A number of studies have examined cytokine and chemokines in SSPE brain or animal model systems. IFNγ and TNF alpha (TNFα) were found to be expressed in endothelial and glial cells (Anlar *et al.*, 2001). Furthermore, MV has recently been shown to up-regulate the virus receptor polio-like receptor-4 (PVRL4, also known as nectin-4) in human brain endothelial cells and cause apoptosis by induction of TRAIL (Abdullah *et al.*, 2013). The inflammatory lesions in SSPE consist of various cell subtypes and cytokines localized in particular regions of the brain, and these show certain associations with clinical course. Using *in situ* hybridization, IL2, IL6, TNF and leukaemia inhibitory factor (LIF) mRNA have been demonstrated in cells in the inflammatory infiltrate as well as in glial cells in foci of infection in cases of SSPE. LIF mRNA was also present in neurons in these areas. In the same study, LIF was also produced after MV infection in a human neuronal cell line (McQuaid *et al.*, 1997). In contrast, the type-I IFN inducible human MxA protein, which exhibits antiviral activity against a number of RNA viruses, including MV, was reported to be highly expressed in the area surrounding, but not in the centre of, MV-antigen-positive lesions in SSPE brains. This pattern of MxA expression indicates that newly infected cells release type I IFN and are demarcated by a protecting barrier of MxA-expressing cells. Expression of MxA was found mainly in the cytoplasm of astrocytes and did not correlate with the presence of infiltrates of inflammatory cells, although some lymphoid cells were positive for MxA (Ogata *et al.*, 2004). Proinflammatory chemokines were found to be produced in a transgenic mouse model of neuron-restricted MV infection as well as in neurons derived from these mice. The chemokines were IFNγ inducible protein (IP10), C–X–C motif chemokine-10 (CXCL10), monokine inducible by IFNγ (Mig), CXCL9 and RANTES (CCL5) (Patterson *et al.*, 2003). Overall, the above studies indicate that neurons as well as glia play important roles in the protective antiviral response to MV in the CNS.

T cell responses The pattern of inflammatory cell infiltration has been studied in the frontal brain biopsies of 28 cases of SSPE by immunohistochemistry. Lymphocytic infiltration and gliosis were common pathologic findings. $CD4^+$ T cells were observed in perivascular areas, and $CD8^+$ lymphocytes in the brain parenchyma. B cells were located in large perivascular cuffs associated with a longer and slower disease course (Anlar *et al.*, 2004). The T cell response in SSPE was also investigated by examining proliferation and cytokine secretion of cells from 35 patients and 42 healthy controls in response to virus and a number of CNS antigens. Proliferation in response to myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and the heat shock protein alpha B-crystallin (HSPB5) did not differ between the groups. IL12 secretion of peripheral blood leukocytes from SSPE patients to MV antigen was lower than in controls, and IFNγ, IL12 and IL10 production was also impaired in SSPE patients. The results did not demonstrate any bystander cellular response against myelin antigens, indicating that the CNS is not a predominant target of an autoimmune response in SSPE (Yentür *et al.*, 2005) unlike post-infection encephalitis.

Virus clearance A number of studies have been carried out to investigate MV clearance from the CNS. Investigation of humans with genetic or acquired deficiencies of either the humoral or cellular arm of the immune system, and rodent models, have implicated T cells in the control of ongoing MV infection. Using a transgenic mouse model in conjunction with depletion and reconstitution of individual B and T cell subsets alone or in combination, it was shown that neither $CD4^+$–$CD8^+$ T cells nor B cells alone are responsible for virus clearance. However, combinations of either $CD4^+$ T cells or B cells, or of $CD4^+$ and $CD8^+$ T cells, are essential but $CD8^+$ T cells with B cells are ineffective in control of virus levels. IFNγ and neutralizing antibodies to MV, but not perforin or TNFα alone, are associated with clearance of infection. TNFα combined with IFNγ is more effective in protection than IFNγ alone (Tishon *et al.*, 2006). A more recent study has shown that primed T cells directly act to clear MV infection in the CNS by using a non-cytolytic IL12 and IFNγ-dependent mechanism and that this mechanism relies upon Janus kinase (Jak)–STAT signalling (Stubblefield-Park *et al.*, 2011). Therefore, lack of an appropriate IFNγ response is likely to be a major factor in establishment of persistence of MV in the CNS. Current treatment for SSPE may slow the disease to some extent and comprises IFNα, ribavirin and inosiplex, either separately or in combination (Solomon *et al.*, 2002). However, clearance when the virus is in a long-term persistent state is unlikely to occur.

The role of regulatory T cells (Tregs) as regulators of the immune response in the brain in MV infection has recently been investigated (Reuter *et al.*, 2012). It was found that persistent CNS infection in mice can be modulated by manipulation of Tregs in the periphery. $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs were expanded or depleted during the persistent phase of the CNS infection,

and the virus-specific immune response and level of virus replication were analysed. Expansion of Tregs increased virus replication and spread in the brain. In contrast, depletion of Tregs induced an increase of virus-specific $CD8^+$ effector T cells in the CNS and caused a reduction of infection.

Henipaviruses

Nipah (NiV) and Hendra (HeV) viruses are the two members of the genus *Henipavirus* and are highly pathogenic, causing fatal encephalitis and respiratory disease in humans. Since the initial outbreak of HeV in Australia (1994) and NiV in Malaysia (1998), they have continued to cause sporadic outbreaks resulting in fatal disease (Mahalingam *et al.*, 2012; Rota and Lo, 2012). The pathological features in human acute henipavirus infections comprise vasculopathy (vasculitis, endothelial multinucleated syncytia and thrombosis), microinfarcts and parenchymal cell infection in the CNS. This pathology also occurs in the lung, kidney and other major organs. Viral inclusions, antigens, nucleocapsids and RNA are readily demonstrated in the blood vessel wall and in numerous types of parenchymal cells. In addition to acute disease, relapsing henipavirus encephalitis is a rare complication reported in less than 10% of survivors. Pathological evidence suggests that this is due to viral reactivation in the CNS (Wong and Tan, 2012).

Bunyaviridae The Bunyaviridae family contains more than 258 viruses and is divided into five genera, four of which comprise animal viruses some of which present serious risks to humans. The family includes Phleboviruses (e.g. Rift Valley fever (RVFV)), Nairovirus (e.g. Crimean–Congo haemorrhagic fever (CHFV)), Bunyavirus and Hantaviruses including some of the haemorrhagic fevers (Charrel *et al.*, 2004). Toscana virus is a newly emerging virus transmitted by phlebotomus sandflies and initially discovered in central Italy, but it has also now been found in other European countries. Hantaviruses (e.g. Hantaan virus) are another, emerging group of viruses causing epidemics in the United States. Bunyavirus is the largest genus (Calisher, 1994), and symptoms caused by members include fever, rash, arthritis, encephalitis and hepatitis. These infections are common in Africa and South America. In the United States, only the California group of viruses are present, one of which is the La Crosse virus. Clinical features of this infection range from a mild febrile illness to aseptic meningitis and fatal encephalitis. Most children with symptomatic disease have signs of encephalitis, with nearly one-half having seizures (Rust *et al.*, 1999).

Lyssaviruses

Viruses in the *Lyssavirus* genus in the family Rhaboviridae have a distinctive bullet-like structure. Rabies (Latin meaning ‘mad’) virus (RABV) is the

type species of the genus *Lyssavirus*, which includes 11 other defined species including the European lyssaviruses, differentiated according to their genomic sequence, plus three further isolates which are awaiting official classification.

Routes of rabies virus transmission Rabies virus cannot penetrate intact skin and thus gains entry to the body through broken skin (a bite or scratch) or through the mucous membranes (eyes, nose and mouth; Fishbein, 1991). Infection via routes other than these is rare in humans and usually either involves an un-noticed bat bite (Warrell, 1995; Messenger *et al.*, 2002) or, in rare cases, occurs following organ transplant (Galian *et al.*, 1981; Hellenbrand *et al.*, 2005).

Clinical course of infection The incubation period ranges from 31 to 90 days (Fishbein, 1991) in naturally acquired cases, but can be longer (Smeloviskii *et al.*, 1950; Charlton *et al.*, 1997). Early signs of disease may include pyrexia, chills, malaise, fatigue, insomnia, anorexia, headache, anxiety and irritability (Toacsen and Moraru, 1985). Other localized signs including burning, numbness, tingling or itching sensations (Schlegel *et al.*, 1985) and can arise at, or remote to, the site of infection. The disease typically occurs in two forms: the encephalitic ('furious') form, which occurs in approximately 80% of patients, and the paralytic ('dumb') form, which occurs in approximately 20% of patients with clinical signs of rabies (Leung *et al.*, 2007). Typically, the histopathological changes observed with lyssavirus infection include non-suppurative meningoencephalitis, accompanied by neuronophagia, focal gliosis and lymphocytic perivascular infiltration (Hicks *et al.*, 2009). With both forms of the disease the patient becomes paralysed and comatose. The cause of death is usually cardiovascular failure (Leung *et al.*, 2007).

Neuropathology It is not understood what differentiates the furious and dumb forms of rabies as both are the result of an acute inflammatory reaction in the CNS, associated with invasion and multiplication of rabies virus in neuronal cells (Figure 7.3). A recent comparison in mice of the clinicopathology of European bat lyssavirus (EBLV) types 1 and -2 to rabies virus was undertaken. The distribution of viral antigen throughout the regions of the brain examined was similar for each of the isolates during the different stages of disease progression, suggesting that antigen distribution was not associated with clinical presentation. However, specific regions of the brain, including the cerebellum, caudal medulla, hypothalamus and thalamus, showed notable differences in the proportion of virus antigen-positive cells present in comparison to other brain regions indicating their importance for development of clinical disease (Healy *et al.*, 2013).

The immune response and vaccination Neutralizing antibodies directed against the glycoprotein are thought to be the main determinant of protection

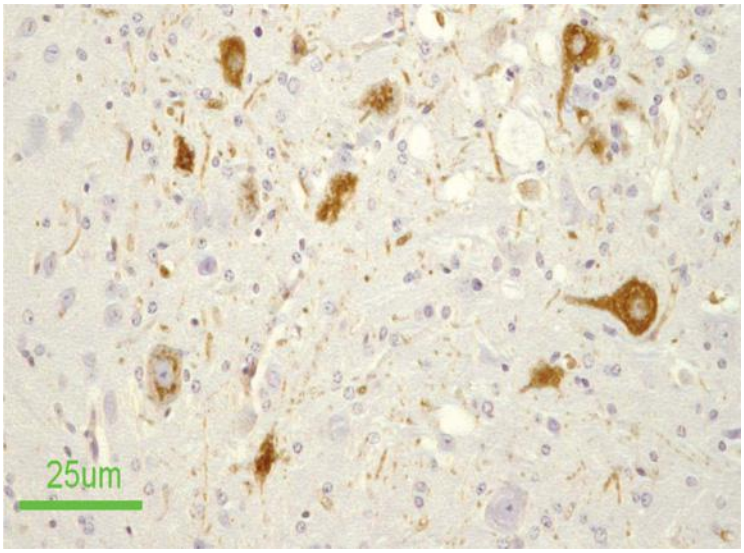

Figure 7.3 Immunohistochemical demonstration of *Lyssavirus* antigen (brown) in the neuronal cells at late-stage disease (animal). (Healy *et al.*, unpublished data.)

against lyssaviruses. The majority of naturally acquired human cases, however, do not have detectable immune responses until after the onset of acute disease (Johnson *et al.*, 2010). Replication of the virus in an immune-privileged site along with the ability of the virus to evade the host immune system using various strategies to prevent apoptosis (Finke and Conzelmann, 2005), to interfere with the transcription of type-1 IFN or to interfere with the STAT pathway (Johnson *et al.*, 2010) allow the virus to persist within the host. The natural immune response to rabies virus is thus severely limited, and without appropriate vaccination an exposure to rabies virus may potentially be fatal. Currently, all rabies vaccines (either human or those for domestic animals) are inactive virus vaccines containing the full-size intact viral proteins G, N, P, M and L. These vaccines must trigger a $CD4^+$ T cell and a humoral B cell response in the host (Herzog *et al.*, 1992). Other live-virus vaccines are restricted to the vaccination of wild animals and involve an additional $CD8^+$ T cell response (Wiktor *et al.*, 1977).

Togaviruses and flaviviruses

The Togaviridae and Flaviviridae families consist of approximately 30 single-stranded positive-sense RNA-enveloped viruses, able to infect various vertebrates such as humans, rodents, fish, birds, larger mammals such as horses as well as invertebrates. Often members of the alphavirus genus, within the *Togaviridae*, cause a rash and arthritis in their infected hosts; however,

Western equine encephalitis virus (WEEV), Eastern equine encephalitis virus (EEEV), Venezuelan equine encephalitis virus (VEEV) and Western encephalitis virus (WEV) (and, more rarely, the Ross river and Chikungunya viruses) cause encephalitis in their infected host (Zacks and Paessler, 2010). Rubella virus is in the *Rubivirus genus* in the Togaviridae and causes a low-grade childhood illness with a maculopapular rash. However, infection in the first trimester of pregnancy can lead to congenital defects. Rubella may rarely cause post-infection encephalitis (Chaari *et al.*, 2014) but is more commonly associated with meningitis.

The *Flavivirus* genus in the Flaviviridae family consists of over 70 small, positive-sense, single-stranded RNA-enveloped viruses that are approximately 11 kb in length. A number of flaviviruses are important human pathogens, such as WNV, tick-borne encephalitis virus (TBEV) and JEV, causing encephalitis in their infected hosts. In the last 20 years, most emerging and re-emerging virus pathogens have been zoonotic RNA viruses (Woolhouse and Gowtage-Sequeria, 2005), and a number have been flaviviruses (Griffin, 2009; Weaver and Reisen, 2010) and alphaviruses (Tsetsarkin *et al.*, 2007). Societal (increased travel) and environmental changes (global warming) coupled with the extensive tropical urbanization that have accompanied globalization mean that flaviviruses especially pose a worldwide danger (Ghosh and Basu 2009; Nett *et al.*, 2009; van den Hurk *et al.*, 2009).

Transmission and pathogenesis Infection occurs after the bite of an infected arthropod. After local replication in the skin and draining lymph nodes, a viraemia occurs and the virus is disseminated to other body tissues. The route of viral entry into the CNS remains unclear. Haematogenous transport and intraneural transport (olfactory nerve) after experimental intranasal inoculation have been described (Hase *et al.*, 1990; Liou and Hsu 1998; Myint *et al.*, 1999; Liu *et al.*, 2008). Infection of the olfactory nerve following virus replication in the periphery and haematogenous spread to the olfactory mucosa has been described in studies with some flaviviruses (Monath *et al.*, 1983; McMinn *et al.*, 1996). Virus transport across the BBB may occur by a 'Trojan horse' mechanism (within infected monocyte cells), by direct infection of vascular endothelial cells and by transcellular transport (Hase *et al.*, 1990; Liou and Hsu 1998; Myint *et al.*, 1999; Liu *et al.*, 2008). Virus transport across the BBB can be facilitated by cytokine-mediated increased permeability of the BBB (Liu *et al.*, 2008).

Immunopathology Virus infection of the CNS causes encephalitis and results in neuronal dysfunction or death. In natural human infections and in mouse models, alphaviruses and flaviviruses infect neurons of the brain and spinal cord (Shieh *et al.*, 2000; Yasui, 2002; Shrestha *et al.*, 2003). The extent of the brain pathogenesis depends on host neuronal maturity, host immune response and virus virulence. This virus infection of the brain stimulates a localized

immune response, which will be summarized in three areas: (i) immune cell infiltration, (ii) antibody production and (iii) production of cytokines, chemokines and other molecules.

Immune cell infiltration Encephalitis caused by these neurotropic flaviviruses and alphaviruses is characterized by the presence of immune cells in the brain parenchyma. Endogenous (e.g. microglia) and/or exogenous (e.g. T cells) cells can infiltrate the CNS as part of the immune response. $CD4^+$ T helper-1 (Th1) and cytotoxic $CD8^+$ T cells infiltrate the brain following JEV infection (Fujii *et al.*, 2008). It is the virus-infected perivascular macrophages and microglia that present viral antigens to these infiltrating T cells; virus-infected neurons do not express MHC class I or class II antigens. Peripheral monocytes and macrophages also infiltrate the brain to limit progression of WNV encephalitis (Rezai-Zadeh *et al.*, 2009). The cellular and molecular mechanisms responsible for immune cell infiltration of the diseased CNS are areas that can be exploited for the development of neuroregenerative processes (see Chapter 5).

Production of antibodies Virus replication is controlled by intraparenchymal production of antibody, but infected neurons are not eliminated which allows viral RNA to persist, requiring continuous intraparenchymal antibody production. Clearance of alphaviruses from the CNS of mice is dependent on antibody (Parsons and Webb, 1989; Levine *et al.*, 1991; Pachnerbrady *et al.*, 2007). In Semliki forest virus (SFV) infection, an initial high-titre plasma viraemia is controlled by immunoglobulin M (IgM) antibodies. Virus enters the brain across cerebral endothelial cells with BBB disruption. Neurons and oligodendrocytes are the cell types most frequently infected. Lesions of inflammatory demyelination require the presence of $CD8^+$ T cells and probably result from destruction by these cells (Fazakerley, 2002). T cell production of IFNγ contributes to alphavirus clearance from neurons (Binder and Griffin, 2001), but viral RNA persists in the CNS (Levine and Griffin, 1992; Tyor *et al.*, 1992). The first stage is clearance of infectious virus by $CD8^+$ T cells and IgM Ab-secreting B cells. Infectivity in the brain can be eliminated by IgG antibodies, although an active T cell response is required for RNA elimination. Maintenance of low levels of viral RNA and prevention of reactivation are controlled by alphavirus-specific plasma cells that secrete IgG (Fazakerley, 2002; Metcalf and Griffin, 2011; Metcalf *et al.*, 2013). Therefore, the recruitment and retention of B cells (including IgG- and IgA-secreting plasma cells) in the CNS are important for virus clearance and prevention of reactivation.

Cytokines, chemokines and other immune molecules Chemokines regulate this leukocyte trafficking during infection by binding and signalling through their cognate receptors, which are found differentially expressed on all leukocytes. The cytokines expressed are primarily Th2 type, which serve the

function of providing B cell help and macrophage deactivation (see Chapter 3). JEV infects neural stem progenitor cells and causes secretion of pro-inflammatory cytokines (IFNγ and IL6) that activate microglia and astrocytes and upregulate cell adhesion molecules on the endothelial cells of the BBB, which recruit further activated T cells and monocytes (Das *et al.*, 2009). JEV infection activates microglia, which also produce pro-inflammatory cytokines IL6 and TNFα as well as other mediators, cytochrome C oxidase-2 (COX2) and inducible nitric oxide synthase (iNOS) and chemokine MCP-1 (CCL2), leading to bystander death of neurons in the CNS (Ghoshal *et al.*, 2007). The TNF receptor (TNFR1) complex is activated in the neurons resulting in mitochondrial-mediated apoptosis (Swarup *et al.*, 2007a,b). JEV infection reduces microglial secretion of anti-inflammatory cytokines (IL10 and IL4) which is associated with increased viral load and tissue pathology (Swarup *et al.*, 2007a,b).

A recent review of neuropathogenic flaviruses shows much is still to be discovered about the role of chemokines (Bardina and Lim, 2012). A study with WNV showed that astrocytes produce chemokines (CXCL10 and CCL5), whereas microglial cells produced pro-inflammatory cytokines (IL6 and TNFα) and chemokines (CXCL10, CCL2 and CCL5) and activated the mitogen-activated protein kinase (MAPK) intracellular signalling pathways. Thus, it is the cytokines and chemokines released from microglial cells that may influence the neuropathogenesis of WNV infection (Cheeran *et al.*, 2005). Clearance of WNV requires the chemokine receptor CCR5, which promotes leukocyte trafficking to the brain (Glass *et al.*, 2005), and this cannot be compensated for by a related receptor, even though there is redundancy in the chemokine system. It is not known if this increased susceptibility due to CCR5 deficiency extends to other viruses (Bardina and Lim, 2012).

Picornaviruses

The enterovirus (EV) genus is part of the Picornaviridae family and includes poliovirus (PV), coxsackievirus (CV) and enterovirus-71 (EV71). Most poliovirus infections are sub-clinical and even where the infection leads to disease, there is an incubation period of 7–30 days. PV causes infection of the motor neurones of the anterior horn of the spinal cord. The global polio eradication programme has eliminated the disease from most of the world, apparently including India, but reversion of the live vaccine is a challenge to complete eradication (Minor, 2012). Infants infected with CV have been shown to be extremely susceptible to meningitis and encephalitis (mortality rate up to 10%). A number of delayed neuropathologies have also been associated with CV infection, including schizophrenia, encephalitis lethargica and amyotrophic lateral sclerosis (Rhoades *et al.*, 2011). EV71 is an important cause of hand, foot and mouth disease in humans but can also cause severe complications of the CNS. Brainstem encephalitis with pulmonary oedema is a

severe complication that can lead to death. Cytokines and chemokines play an important role in the pathogenesis of EV71 brain stem encephalitis. Treatment with intravenous immunoglobulin and milrinone, a phosphodiesterase inhibitor, has been shown to modulate inflammation, to reduce sympathetic overactivity and to improve survival in patients with EV71 autonomic nervous system dysregulation and pulmonary oedema (Wang *et al.*, 2012).

Retroviruses

Infection with the retroviruses human immunodeficiency virus type 1 (HIV1) and human T cell leukaemia virus type 1 (HTLV1) can lead to an immunodeficiency associated with depletion of $CD4^+$ T cells and eventually, if untreated, to neurodegenerative diseases. HIV infection can give rise to HIV-associated dementia (HAD) or neuroAIDS, while HTLV1 can cause HTLV1-associated myelopathy and tropical spastic paraparesis (HAM–TSP) (Irish *et al.*, 2009). HAD is increasing in prevalence as use of highly active antiretroviral therapy (HAART) increases survival lengths and there are more people living with HIV (McArthur *et al.*, 2005).

Human immunodeficiency virus

HIV enters into the CNS by the 'Trojan horse' mechanism (i.e. within infected cells of the monocyte–macrophage lineage), which cross the BBB and reach the brain parenchyma where they may pass infection to other CNS cells. Entry of HIV1 into T cells or macrophages occurs after binding of the viral envelope protein gp120 to chemokine receptors CXCR4 and CRCR5 in conjunction with CD4. Microglial cells have also been reported to be immunopositive for these major receptors and co-receptors for HIV (Kaul *et al.*, 2001). Individuals who are homozygous for a 32 bp CCR5 gene deletion are resistant to HIV1 infection (Liu *et al.*, 1996), while the influence of heterozygosity for this gene on progression of HIV infection has been controversial. A recent meta-analysis in 12,000 subjects concludes that there is no significant effect in susceptibility to infection (Liu *et al.*, 2012). However, specific effects in the CNS of infected CCR5 gene heterozygotes have not been investigated. HIV-induced abnormalities in the BBB have also been observed, but the mechanism of brain endothelial cell infection is not clear. These cells express CCR5 and CXCR4, while expression of CD4 is contradictory (Kaul *et al.*, 2001).

Although the majority of CNS cells are not permissive for HIV replication with the exception of microglia, perivascular macrophages and astrocytes, activation of the monocytic cells in the brain due to infection by HIV1, viral proteins or virus-induced inflammatory mediators is thought to result in the release of neurotoxic viral factors. These ultimately lead to astrocytic and neuronal dysfunction (Verma *et al.*, 2010). Neurotoxic viral proteins including Tat,

VPr, Nef and gp120 are known to stimulate neurons, resulting in excitotoxicity and the loss of cellular functions, similar to other neurodegenerative diseases (Kaul *et al.*, 2001; Irish *et al.*, 2009). Phylogenetic studies have shown that viruses present in the CNS are closely related to each other which indicates a slow mutation rate in the brain. The reasons for this has been suggested to be due to (i) low penetrance of antiretroviral drugs to the CNS, limiting drug resistance; (ii) reduced production of neutralization antibody in the CNS; and (iii) reduced response of cytolytic T cells in the CNS (Verma *et al.*, 2010).

In AIDS, opportunistic infections in the CNS are a major problem in addition to direct infection with HIV. Several of these opportunistic organisms are already present in the CNS or the periphery but do not cause clinical disease prior to immunosuppression. The most common opportunistic infections are CMV, toxoplasmosis, cryptococcosis and PML. Other infections including herpetic encephalitis, tick-borne encephalitis, herpes zoster multifocal encephalitis, bacterial (metastatic encephalitis connected with heart valvular changes) and fungal (candidiasis) infections, aspergillosis and leptomeningeal tuberculosis are less frequent or rare (Zelman and Mossakowski, 1998). With the success of HAART, a relatively new phenomenon of CNS–immune reconstitution inflammatory syndrome (CNS-IRIS) has developed in some individuals after the initiation of therapy. This is characterized by an intense inflammatory reaction to dead or latent organisms or to self-antigens due to a heightened but dysregulated immune response (Post *et al.*, 2012).

Human T cell lymphotrophic virus-1 (HTLV1)

HTLV1 neurological disease is poorly understood with little or no treatment available. It is not clear as to what drives an infected individual to develop adult T cell leukaemia and lymphoma rather than HTLV1-associated myelopathy and tropical spastic paraparesis (HAM–TSP). As with HIV, the viral Tax protein plays an important role in neuropathogenesis. The levels of protein secreted and its presentation by dendritic cells to T cells influence disease severity. It has also been determined that Tax, the viral transactivator once in the extracellular environment, may cause functional alterations in cells of the CNS as well as cells in peripheral blood and lymphoid organs. These extracellular biological activities of Tax are likely very relevant to the neuropathogenesis of HTLV1 and have been suggested to represent attractive targets for therapeutic intervention over other viral proteins (Irish *et al.*, 2009). It is becoming clear that complex virus–host interactions and the host immune response play an important role in the pathogenesis of HAM–TSP. Especially, the efficiency of an individual's cytotoxic T cell response to HTLV1 limits the HTLV1 pro-viral load and the risk of HAM–TSP (Saito and Bangham, 2012). Consequently, this immunologic pathway may provide an opportunity to identify novel therapeutic interventions.

JC polyoma virus

Progressive multifocal leukoencephalopathy (PML) is a debilitating and frequently fatal CNS demyelinating disease caused by JCV. JC (the initials of the patient, John Cunningham, from which the virus was isolated) virus is widespread in populations worldwide but is only associated with disease in immunocompromised individuals (Tavazzi *et al.*, 2012). The incidence of PML increased dramatically with the onset of the AIDS pandemic. Approximately 3 to 5% of HIV-infected individuals develop PML, which is classified as an AIDS-defining illness. Before AIDS, PML was a relatively rare disease, reported primarily in those with underlying neoplastic conditions affecting immune function and, more rarely, in allograft recipients receiving immunosuppressive drugs (Ferenczy *et al.*, 2012). The recent advances in treatment with natalizumab, a humanized monoclonal antibody against the adhesion molecule VLA4 for the treatment of MS and other autoimmune diseases such as Crohn's disease, has also led to an increased risk of PML as a side effect of the immunosuppressive immunotherapy (Meuth *et al.*, 2012).

Lytic infection of oligodendrocytes by JCV in the brain leads to their eventual destruction and progressive demyelination, resulting in multiple foci of lesions in the white matter of the brain. Until recently, oligodendrocytes were the only cells to be infected by JCV, but it is now known that infections of astrocytes and neurons may also occur. Furthermore, JCV-infected glial cells are frequently located at the grey matter–white matter junction or within the grey matter, causing demyelinating lesions within cortical areas. It has also been shown that JCV variants can infect neurons, leading to the recognition of two distinct clinical entities: JCV granule cell neuronopathy and JCV encephalopathy (Gheuens *et al.*, 2013).

Prion diseases

Prions (infectious proteins) cause a number of human diseases known as transmissible spongiform encephalopathies (TSEs). However, the existence of accessory factors has not been excluded. TSEs are invariably fatal conditions that include Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS), fatal familial insomnia (FFI), kuru and variant CJD (vCJD) in humans. There are also a number of veterinary TSEs: scrapie in sheep, bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy and chronic wasting disease in deer (Colby and Prusiner, 2011).

Pathogenesis

Human TSEs are associated with a range of clinical presentations (Head and Ironside, 2012). Approximately 85% of human prion diseases occur

sporadically with 1–2 sporadic CJD cases per million of the worldwide population per year. Around 15% of human prion disease is associated with autosomal dominant pathogenic mutations in the prion protein gene (PRNP) with over 30 mutations described. As for the other prion diseases, inoculation of brain material from these individuals results in disease in susceptible transgenic mice strains (Windl *et al.*, 1999). Inherited prion diseases are caused by mutations in *PRNP* and display marked phenotypic heterogeneity within families despite carrying the same mutation. A proline-to-leucine substitution at prion protein (PrP) residue 102 (P102L) is associated with the GSS phenotype but also gives rise to a CJD phenotype (Wadsworth *et al.*, 2006).

Iatrogenic CJD has arisen as a result of transmission of CJD prions through treatment with pituitary hormones derived from human cadavers, implantation of *dura mater* grafts, corneal transplantation and the use of contaminated electroencephalographic electrodes. New sources of iatrogenic disease have not been identified, and current practices, which combine improved recognition of potentially infected individuals with new disinfection methods for surgical instruments and biological products, should continue to minimize the risk (Brown *et al.*, 2012).

Acquired prion disease in humans as a result of ingestion was first described for kuru. This was caused by cannibalism among the Fore linguistic group of the Eastern Highlands in Papua New Guinea. Kuru demonstrated that incubation periods of infection with human prions can exceed 50 years. More recently, vCJD arose in the United Kingdom and other countries due to human exposure to BSE prions in infected beef products. The histopathological features of vCJD are distinguished from other human TSEs as there are characteristically large numbers of PrP-positive amyloid plaques that differ in morphology from the plaques seen in kuru and GSS in that the surrounding tissue takes on a micro-vacuolated florid appearance (Head and Ironside, 2012). Although contaminated food products have been removed from the market, it has been established that the transfusion of blood from vCJD-infected individuals can transmit the disease. This remains a major public health concern as the number of asymptomatic infected donors remains unresolved, and therefore the risk of inter-individual vCJD transmission through blood and blood-derived products cannot be determined (Andréoletti *et al.*, 2012).

Prion replication

The primary structure of PrPsc (the infectious form of the protein) corresponds to a normal cell protein, PrPc, which is encoded by the *Prnp* gene and exhibits a high homology throughout various species (Harrison *et al.*, 2010). PrPc is constitutively expressed in both neurons and glia in specific brain regions. PrPc is also found in blood cells, lymphocytes and the gut (Aguzzi and Polymenidou, 2004; Wadsworth *et al.*, 2006; Colby and Pruisiner, 2011).

Propagation of PrP^{Sc} takes place through a catalytic conversion of PrP^{C} to PrP^{Sc} by binding of PrP^{C} to pre-existing PrP^{Sc} seeds (Bieschke *et al.*, 2004). However, it is not clear how the initial PrP^{Sc} seeds are generated in TSEs other than the acquired cases. It has been shown that the phenotypic heterogeneity observed in inherited prion disease (P102L) is associated with differential propagation of protease-resistant wild-type and mutant prion protein (Wadsworth *et al.*, 2006). The replication of PrP^{Sc} produces protofibrils, which elongate subsequently into amyloid fibrils. A species barrier exists for the amino acid sequences of PrP, but this cannot be simply explained by mismatches in the amino acid sequences at specific sites in the protein. However, polymorphisms at codon 129 in PrP^{C} in the human population have determined the susceptibility to infection with BSE. Until recently, all of the vCJD-affected individuals were identified to express methionine homozygous at this site. A single case of transfusion-related vCJD in a patient heterozygous at codon 129 (Met/Val) has been confirmed, raising the possibility of a second wave of 'mad cow'–related deaths (Kaski *et al.*, 2009).

Transmission from the gastrointestinal tract to the CNS

In vCJD, PrP^{SC} enters the body in contaminated food products and can spread from the intestinal entry site to the CNS by intercellular transfer by follicular dendritic cells (FDCs) from the lymphoid tissue to the peripheral nervous system. Prion accumulation in lymph nodes was found to be dependent on the lymphotoxin-β receptor signalling, as loss of this factor was concurrent with the dedifferentiation of high endothelial venules (HEVs) required for lymphocyte entry into lymph nodes. It is suggested that prions may enter lymph nodes by HEVs and accumulate or replicate in the absence of mature FDCs (O'Connor *et al.*, 2012). With regard to the intercellular spread of prions, tunnelling nanotubes (TNTs) have been shown to be important as infection can be efficiently transmitted only from infected DCs to primary neurons in co-culture conditions that are permissive for TNT formation. Prions appear to traffic through these structures between infected and non-infected cells (Gousset and Zurzolo, 2009).

Immunopathology

Prions are host-coded proteins, and not surprisingly they do not appear to induce measurable systemic immune responses. However, prion infections of the CNS give rise to an initial reactive gliosis, with subsequent degeneration of neuronal tissue giving the characteristic spongiform appearance. The activation of glial cells is likely to be initially caused by the deposition of misfolded, in part proteinase K-resistant, isoforms of PrP^{C} in the brain. Proinflammatory cytokines and chemokines released by PrP^{Sc}-activated glial cells and

stressed neurons may contribute directly or indirectly to the disease development, causing generalized gliosis and cytotoxicity for neurons. Furthermore, recent studies have illustrated that interfering with inflammatory responses may represent a therapeutic approach for treatment of prion diseases (Riemer *et al.*, 2009).

Recent data suggest that cytokines are involved in scrapie progression. Microglia in TSEs have been found to produce only IL12p40 and CXCL10, whereas astroglia produced these cytokines plus CCL2, CCL3, CCL5, CXCL1, GCSF, IL1β, IL6, IL12p70 and IL13 (Tribouillard-Tanvier *et al.*, 2009). IFNγ has also recently been detected in the brains of prion-infected animals (Moody *et al.*, 2011). In a recent study, brain tissue, mesenteric lymph nodes, splenic tissue and serum from ovinized mice were screened for 62 cytokine and cytokine-related proteins at pre-clinical and clinical points of infection. Expression patterns were compared to brain histology and clinical presentation. Increased cytokine expression in both the brain and periphery were noted in PrP^{Sc}-positive animals before histologic changes or clinical signs were evident. Only IL10 and tissue inhibitor of metalloproteinase-1 (TIMP1) were consistently expressed at increased levels in the serum throughout infection (Newsom *et al.*, 2011).

A novel interplay between the nervous, endocrine and immune systems has been found to moderate both the expression and function of PrP^{C} in neutrophils, which may have a broad impact upon the physiology and pathology of various organs and systems. Lipopolysaccharide (LPS) was found to induce transcription and translation of PrP^{C} in mouse neutrophils. Furthermore, glucocorticoids (GC) and transforming growth factor beta (TGFβ), either alone or in combination, directly up-regulate PrP^{C} in neutrophils, and the resulting blockade of GC receptors curtails the LPS-induced increase in the content of PrP^{C}. Neutrophils with up-regulated PrP^{C} presented enhanced peroxide-dependent cytotoxicity to endothelial cells (Mariante *et al.*, 2012). It has also been suggested that truncated cyclophilin A (CyPA) detected in brain following prion infection may have an important role in the activation of brain-derived primary astroglia and microglia and perhaps in other neurodegenerative or neuroinflammatory diseases (Tribouillard-Tanvier *et al.*, 2012).

Conclusions

In this chapter, we have focused on virus and prion infections of the CNS. Viral encephalopathies are caused by many different types of virus, and they can be acute with virus clearance from the CNS and recovery or have a fatal outcome. There is a fine balance in the inflammatory response where failure to elicit an adequate T cell response can lead to uncontrolled virus replication whereas bystander effects of inflammation can exacerbate pathology such as demyelination and BBB breakdown. In subacute encephalopathies, the immune response may facilitate a virus persistent state and clearance of

viral RNA in such cases presents a major challenge. Inhibiting reactivation of latent CNS infections in immunodeficient individuals (possibly including ageing) is an equally difficult goal. Therefore, an in-depth understanding of the neuroimmunological processes in viral CNS infections and how these can be modulated is crucial for development of new therapies. These could be targeted either to prevent infectious agents initially crossing the BBB or to clear infection or prevent virus reactivation once established. These will be major challenges for the future.

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8

Neuroimmunology of Amyotrophic Lateral Sclerosis

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Overview of amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS), first described by Jean-Marie Charcot in the 19th century and now colloquially referred to as Lou Gehrig's disease, is a member of a group of adult-onset anterior horn disorders that cause selective, rapidly progressing and irreversible neurodegeneration of the patient's upper and lower motoneurons (Naganska and Matyja, 2011). The motoneurons in the cortex, brainstem and spinal cord die, resulting in various degrees of weakness, limb musculature atrophy, spasticity, dysarthria (slurred speech) and dysphagia (difficulty with swallowing); the neuromuscular system collapse caused by this devastating disorder ultimately culminates in respiratory failure and death, usually within 4 to 6 years of symptom onset. ALS initiates focally and then spreads to neighbouring structures (Ferraiuolo *et al.*, 2011). Prior to motoneuron cell body loss in the spinal cord, denervation at the neuromuscular junction is observed, followed by a 'dying back' of distal

* This chapter is dedicated to the memory of the late Dr. Jenny S. Henkel. May this chapter add to her brilliant and lasting legacy.

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branches (Fischer *et al.*, 2004). At autopsy, an obvious loss of motoneurons in the central nervous system (CNS) is evident, while the remaining neurons contain numerous inclusions comprising misfolded proteins, swelling of the perikaryon and proximal axon, mitochondria swellings, vacuoles and neurofilament accumulations. In general, this accumulation of intracellular or extracellular misfolded proteins in the CNS is a common feature of neurodegenerative disorders. In addition to the well-described motoneuron pathologies, the surrounding glia are also affected; proliferating microglia and astrocytes are highly activated and contain numerous inclusions.

The worldwide incidence of ALS is approximately 2 per 100,000 individuals, and is fairly uniform except for a few high-incidence regions such as the Kii Peninsula and Guam (Logroscino *et al.*, 2010). The mean age of onset is between 55 and 60 years of age, and it commonly affects more men than women until after age 70, when the ratio approaches 1 to 1. ALS was traditionally considered to be a pure motoneuron disorder (MND); however, recent findings in subsets of patients have highlighted the involvement of the frontal and temporal lobes and, to a lesser extent, sensory and spinocerebellar pathways, as well as the substantia nigra and hippocampal dentate gyrus. Thus, ALS is now regarded as a multisystem disorder in which motoneurons tend to be affected the earliest and the most severely.

The pathogenic processes underlying ALS are multifactorial and, at present, not fully understood. Most forms of ALS are idiopathic, having no obvious genetic basis for the disease, and are known as sporadic ALS (sALS), whereas approximately 10% of patients have a heritable form of ALS, referred to as familial ALS (fALS) (Andersen and Al-Chalabi, 2011). In sALS, recent studies have documented the presence of hexanucleotide repeats in chromosome 9, open reading frame 72 (C9ORF72) in 6–10% of the cases (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). Several other, less common mutations have been described in non-overlapping lists of candidates that include Ataxin-2 and the Unc-13 vesicular protein. It is likely that sALS, a phenotypically variable syndrome, is a genetically heterogeneous disease with multiple genes initiating motoneuron degeneration through separate but convergent biological pathways. Thus, definitive diagnosis of ALS relies upon a combination of primary clinical findings, systematic inclusion and exclusion of potential genetic factors, and environmental explanations. Unfortunately, the present therapies are mainly symptomatic and fail to halt disease progression; Riluzole, the first and only US Food and Drug Administration (FDA)-approved medication for ALS, only modestly prolongs survival. The absence of an effective treatment can be explained in part by the complex and heterogeneous genetic, biochemical and clinical features of ALS. While ALS accounts for the majority of the motoneuron diseases, the recognition of disease variants and mimic syndromes may lead to further insights into possible causes for the generality of ALS. From a biochemical perspective, the process of motoneuron degeneration is complex and the multifactorial influences

and potential biomarkers of ALS have never been assessed in the light of the clinical heterogeneity of ALS.

Approximately one-tenth of ALS cases are hereditary, but even within this subset there are currently 13 confirmed Mendelian gene mutations encoding proteins in disparate pathways that appear to be minimally interconnected; however, the genetic penetrance of these genes is not 100%. More significant is the fact that most of the genes associated with these fALS cases code for proteins involved in cellular mass transport (either axonal transport or vesicle trafficking; i.e. spatacsin or vesicle-associated membrane protein (VAMP)); abrogating or reducing cellular oxidative stress (superoxide dismutase); RNA transcription, processing and function (angiogenin, TAR DNA binding (TDP43) and fused in sarcoma (FUS)); endosomal trafficking (alsin and FIG4 homolog); and protein degradation pathways (valosin-containing protein and ubiquitin-2). Interestingly, these genes often code for proteins whose mutation or malfunction results in macromolecular cellular aggregates. Most importantly, in 30–50% of all fALS cases, there is an uncharacterized gene product of C9ORF72 that codes for a protein involved with RNA splicing and formation of nuclear RNA foci.

The first identified gene accounting for 20% of the known mutations in fALS is the SOD1 gene encoding the Cu^{2+}–Zn^{2+} superoxide dismutase 1 (SOD1) enzyme (Rosen *et al.*, 1993). Mutations in SOD1 (mSOD1) account for approximately 20% of the familial cases and were first associated with fALS in 1993. To date, over 165 mutations have been identified in SOD1 that induce disease by a toxic gain of function and not by a loss of enzymatic activity.

The most common cause of fALS is the recently identified expanded GGGGCC repeat that is located on 9p21 in the noncoding region of C9ORF72 and accounts for approximately 30–50% of all familial cases. C9ORF72 is a gene that is highly conserved across species and that encodes an uncharacterized protein with no known function. The hexanucleotide repeat expansion prevents expression of the C9ORF72 transcript variant 1 from the mutant allele; however, the defect may also impair RNA processing in general. While little is known about the C9ORF72-encoded protein, it appears to be expressed in the regions of affected neurons in cytoplasmic and synaptic localizations; the proposed C9ORF72 protein is structurally related to DENN domain proteins, which are highly conserved guanosine diphosphate (GDP)–guanosine triphosphate (GTP) exchange factors for Rab GTPases (Levine *et al.*, 2013). The mechanism of disease mediated by the GGGGCC repeat is still unresolved, but it has been attributed to haplo-insufficiency, a toxic gain of function or the synthesis of toxic di-peptide repeat proteins (Renoux and Todd, 2012).

With regard to the di-peptide hypothesis, a repeat associated non-ATG translation (RAN translation) across expanded GGGGCC repeats, an unconventional mode of translation in the absence of an initiating ATG, may

produce three two-amino-acid alternating copolymers – (glycine–alanine)n, (glycine–arginine)n and (glycine–proline)n – similar to the polyglutamine, polyserine or polyalanine tracts found with expanded CAG repeats. High-molecular-weight insoluble accumulations of poly-(glycine–proline) peptides are detected, with little or no poly-(glycine–arginine) or poly-(glycine–alanine) peptides, in brain homogenates of C9ORF72 ALS patients with frontotemporal dementia; poly-(glycine–proline) peptides appear specific for C9ORF72 ALS because none of the three possible two-amino-acid copolymers were found in the CNS of patients with other neurodegenerative diseases (Ash *et al.*, 2013). However, it remains to be determined whether the polypeptides generated by RAN translation are neurotoxic.

The third most common cause of fALS involve mutations in the related genes TARDBP and FUS encoding the DNA- and RNA-binding proteins, TDP43 and FUS–translocated in liposarcoma (TLS), respectively (Mackenzie *et al.*, 2010). Mutations in these proteins, which are associated with fALS, were first identified in 2008 and 2009, respectively. Since their initial discovery, many other mutations in TARDBP and FUS have been found and comprise approximately 4–5% of all fALS cases.

Although ALS has been traditionally viewed as a motoneuron cell-autonomous disorder, the current dogma suggests that ALS is a non-cell-autonomous neurodegenerative disorder, in which neurons do not die alone, but numerous cell types (both neuronal and non-neuronal) play a role in disease initiation and progression. There is now the emerging and generally accepted concept that both the innate and adaptive immune systems play early and important neuroinflammatory roles at the neuromuscular junction and the distal axonal compartment, and in the CNS compartment, in the disease pathophysiology of both fALS and sALS; there is an increasingly recognized role of macrophages, neighbouring CNS microglia and infiltrating lymphocytes and their particular molecular signals, in motoneuron survival and cell death. Even though the CNS has traditionally been considered immunologically privileged, and in light of this emerging concept, there has been a re-evaluation of this tenet and neuroinflammation is now recognized to be a prominent feature of many classic neurodegenerative disorders, including ALS. Furthermore, denervation at the neuromuscular junction is observed prior to motoneuron cell body loss in the spinal cord, suggesting that motoneuron pathology begins at the distal axon and proceeds in a ‘dying back’ process. Thus, in ALS and other neurodegenerative disorders, microglial activation and T cell infiltration are pathological hallmarks at sites of neuronal injury, yet such neuroinflammation has been considered the consequence and not the cause of neuronal injury; both innate and adaptive immune systems respond to, as well as contribute to, the pathology and tissue destruction (see Figure 8.1). However, more recent evidence challenges this belief and suggests that at an early phase of disease, the immune system may contribute to neuroprotective functions (see Figure 8.2).

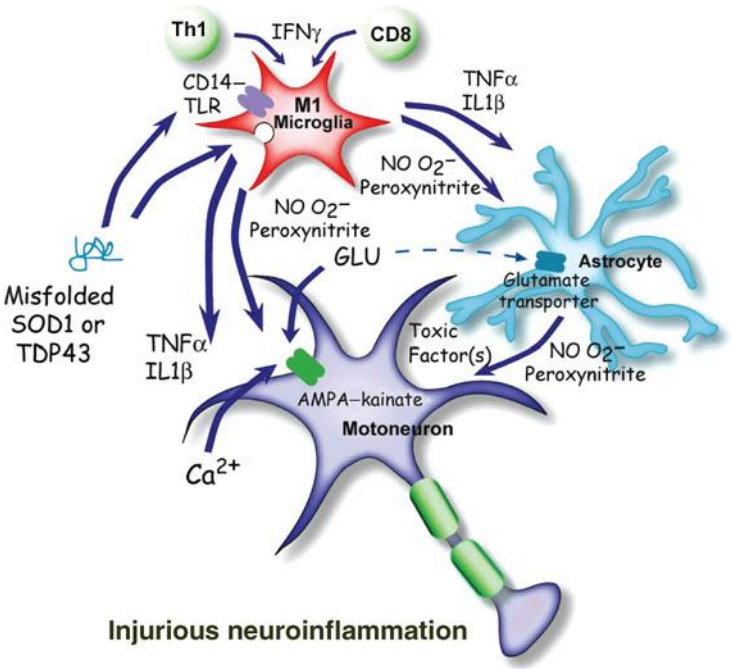

Figure 8.1 An injurious neuroinflammatory response. M1 microglia, ROS and aberrant astroglia contribute to motoneuron injury. Mutant or misfolded SOD1 or TDP43 proteins interact with CD14–TLR, inducing M1 microglia, secretion of proinflammatory cytokines and ROS. ROS increase the number, or Ca^{2+} permeability, of AMPA receptors and reduce glutamate transporter function on astrocytes, allowing excessive activation of AMPA receptors. Astrocytes have reduced trophic factors and up-regulate the release of toxic factors. The result of this is a transformation of microglia from an M2 to an M1 phenotype, which promotes neurotoxicity.

The controversial role of T cells in ALS was resolved by the demonstration that their presence in mSOD1 mice plays an endogenous neuroprotective function by augmenting the protective potential of microglia and attenuating their toxic responses (Beers *et al.*, 2008; Chiu *et al.*, 2008). Another study using mSOD1 mice bred with different T cell–deficient mice reached a similar conclusion that T cells mediate a beneficial response; in both studies, T cell deficiency led to the attenuation of microglial protective responses and survival. Banerjee *et al.* (2008) also concluded that the passive transfer of *ex vivo*–activated $CD4^+$ T cells into mSOD1 mice improved neurological function and life expectancy. More recently, a costimulatory pathway was demonstrated to be up-regulated in T cells in the blood of patients with ALS; modulating this costimulatory pathway in ALS mice delayed disease onset and reduced toxic microglial inflammatory responses (Lincecum *et al.*, 2010).

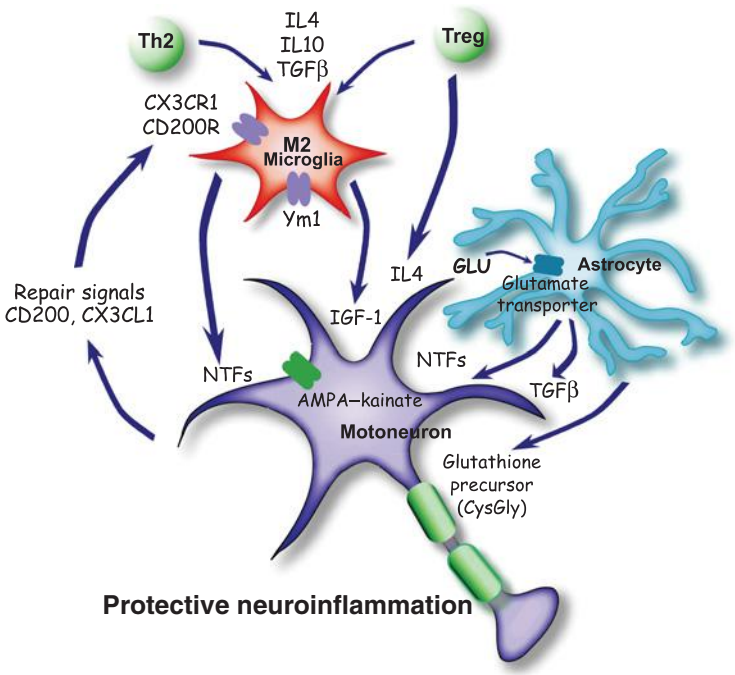

Figure 8.2 A protective neuroinflammatory response. Injury to motoneuron cell bodies, or perhaps injury in the periphery, induces the release of repair signals from motoneurons, which promotes an M2 microglial protective phenotype. M2 microglia secrete increased levels of neurotrophic factors (NTFs), thereby exerting a neuroprotective function. M2 microglia also release anti-inflammatory cytokines to inhibit the production of pro-inflammatory responses. Astrocytes also participate in the neuroprotective process with secretion of NTFs and uptake of excess glutamate from synaptic clefts. Astrocytes also enhance the antioxidant capacity of neurons by releasing the glutathione precursor which is taken up by motoneurons for the synthesis of glutathione.

There was also controversy as to whether the proliferation and activation of microglia in ALS promoted survival or exacerbated neuronal death. Several groups have demonstrated that wild-type (WT) microglia or microglia expressing less mSOD1 promoted neuroprotection and extended survival of mSOD1 mice (Beers *et al*., 2006; Boillée *et al*., 2006). *In vitro* studies utilizing primary microglia–motoneuron co-cultures provided evidence that WT microglia were less neurotoxic than mSOD1 microglia due to their enhanced release of neurotrophic factors (NTFs) and attenuated release of free radicals and proinflammatory cytokines (Xiao *et al*., 2007). Marden *et al.* (2007) determined that dysregulated redox stress in ALS mice caused by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits NOX1 and NOX2, expressed in microglia, significantly influenced the progression of motoneuron disease caused by mSOD1 expression; deletion of either Nox gene slowed disease progression and improved survival. Therefore, T cells

and microglia, through their distinctive temporal and spatial contributions, can have both neuroprotective and cytotoxic functions depending on their different phenotypic activation states and the physiological conditions they encounter.

Mutant superoxide dismutase animal model of ALS

SOD1 is a ubiquitously expressed cytosolic enzyme that converts highly reactive and toxic superoxide radicals ($O_2^{\bullet -}$) to hydrogen peroxide (H_2O_2), which is subsequently converted to water and oxygen by a catalase. Mutations in the SOD1 gene are known to cause fALS. After the SOD1 mutations responsible for fALS were identified, researchers overexpressed these SOD1 mutations in transgenic mice and rats that subsequently developed a chronic progressive motoneuron disease. This disease reflected many clinical and pathological features of ALS including tremulousness, muscle weakness and atrophy, spasticity, progressive paralysis and ultimately death (Gurney *et al.*, 1994). Similar overexpression of WT SOD1 did not induce an ALS-like phenotype, nor did a deletion of SOD1 gene, suggesting that the toxicity is not due to a loss or gain of SOD1 enzymatic activity but rather to a toxic gain of function. Genetic and chimeric mouse studies indicate that the mechanisms of motoneuron injury are 'non-cell-autonomous', a phrase that denotes that other cell types are involved in the pathoprogression of the disease. Mice chimeric for mSOD1 revealed that WT motoneurons, when surrounded by mSOD1-expressing glia, were injured, while mSOD1-expressing motoneurons surrounded by WT glia appeared normal (Clement *et al.*, 2003). The lack or reduction of mSOD1 expression in microglia slowed disease progression and prolonged survival of mSOD1 mice, while disease onset was not altered. Reduced mSOD1 expression in astrocytes also slowed disease progression and prolonged survival, while not affecting disease onset (Yamanaka *et al.*, 2008). The only cell type where mSOD1 expression was beneficial was the Schwann cell, where reduced expression of an enzymatically active mSOD1 in Schwann cells accelerated disease progression and reduced survival of mSOD1 mice (Lobsiger *et al.*, 2009). While early attempts at overexpressing mSOD1 predominantly in neurons were unsuccessful at inducing motoneuron injury, high homozygous $mSOD1^{G93A}$ overexpression in mice did induce a motoneuron disease, although disease was dramatically delayed (Jaarsma *et al.*, 2008). As in patients with ALS, neuroinflammation is a prominent pathological feature of mice expressing mSOD1, and again is characterized by activated microglia, infiltrating T cells, immunoglobulins and dendritic cells (DCs) in the spinal cords of these mice. Activated microglia are observed at an early age prior to signs of disease onset. Numerous studies of mSOD1 mice also suggest immune activation of microglia with increased expression of inducible nitric oxide synthase (iNOS), the catalytic subunit of the phagocyte NADPH oxidase (NOX2), interleukin-10 (IL10), IL6, tumour necrosis factor alpha (TNFα) and

CCL2. Although it is not clear if these responses are protective or injurious, there is clear evidence of an early immune response in the transgenic mSOD1 mice. Combined, these results indicate that glial cells contribute significantly to ALS progression and that the disease is non-cell-autonomous, involving both motoneurons and surrounding glia.

TDP43 animal model of ALS

TDP43 is a multifunctional DNA- and RNA-binding protein involved in RNA processing, stabilization and transport. Its involvement in ALS pathogenesis was originally recognized when TDP43 was identified as a primary component of ubiquitinated inclusions in neurons and glial cells in sALS tissue, and it was then reinforced when TDP43 mutations were identified in fALS patients (Neumann *et al.*, 2006). WT TDP43 and several TDP43 mutations have been overexpressed in neurons of mice and rats using the Prp, Thy1 or CaMKII promoters, which subsequently resulted in growth retardation, gait abnormalities, degeneration of motoneurons and pyramidal neurons in layer 5 of the frontal cortex, microglial and astroglial activation, cognitive impairments and premature death; the severity of the phenotype correlated with the levels at which the transgene accumulated in the neuron. In addition, abnormal nuclear inclusions composed of TDP43 and FUS–TLS are found in motoneurons, with substantial accumulations of mitochondria in cytoplasmic inclusions, a lack of mitochondria in axon terminals and immature neuromuscular junctions. TDP43 deletions are embryonically lethal, and a conditional deletion of a floxed TDP43 by a tamoxifen-induced Cre recombinase resulted in dramatic weight loss and death of the mouse in 9 days. Moderate overexpression of mTDP43 or truncated TDP43, as compared with equivalent expressions of WT TDP43, resulted in up-regulated cytoplasmic and nuclear ubiquitin and aggregates of phosphorylated TDP43 with enhanced motoneuron degeneration. Currently, less is known about the TDP43 rodent models than the SOD1 models due to their recent development.

Proposed mechanisms of motoneuron injury in ALS

Different mechanisms of motoneuron injury and death in ALS have been proposed. These mechanisms include misfolded protein toxicity – particularly misfolded SOD1 or TDP43 toxicity; glutamate- and calcium-mediated excitotoxicity; mitochondrial dysfunction; neurofilament and cytoskeletal alterations affecting axonal transport; RNA processing and handling defects; endoplasmic reticulum (ER) and Golgi dysfunction, resulting in ER stress and protein degradation malfunction; neuroinflammation with subsequent neuroprotection and/or neurotoxicity; and astroglial alterations with reduced

neuroprotection and direct neural toxicity. These mechanisms of neural injury have been proposed because all pathological alterations are present in the motoneuron and/or surrounding glia, and all contribute to neurodegeneration, although the initiating mechanism is not clear. In addition, these observed pathological processes interact with each other, aggravate one another and may even have a synergistic injurious effect. In this chapter, the currently proposed mechanisms of motoneuron protection and injury, induced by the accompanying neuroinflammation, will be discussed.

Neuroinflammation and mSOD1 mice

Although the mechanisms whereby mSOD1 causes disease are unknown, there is compelling evidence suggesting immune system involvement in mSOD1-mediated motoneuron injury. Several studies have demonstrated the presence of activated microglia, T cells and DCs in the spinal cords of mice expressing the G93A form of mSOD1, with elevated transcripts in the mSOD1 spinal cord beginning at 110 days (Henkel *et al.*, 2006). CCL2 mRNA and immunoreactivity were up-regulated in neuronal and glial cells as early as 15 days prior to any evidence of microglial activation. Zhong *et al.* (2008) also demonstrated an increase in CCL2 mRNA and suggested it was correlated with a breakdown in the blood–brain barrier. In addition, at 39 days of age, before evidence of disease onset, CD68 immunoreactivity, a myeloid-specific LAMP protein expressed by phagocytic cells, was present in lumbar spinal cords of mSOD1 mice. Other studies also indicate an immune activation in the mSOD1 mice, including activated microglia, increased iNOS expression, increased IL1β expression and increased IL6, TNFα and CCL2 expression. Although it is not clear if these responses were protective or injurious, there is clear evidence suggestive of an early immune response in the transgenic mSOD1 mouse model of fALS, as was shown in patients with ALS.

Immunologic aspects of ALS: part 1 – microglia

As has been found in other models of neuronal injury, microglia activation may be a double-edged sword with the abilities to promote either neuronal protection or injury. In ALS patient autopsy tissues, the neuroinflammation is observed specifically at sites of motoneuron injury, highlighted by the presence of activated and proliferating microglia and infiltrating DC (Henkel *et al.*, 2009; Philips and Robberecht, 2011). Positron emission tomography using [11C](R)-PK11195 enabled the detection of cortical microglial activation throughout disease in ALS patients (Turner *et al.*, 2004). This microglial activation, which was not present in healthy controls, correlated with the severity of upper motoneuron damage. In addition, granular aggregates of misfolded SOD1 were regularly detected in ventral horn microglia in ALS

autopsy tissues, regardless of whether the patients were carrying or lacking SOD1 mutations (Forsberg *et al.*, 2011).

ROS play a significant role in exacerbating disease in ALS by aggravating the pathological mechanisms of motoneuron injury. As evidence of damage, cerebrospinal fluid (CSF), serum and urine from ALS patients all contain elevated biomarkers of free radical damage. The reactive species involved in ALS include NO, $O_2^{\bullet-}$, H_2O_2, hydroxyl radical ($^{\bullet}OH$) and peroxynitrite, the latter two being particularly toxic. $O_2^{\bullet-}$ is generated from two sources: the enzyme NADPH oxidase and mitochondrial respiration. About 15% of $O_2^{\bullet-}$ produced by mitochondria goes towards formation of peroxynitrite and the other 85% is converted to H_2O_2. NO is produced by the enzymes nNOS (expressed in neurons, and can be expressed in activated astrocytes), eNOS (expressed in endothelial cells) and iNOS (expressed in activated microglia); however, iNOS is also seen in motoneurons in early stages of disease in the mSOD1 mouse and in astrocytes as the disease progresses. While the direct toxicity of NO is modest, its toxicity is greatly enhanced by reacting with $O_2^{\bullet-}$. NO readily diffuses into the mitochondria, where it rapidly reacts with $O_2^{\bullet-}$ to form the highly toxic peroxynitrite. NO can capture $O_2^{\bullet-}$ three times faster than SOD1, and in fact, NO is the only biological molecule produced in high enough concentrations to outcompete SOD1 for $O_2^{\bullet-}$. NO and $O_2^{\bullet-}$, released by activated microglia, can also interact outside the microglia. The resulting peroxynitrite can react with and modify DNA, proteins and lipids, inhibiting their function, inducing misfolding or aggregation and resulting in significant motoneuron and glial alterations. *In vitro*, extracellular mSOD1 or oxidized or misfolded SOD1 proteins induced a functional and morphological activation of primary microglia, enhancing their release of ROS and pro-inflammatory cytokines (Zhao *et al.*, 2010). Only when motoneurons were co-cultured with microglia did mSOD1 protein induce motoneuron injury. Adding TLR2 and TLR4 blocking antibodies, or CD14 blocking antibodies, or using CD14 knockout microglia and motoneurons partially inhibited the activation and neurotoxicity, indicating that the toxicity was mediated in part through TLR–CD14 receptors on microglia. Additionally, blocking NADPH oxidase or iNOS with apocynin or L-NIL, respectively, reduced the production and release of $O_2^{\bullet-}$ and NO, and protected motoneurons, indicating that the toxicity was mediated via up-regulated NOX2 and iNOS expression and subsequent enhanced $O_2^{\bullet-}$ and NO production (Figure 8.1).

In vitro, microglia can be activated by the addition of lipopolysaccharide (LPS) to the cultures. LPS interacts with microglial cell surface CD14 receptor and TLR2/4, thereby initiating the release of the free radicals $O_2^{\bullet-}$ and NO, which combine to form the toxic peroxynitrite ($ONOO^-$). The peroxynitrite in turn interacts with motoneurons and promotes cell death by sensitizing cells to the excitotoxic effects of glutamate and extracellular calcium. When microglia from mSOD1 mice were studied, they were noted to be more activated and more responsive to LPS activation than microglia from their

non-transgenic WT littermates; when compared with WT microglia, mSOD1 microglia caused more motoneuron injury in microglia–motoneuron co-cultures. mSOD1 microglia expressed more iNOS and released more $O_2^{\bullet -}$ and NO relative to that produced and released by WT microglia. The interaction of $O_2^{\bullet -}$ and NO to form $ONOO^-$ caused oxidative injury to cell lipids and proteins, and also mediated neurotoxicity in rat cortical microglia–neuronal co-cultures. Further evidence for the toxicity of secreted NO was provided by the demonstration that motoneuron survival was inversely correlated with nitrate (NO_3^-) + nitrite (NO_2^-) concentrations; the more $NO_3^- + NO_2^-$ produced, the fewer motoneurons survived in WT or mSOD1 microglia–motoneuron co-cultures. Furthermore, pre-treatment with L-NIL prior to LPS treatment significantly decreased $NO_3^- + NO_2^-$ concentrations and increased motoneuron survival in microglia–motor neuron co-cultures.

Glutamate is the most abundant excitatory neurotransmitter in the CNS, and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)–kainite receptors are the major glutamate receptors on motoneurons. Glutamate is normally nontoxic, but excessive levels of glutamate and modification of AMPA–kainate receptors result in increased influx of calcium and neuronal death (Figure 8.1). Activated microglia can induce motoneuron cell death *in vitro* by releasing free radicals and increasing the susceptibility of the motoneuron AMPA–kainate receptors to the toxic effects of glutamate. Furthermore, microglia that express mSOD1 are more readily activated and induce more motoneuron cell death than WT microglia.

During the early, slowly progressing stage of disease in mSOD1 mice, microglia have a predominantly M2 phenotype that mediates neuroprotection, while later, during the rapidly progressing stage, microglia are transformed into predominantly neurotoxic cells with an M1 phenotype. What triggers the neurotoxic microglial phenotype in mSOD1 mice has not been definitively established. Not only has misfolded and aggregated mSOD1 been demonstrated to activate microglia and mediate motoneuron injury *in vitro*, but also misfolded mSOD1 accumulates as aggregates in motoneurons *in vivo*; the misfolded mSOD1 has been shown to enhance mitochondrial dysfunction and induce ER stress. In addition, although mSOD1 protein does not directly injure motoneurons, the misfolded mSOD1 protein or related signals released from motoneurons could activate microglia, again through CD14 and TLR2/4, shifting them from an M2 phenotype, associated with the release of cytokines that suppress inflammation, towards a proinflammatory neurotoxic M1 phenotype. These neurotoxic M1 microglia in turn could release ROS and pro-inflammatory cytokines, further increasing motoneuron stress and cell injury, and thus initiating a self-propagating cycle of motoneuron injury and death. In the mSOD1 model of ALS, the signal could be mSOD1 itself, because mSOD1 protein bound to chromogranin (a secretory granule protein) is secreted from neural cells and triggers microgliosis and neuronal death in mixed spinal cord cultures. iNOS was also found to be up-regulated

in mSOD1 mouse in early symptomatic stages through end-stage disease. Up-regulation of iNOS may in turn stimulate NO production, which, as previously mentioned, plays a deleterious role in the pathogenesis of ALS. NO can also induce Fas signalling in mSOD1 motoneurons, resulting in cell death; up-regulation of nNOS was required in this Fas-triggered motoneuron death. Of possible relevance to sALS, WT SOD1 acquires binding and toxic properties of mSOD1 through oxidative damage, and it might activate microglia and induce motoneuron death in spinal cord cultures. Furthermore, an altered SOD1 species has been detected within the spinal cords of sALS patients that must have originated from misfolded WT SOD1, possibly unifying a shared pathophysiological pathway between sALS and fALS.

In addition to the neurotoxic effects, microglia may also have neurotrophic effects on surrounding motoneurons. Resting microglia promote neuronal proliferation *in vitro* by secreting NTFs. Among these NTFs, insulin like growth factor-1 (IGF1) has been shown to have a protective effect on motoneurons; IGF1 is a potent neurotrophic and survival factor, and can enhance motor nerve regeneration after neonatal sciatic nerve axotomy. *In vitro* studies utilizing primary microglia–motoneuron co-cultures provided supportive evidence that microglia may be neuroprotective. WT microglia, after LPS activation, were less neurotoxic than mSOD1 microglia due to the enhanced release of IGF1 and the attenuated release of free radicals and proinflammatory cytokines (Zhao *et al.*, 2006). Additionally, in microglia–motoneuron co-cultures, treatment with IL4 suppressed M1 microglial activation promoting an M2 phenotype, reduced the release of the ROS, enhanced IGF1 secretion and improved motoneuron survival. In addition to neurons, the enhanced IGF1 secretion may affect other CNS glial cells, resulting in further neuroprotection. Furthermore, fractalkine receptor (CX_3CR1) has been identified as a regulator of microglial neurotoxicity *in vivo*. Based on complementary expression of fractalkine (CX_3CL1) from neurons and CX_3CR1 on microglia, it has been proposed that neuron signalling to microglia might be mediated through this CX_3CR1. These data suggest that WT microglia, IL4-treated microglia or CX_3CL1-treated microglia exhibit more of an M2 phenotype which may promote neuroprotection in ALS mice (Figure 8.2).

In contrast, free IGF1 levels in ALS patients' spinal cords and serum were shown to be reduced and were accompanied by increased levels of inhibitory binding proteins, suggesting that down-regulation of the IGF1 trophic support may lead to degeneration of motoneurons. IGF1 has been reported to prevent glutamate-induced embryonic rat spinal cord motoneuron death and has successfully prolonged life and delayed disease progression in mSOD1 mice; intrathecal injection of IGF1 into the lumbar spinal cord of mSOD1 mice delayed disease onset and extended survival.

The importance of microglia in the pathogenesis of motoneuron injury in ALS was derived from studies using PU.1 knockout ($PU.1^{-/-}$) mice that, at

birth, lack macrophages, neutrophils, T and B cells and microglia, and require bone marrow transplantation (BMT) to survive; without BMT, PU.1$^{-/-}$ mice die due to sepsis within 3 to 5 days after birth (Beers *et al.*, 2006). The donor bone marrow cells colonize the CNS and resemble resident microglia, and their genotype and phenotype dictate microglia-mediated neuroprotection or neurotoxicity. Following transplantation of PU.1$^{-/-}$ mice with mSOD1 mice–derived bone marrow, the CNS microglia expressed mSOD1 but did not produce motoneuron injury or clinical signs of motoneuron disease; these data support the importance of the non-cell-autonomy hypothesis such that mSOD1 microglia *per se* cannot initiate motoneuron disease. Cross breeding the PU.1$^{-/-}$ mice with mSOD1 mice results in the expression of mSOD1 in motoneurons, but these animals still required BMT for viability. When WT mice donor bone marrow was used to transplant doubly transgenic mice (mSOD1–PU.1$^{-/-}$), the CNS microglia had a WT phenotype and motoneuron loss was decreased, and disease duration and survival were prolonged when compared with mice receiving mSOD1 mice donor–derived bone marrow. Furthermore, when another independent laboratory generated and employed transgenic mice with a different SOD1 mutation, and a different technique to reduce the expression of mSOD1 (i.e. the Cre–Lox system), a similar conclusion was reached, namely, that the reduction of mSOD1 in microglia prolonged disease duration and survival. A further suggestion from this latter study was that the onset of disease may be more related to the expression of mSOD1 in motoneurons, while, in accord with the previous study, the duration of disease may be related to expression of mSOD1 in microglia. Thus, the *in vivo* data demonstrate that the neurotoxicity and neuroprotection noted *in vitro* may be induced by a decrease in specific neurotoxic substances and an increase in a neurotrophic molecule from WT microglia relative to mSOD1 microglia, suggesting potential mechanisms for how WT microglia, by either the reduction or the elimination of mSOD1 expression, are less toxic *in vivo*.

Immunologic aspects of ALS: part 2 – T cells

Research linking immunity and neurodegeneration has focused primarily on microglia and innate immunity. However, infiltrating T cells of the adaptive immune response are also present in areas of CNS motoneuron degeneration. Depending on their phenotype and activation status, T cells can crosstalk with neurons and microglia, and either protect or damage neurons from stressful stimuli.

Several studies have addressed T cell infiltration of post-mortem material from ALS patients and identified substantial T cells in 38 of 48 spinal cords from ALS patients; T cells were identified along the vessel walls in the precentral gyrus and extending into the areas of neuronal injury in all eight ALS patients who were examined (McGeer and McGeer, 2002). Others have found perivascular and intraparenchymal T cell infiltrates in the corticospinal tracts

and ventral horns of 18 of 27 consecutive ALS autopsy cases compared to 1 of 11 control brains; no B cells were detected. $CD4^+$ T cells were present in the proximity of degenerating corticospinal tracts, whilst both $CD4^+$ and $CD8^+$ T cells were demonstrated in ventral horns. Another study reported perivascular infiltration of T cells in the spinal cords of all six ALS patients studied.

Of importance is the fact that mSOD1 mice show a similar T cell response that is observed in sALS patients. Alexianu *et al.* (2001) demonstrated the presence of $CD3^+$ T cells in the spinal cords of mSOD1 mice at end-stage disease. At early stages of disease, only $CD4^+$ T cells are found in lumbar spinal cords of mSOD1 mice (Henkel *et al.*, 2006; Beers *et al.*, 2008; Chiu *et al.*, 2008). $CD8^+$ T cells were observed only at end-stage disease, but even at this stage, $CD4^+$ T cells still predominated, accounting for approximately 60% of the total T cell population while remaining T cells were $CD8^+$. The fact that $CD4^+$ T cells first enter the spinal cords of mSOD1 mice, and that their numbers increase as disease progresses, suggests that they are active participants throughout the course of disease in these mice.

To determine the role of T cells in mSOD1 transgenic mice, mSOD1 mice were bred with RAG2 knockout mice that lack functional T and B cells. Interestingly, double transgenic mice ($mSOD1^+/RAG2^{-/-}$) had an accelerated disease course and the mice died earlier than the heterozygous ($mSOD1^+/RAG2^{+/-}$) control mice, indicating that T cells, and possibly B cells, contribute to a neuroprotective environment (Beers *et al.*, 2008); the disease course of $mSOD1^+/RAG2^{+/-}$ mice was identical to that of mSOD1 mice. Similar results were obtained when mSOD1 mice were bred with CD4 knockout mice, indicating that $CD4^+$ T cells contribute to the prolongation of disease duration. These results were confirmed when mSOD1 mice were bred with mice deficient for the T cell receptor β chain; the specific ablation of T cells led to accelerated disease progression and shorter lifespans. The participation of $CD4^+$ T cells has been further supported by the evidence that the passive transfer of *ex vivo*–activated $CD4^+$ T cells improves the neurological function and lifespan of mSOD1 mice. Taken in its entirety, these results established that the lack of T cell recruitment, through either the loss of CCR2 or developmental inhibition, accelerates disease progression and the demise of mSOD1 mice. Therefore, adaptive immune responses mediated by $CD4^+$ T cells serve an important neuroprotective function in the ALS mouse.

The neuroprotective immunity associated with $CD4^+$ T cells is mediated by interactions with microglia and astrocytes (Figure 8.2). When T cells are depleted in mSOD1 mice, a more aggressive disease course occurred and was accompanied by increased proinflammatory cytokines and NOX2, and decreased levels of trophic factors and glutamate transporter; bone marrow transplantation reconstituted mice with functional T cells, restored neuroprotective factors and reduced toxic pro-inflammatory responses. Chiu *et al.* (2008) also reported that deficiency of T cells in mSOD1 mice resulted

in decreased IGF1 expression in microglia. These results suggest that $CD4^+$ T cells provide neuroprotection by modulating the trophic–cytotoxic balance of glia.

T cells have been shown to directly communicate with microglia and macrophages, and infiltrating T helper (Th) cells can be both toxic and protective. Although an oversimplification, Th cells can be subdivided into Th1, Th2, Th17 and Treg cells. Prompted by different types of cytokines produced by antigen-presenting monocytes, macrophages and microglia, as well as by DC and other cell sources, undifferentiated Th cells develop into the Th1 or Th2 lineages (see Chapter 3).

Recent studies have systematically evaluated the dynamic changes that occur in the $CD4^+$ subsets – Th1, Th2, Th17 and Tregs – during progression of disease in mSOD1 mice. In lumbar spinal cords of mSOD1 mice at early, slowly progressing stages, Tregs were increased accompanied by increased levels of IL4, IL10 and M2 microglia, and then decreased when disease rapidly accelerated. During the rapid stage of disease, there was an increased expression of mRNA for Th1 cells and decreased expression of mRNA for Th2 cells; microglia were predominantly M1 (Figure 8.1). IL17 expression was not detected at any time in the course of disease, suggesting that Th17 may not be involved in the pathogenesis of ALS in the mouse model (Beers *et al.*, 2011a). At early stages of disease in mSOD1 mice, increased expression of IL4 and IL10 was noted, suggesting that these cytokines were able to skew microglia towards an M2 phenotype (Figure 8.2). Although Th2 cells are one of the major sources of IL4, Th2 are not increased in the lumbar region of ALS mice spinal cords. Tregs are the likely source of IL4; our own studies as well as those from other laboratories have documented that Tregs are capable of releasing IL4 and have the potential to maintain the protective M2 phenotype (Tiemessen *et al.*, 2007; Beers *et al.*, 2011a). In addition, passive transfer of early-phase mSOD1 Tregs prolonged the slow phase of disease in mSOD1 mice, augmented M2 markers and suppressed M1 markers and their pro-inflammatory cytokines. It was further demonstrated that Tregs, through their secretion of IL4, can directly suppress the toxic properties of microglia (Zhao *et al.*, 2012); we previously showed that suppressing the toxic attributes of microglia leads to prolonged survival in this model of ALS. Moreover, M2 cells also have the ability to induce $CD4^+$ Tregs with a strong suppressive function (Savage *et al.*, 2008). mSOD1 microglia induced more IL4-expressing Tregs from mSOD1 mice. Thus, Tregs during the early stages of disease are immunocompetent and actively contribute to neuroprotection through their interactions with microglia. As disease progresses, a transformation occurs from a supportive Treg-M2 response to an injurious Th1–M1 response. It is known that Th1 cells produce interferon gamma (IFNγ), which promotes M1 microglial activation, and M1 cells can promote proliferation and function of Th1 cells. This vicious cycle is believed to be a significant driving force

for acceleration of disease course. Therefore, the dialogue between T cells and microglia modulates their phenotypic profiles and subsequently drives disease progression.

When disease entered the rapidly progressing stage, transplantation of Treg cells could not reverse the acceleration of disease. What causes the transformation and dysfunction of Tregs remains unknown, but cytokines released from activated microglia, astrocytes and T cells are likely candidates. Both Th1 cells and M1 microglia release TNFα, which has been recently demonstrated to induce the dysfunction of Tregs by inhibiting phosphorylation of FoxP3 (Nie *et al.*, 2013). IL1β was required to drive the conversion of Tregs to Th17-producing cells (Li *et al.*, 2010). Moreover, IL6 has been reported to inhibit the generation of FoxP3$^+$ Tregs (Bettelli *et al.*, 2006). In the mSOD1 mouse model, the transition from protection to toxicity coincided with increased expression of IL6; IL6 can be produced by activated microglia, astrocytes and Th1 cells. Nevertheless, it is unlikely that any single cytokine such as IL6 is solely responsible for the transformation from neuroprotective Treg–Th2–M2 cells to cytotoxic Th1–M1 cells, and it appears much more likely that multiple pro-inflammatory cytokines mediate the transition.

Although Th2 cells did not increase in lumbar spinal cord, more Th2 cells exist in the cervical spinal cord where disease starts later and progresses more slowly than in lumbar cord (Beers *et al.*, 2011b). Th2 cells infiltrate the cervical region of ALS mice with enhanced IL4; Tregs were also increased and sustained at elevated levels for a longer period in cervical than lumbar cord, suggesting that the higher levels of IL4 in the cervical region are attributable to both Tregs and Th2. The protective M2 response is maintained in the cervical spinal cords of these mice even when disease progressed rapidly. Thus, distinctly different regional and temporal neuroinflammatory responses are present in the lumbar cord compared with the cervical cord, and may account for the earlier loss of function in hindlimbs than forelimbs.

The documented changes in T cell populations that occur in mSOD1 mice were also examined in ALS patients. Diminished levels of naïve (CD45RA) T cells and increased levels of memory (CD45RO) cells within the CD4$^+$ T cell subset were reported in peripheral blood of ALS patients. Henkel *et al.* (2013) found that there were T cell alterations present in the peripheral blood and spinal cord tissues of ALS patients. The numbers of CD4$^+$CD25^{high} Treg, FoxP3 and CD25 mRNA levels were reduced in ALS patients with rapidly progressing disease. Similarly, Gata3, transforming growth factor beta (TGFβ) and IL4 mRNA levels were also reduced in rapidly progressing patients. The levels of these Treg and Th2 markers inversely correlated with the rate of disease progression. Similar results on peripheral Tregs were reported in a recent study (Rentzos *et al.*, 2012). Furthermore, in post-mortem spinal cord tissues of ALS patients, Tbx21, IFNγ and NOX2 levels were up-regulated in rapidly progressing patients and FoxP3 expression was decreased. These data suggest that decreased Treg–Th2 and enhanced Th1–M1 cells contribute to rapid

disease progression. Most importantly, low FoxP3 mRNA levels early in disease predicted a rapid progression and reduced survival. For the first time we may be able to predict rapid progression of disease in ALS patients, and such predictive ability may be of value in stratification for enrolment in clinical trials.

Another population of T cells that participates in the systemic immune pathology of ALS are natural killer T (NKT) cells, which share properties of both T cells and NK cells. NKT cells recognize lipids and glycolipids presented by CD1d molecules. Upon activation, these cells modulate different immune responses by rapidly releasing cytokines, such as IL2, IFNγ, TNFα, IL13 and IL4. It has been shown that NKT cells were increased in peripheral blood of ALS patients. NKT cell levels and activation state were also increased in the spinal cords, spleens and livers of mSOD1 mice. After treatment with PBS57, a ligand analogue that induced hypo-responsiveness of NKT cells, mSOD1 mice had a delayed onset and extended lifespan as well as a reduction in motoneuron loss and astrogliosis (Finkelstein *et al.*, 2011). The evidence of early recruitment of T cells to the spinal cord after down-regulation of NKT cell activity by PBS57 suggests that NKT cells may contribute to the suppression of protective T cell responses in ALS.

Immunologic aspects of ALS: part 3 – B cells

Autoantibodies to neural antigens have been identified in CSF and serum of ALS patients. However, it is still unclear if these autoantibodies have a pathological role in motoneuron degeneration or represent a secondary immunological consequence of neuronal death. The lack of mature B cells did not change disease development in mSOD1 mice, arguing against an essential role for B cells in ALS. Using antibodies to CD19, a B cell marker, B cells were not identified in lumbar spinal cord sections examined from 105- and 140-day-old mSOD1–RAG2$^{+/-}$ mice and mSOD1–RAG2$^{-/-}$ mice with BMT (Beers *et al.*, 2008). Furthermore, at end-stage disease, there was no consistent and convincing evidence for the presence of B cells.

Immunologic aspects of ALS: part 4 – astrocytes

As the largest glial cell population in the CNS, astrocytes provide structural, trophic and metabolic support to neurons and influence neuronal excitability. In addition, astrocytes help protect neurons from excitotoxicity. Although astrocytes are not immune cells *per se*, they actively contribute to the immune response and motoneuron degeneration in ALS. Analyses of astrocytes in post-mortem tissue from both fALS and sALS patients revealed a set of 22 up-regulated genes encoding chemokines, pro-inflammatory cytokines as well as components of the complement cascade (Haidet-Phillips *et al.*, 2011). Human mSOD1 astrocytes were shown to induce motoneuron toxicity that

was correlated with an increased astroglial inflammatory response; mSOD1 astrocytes up-regulated NOX2 to produce $O_2^{\bullet -}$, and apocynin prevented motoneuron loss caused by mSOD1 astrocytes. The transplantation of astroglial precursor cells into spinal cord of mSOD1 mice to establish healthy astroglial pools, which resulted in extended survival, attenuated motoneuron loss and improved motor function (Lepore *et al.*, 2008). Most importantly, reduced microgliosis was observed in these transplanted mice, suggesting that astrocytes may modulate the immune response elicited by microglia. mSOD1-derived astrocytes transplanted into the cervical spinal cord of WT rats induced reactive astrocytosis with reduced excitatory amino acid transporter-2 (EAAT2)–GLT1 transporter expression, forelimb motor and respiratory dysfunction, ubiquitination, and death of host motoneurons. The mSOD1 astrocyte-induced motoneuron death was, in part, mediated by host microglial activation and oxidative stress. These mSOD1 astrocytes that released the toxic factor(s) may have an aberrant phenotype with enhanced proliferative capacity. While the identity of the toxic factor(s) is unclear, several toxic factor(s) have been proposed; ROS-induced dysregulation of the ratio of pro-NGF to mature NGF, and a sumoylated CTE fragment of EAAT2–GLT1. The toxic factor(s) may induce activation of an apoptotic pathway in motoneurons, as Bax inhibition provided strong neuroprotection. Additionally, the toxic factor(s) may reduce GluR2 expression in motoneurons. Thus, astrocytes participate in immunological and inflammatory events, which ultimately mediate motoneuron toxicity in ALS.

The NTFs provided by astrocytes include glial-derived neurotrophic factor, brain-derived neurotrophic factor, IGF1, ciliary neurotrophic factor and vascular endothelial growth factor. Trophic factors not only are directly neuroprotective for motoneurons but also can reduce microglial and astroglial activation. However, ALS astrocytes may provide insufficient support, which could be contributing to motoneuron injury. Additionally, astrocytes expressing mSOD1 expressed reduced lactate efflux transporter. While up-regulating these trophic factors slowed disease progression in the mouse model, they have not provided neuroprotection in the ALS patient (Vargas and Johnson, 2010). Excess glutamate released into the synaptic cleft is normally taken up by astrocytes through the excitatory amino acid transporters which tightly regulate glutamate concentration in the synaptic cleft. EAAT2–GLT1, expressed predominantly in astrocytes, is one of the major glutamate transporters responsible for 90% of total glutamate uptake. If the extracellular glutamate is not removed, sustained AMPA receptor activation and calcium influx can initiate a cascade of events that lead to motoneuron death. In ALS patient autopsy tissue, cytoplasmic hyaline inclusions and other indicators of ROS stress are present in reactive astrocytes surrounding regions of motoneuron injury. In addition, granular aggregates of misfolded SOD1 are regularly present in activated astrocytes regardless of whether the ALS patient had a SOD1 mutation; negligible staining of astrocytes was observed in other neurodegenerative and non-neurological controls. These reactive astrocytes often

express reduced levels of EAAT2 in the motor cortex and spinal cord, which results in elevated glutamate levels in the CSF of many patients. ROS inactivated the EAAT2 transporter when co-expressed with mSOD1, but not WT SOD1. Consequently, the escalating glutamate concentrations further exacerbate motoneuron injury. Normally *in vitro*, astrocytes provide sufficient support to enable motoneuron survival; however, 40% of the motoneurons undergo apoptosis when plated on astrocytes pre-treated with peroxynitrite or LPS, even with the addition of NTFs.

While mSOD1 expression in astrocytes alone induced astrogliosis, it did not induce disease in the mouse (Gong *et al.*, 2000). However, levels of mSOD1 expression in astrocytes affected disease progression; a lack or reduction of mSOD1 expression in astrocytes slowed disease progression and prolonged survival of mSOD1 mice (Yamanaka *et al.*, 2008; Wang *et al.*, 2011). Astrocytic inclusions staining positive for SOD1 and ubiquitin were observed in mSOD1 mice prior to or at disease onset, depending on the mutation, and they increased with disease progression. Reactive astrogliosis also intensified as the disease progressed, concurrent with a reduction in EAAT2–GLT1 expression. While a reduction in EAAT2–GLT1 expression was present in both ALS patients and the animal models of ALS, and over expression of EAAT2–GLT1 in mSOD1 mice under control of the human GFAP promoter delayed the onset of motor symptoms and aggregate formation, it did not delay the onset of paralysis or prolong survival.

In addition to the reduction of EAAT2–GLT1 glutamate transporter and trophic factor support, ALS astrocytes release factor(s) that are directly toxic to motoneurons. *In vitro*, astrocytes isolated from post-mortem sALS and fALS patient autopsy tissues induced similar motoneuron toxicity. The toxicity from both sALS and fALS astrocytes was reduced with SOD1 knockdown, and human and mouse primary mSOD1 astrocytes were toxic to primary or embryonic stem cell–derived motoneurons with or without mSOD1 expression.

Immunologic aspects of ALS: part 5 – cytokines, chemokines and other markers of inflammation in ALS

Levels of numerous pro- and anti-inflammatory cytokines and chemokines are increased in ALS patients as well as in ALS mouse models. The pro-inflammatory mediators include IL6, TNFα, IL1β, IL12, IL17, IL23 and IFNγ, whereas the anti-inflammatory cytokines include IL4, IL10 and TGFβ, most of which derive from activated glia, monocytes and macrophages, T cells or DCs. IL17 and IL23 were found to be elevated in serum and CSF of patients with ALS, and it has been suggested that these elevations are a reflection of Th17 cell activation.

None of these cytokines are specific for ALS or ALS models, and several such as TNFα and TGFβ can contribute to either protection or toxicity. Furthermore, the multiplicity of such cytokines and pathways suggests that no single factor *per se* mediates either protection or toxicity; deletion or inhibition of no single factor can dramatically change the course of disease. Yet, collectively, their presence supports the involvement of immune and inflammatory processes in the pathogenesis of disease.

Chemokines, such as CX_3CL1 and CCL2, have a role in modulating neuroinflammation in ALS that promotes neuroprotection or propagation of inflammation. Microglia are the only CNS cells that express CX_3CR1. In the absence of CX_3CR1, M1 neurotoxic microglial activation is increased and is associated with extensive neuronal loss in mSOD mice (Cardona *et al.*, 2006). CCL2 is expressed mostly in astrocytes but also in neurons, microglia and macrophages following diverse injury. Higher levels of the chemokine CCL2 are present in ALS spinal cord tissue and in the CSF of ALS patients compared with patients with non-inflammatory neurological disease. ALS patients with higher CCL2 values tended towards a shorter diagnostic delay and a shorter survival time. The association of higher CCL2 with faster disease progression suggests that enhanced trafficking of activated monocytes and macrophages might contribute to the pathogenesis of disease. It has also been reported that the CCL2 receptor, CCR2, was reduced on circulating monocytes in ALS and associated with a slower rate of disease progression (Zhang *et al.*, 2006). Thus, the loss of CCR2 expression might be an essential protective reaction of the host immune response to macrophage-mediated CNS damage in ALS.

Other markers of inflammation noted in ALS include those of the classical complement pathway. Levels of mRNA and proteins of C1q and C4, as well as the downstream complement components C3 and C5b9, were found to be elevated in ALS samples in comparison with controls (Sta *et al.*, 2011). There is also evidence of low-level systemic inflammation with increased levels of C-reactive protein and raised erythrocyte sedimentation rate (ESR) in subjects with ALS compared with controls, and the levels correlate with levels of disability as measured by the ALS Functional Rating Scale.

Immunologic aspects of ALS: part 6 – dendritic cells

Immature and activated or mature blood-derived DCs are present in the ventral horn and corticospinal tracts of ALS patients as well as the spinal cord of late symptomatic mSOD1 mice (Henkel *et al.*, 2004, 2006). Furthermore, increased expression of DC transcripts, but not monocytic–macrophage–microglial, cytokine or chemokine transcripts, appeared to correlate with

more rapidly progressing disease, thus suggesting that DCs may exacerbate motoneuron injury in ALS. In a subsequent report, Sta *et al.* (2011) also found higher numbers of DCs in ALS patient tissue compared with control tissue, and found that rapidly progressing ALS patients had more DCs than slowly progressing ALS patients. Increased DCs were also seen in the spinal cord tissue of the mSOD1 mouse model (Henkel *et al.*, 2004, 2006).

Degenerating and electrically silent motor neurons express MHC class I and induce MHC class II on surrounding glial cells (Neumann, 2001). Moreover, microglia from mSOD1 mice significantly up-regulate the DC markers CD11c, CD86, CD54 as well as MHC class II, suggesting that mSOD1 microglia acquire DC features. All of these studies indicate that the fundamental conditions for efficient antigen presentation may be present in degenerating areas of ALS CNS tissues. However, the exact functions of DCs and DC-like microglia in ALS, especially their interactions with T cells, and the ALS-specific antigens have not yet been identified.

Conclusions

Neuroinflammation, which is characterized by activated microglia and infiltrating T cells, is a prominent pathological feature in the neurodegenerative disease ALS. Experimental models suggest that activated microglia and $CD4^+$ T cells contribute significantly to disease progression, but probably do not initiate disease. Data from rodent axotomy models and early stages of disease in mSOD1 mice indicate that the first response to injury, provided by the surrounding predominantly M2 microglia and infiltrating $CD4^+$ T cells, is neuroprotective. However, at later stages of disease in mSOD1 mice, this neuroprotective response is transformed into a cytotoxic response, possibly because of continued neuronal stress and signalling. It is unknown at present why and how neuroprotection is transformed into cytotoxicity. In mSOD1 mice, misfolded and aggregated proteins, mutant as well as oxidized SOD1, are secreted from neurons, which promotes pro-inflammatory M1 microglia and cytotoxic T cells and amplifies neuronal injury. In sALS, it is unknown what initiates disease onset, but a similar set of temporal and mechanistic events could transform neuroprotective microglia and T cells into cytotoxic cells, thereby accelerating disease progression. A greater understanding of what dictates the presence of cytotoxic or neuroprotective immunomodulation, and how to limit cytotoxicity and enhance neuroprotection, would help identify appropriate targets for immune-based therapy in neurodegenerative diseases. Thus, therapies that can down-regulate the harmful responses of innate and adaptive immune cells (M1 and Th1), and up-regulate the beneficial responses (M2 and Treg) may slow the progression of ALS and provide meaningful hope for patients with ALS.

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9
Demyelinating Disorders of the CNS

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Introduction

Demyelination is observed in a number of inflammatory pathological conditions, including viral infections, autoimmune disorders and other idiopathic diseases. In general, these diseases are acquired demyelinating syndromes, which distinguishes these conditions from hereditary metabolic, toxic or traumatic disorders of myelin. Immune-mediated damage to myelin may occur as a primary inflammatory event or following neuronal damage (Amor *et al.*, 2010). In the former case, such damage is termed primary demyelination and exemplifies the 'outside-in' model, which refers to myelin damage preceding axonal damage (Kipp *et al.*, 2012a). In contrast, myelin loss can also be observed following axonal damage and neuronal degeneration, which is frequently associated with inflammation. This damage is termed Wallerian degeneration, exemplifying the 'inside-out' model, in which neuronal and axonal damage occurs prior to myelin loss. This mechanism operates, for example, in Theiler's murine encephalomyelitis virus infection due to a direct autoimmune attack on axons. Acquired, inflammatory or infectious

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demyelinating diseases encompass a range of disorders, but for the purpose of this chapter we have limited the discussion to those listed in Table 9.1. For more detailed information on virus infections of the CNS that lead to demyelination, see Chapter 7 and Amor *et al.* (2010).

Table 9.1 Inflammatory demyelinating diseases of the central nervous system

Disease	Characteristics	Variants
Acute haemorrhagic leucoencephalopathy (AHL)	Rare, rapidly progressive disease of cerebral white matter; high fatality	
Acute disseminated encephalomyelitis (ADEM)	Acute inflammatory disease damaging the CNS white matter; commonly described in young children; clinical course monophasic and multifocal	Post infectious Post vaccination Post immunization
Clinically isolated syndrome (CIS)	The initial clinical presentation of demyelination	
Neuromyelitis optica (NMO)	Once classified as a variant of multiple sclerosis (see below), it is now a disease in its own right in which there is selective damage to the optic nerves and spinal cord. Disease is due to autoimmunity to the water channel aquaporin-4 (AQP4).	Patients are characterized as AQP4 seropositive or negative, although there is no distinction clinically or pathologically.
Multiple sclerosis (MS)	Acute disease with rapid deterioration in clinical condition	Marburg
	Clinically presents with rapid deterioration. Pathology shows concentric rings of demyelination.	Balo's concentric sclerosis
	Rapid decline; pathology of large diffuse areas of damage	Schilder's diffuse sclerosis
	Large rapidly expanding lesions in the brain suggestive of a tumour	Tumefactive MS
	Classical multiple sclerosis	Benign, relapsing-remitting, secondary progressive, primary progressive
Optic neuritis (ON)	Inflammation of the optic nerve is mostly idiopathic but also associated with demyelinating disease such as MS and infections such as Lyme disease.	Also observed in Leber's hereditary optic neuropathy (LHON)

An overlap of symptoms and diseases is frequently observed in acquired demyelinating disease. For example, optic neuritis is seen in multiple sclerosis (MS) as well as neuromyelitis optica (NMO) and acute disseminated encephalomyelitis (ADEM), while a clinically isolated syndrome (CIS) may be the initial sign of either MS or NMO.

Here we discuss aspects of inflammatory autoimmune and idiopathic inflammatory demyelinating diseases, including clinical manifestations, pathology and therapeutic strategies. We also discuss the animal models used to study human diseases, and the pathogenic mechanisms with particular reference to the role of the immune response.

Specific diseases

Acute disseminated encephalomyelitis and acute haemorrhagic leucoencephalopathy

Acute disseminated encephalomyelitis is a group of demyelinating disorders that occur with a temporal relationship to viral infections (post-infection encephalomyelitis) or following vaccination (post-vaccination encephalomyelitis). The annual incidence of ADEM is between 0.2 and 0.8 per 100,000 and varies with geographic location (Parrish and Yeh, 2012) with a higher incidence in children. Diagnosis is based on a polysymptomatic clinical event with acute or subacute onset that includes encephalopathy, defined by behavioural changes or altered consciousness, and on the presence of multifocal lesions as detected by magnetic resonance imaging (MRI).

The more severe form of ADEM is acute haemorrhagic leucoencephalopathy, first described by Hurst in 1941. In AHL, myelin loss is accompanied by haemorrhages and fibrin deposits around blood vessels, due to vessel wall necrosis. Both ADEM and AHL are monophasic, although relapsing forms have been reported. AHL has a higher morbidity and mortality than ADEM. Diagnosis is supported by MRI, the presence of elevated levels of protein and oligoclonal bands in the cerebrospinal fluid (CSF), and the temporal association with infections.

Examination of the CNS of patients with ADEM reveals perivenous inflammation and demyelination in the brain and spinal cord. In addition to inflammation, large areas of demyelination are observed in cortical regions that are also perivenous. The immune component is reflected by the presence of T cells, B cells and plasma cells associated with areas of demyelination, as well as of lipid-laden macrophages indicative of ongoing myelin damage. In the more aggressive AHL, haemorrhages are observed around blood vessels. In the CSF, mild lymphocytic pleocytosis is present, while oligoclonal immunoglobulin bands are not always present. ADEM and AHL are observed in males and females and, while more frequently reported in young patients, both are

also reported in older patients. In severe cases of both AHL and ADEM, therapies include the use of high-dose steroids, intravenous immunoglobulins and plasmapheresis.

Optic neuritis

Optic neuritis (ON), defined as inflammation of the optic nerve, is often idiopathic in nature but is also associated with autoimmune diseases, viral and bacterial infections, other inflammatory conditions and vaccinations (Hoorbakht and Bagherkashi, 2012). ON is most frequently associated with MS and NMO, and is often the first clinical signs of these disorders. ON can be monophasic or recurrent, and may occur in the same or the other eye, the latter being more predictive of developing MS. Like MS, ON is more common in females. Clinically, ON is diagnosed by sub-acute unilateral pain and visual symptoms including periocular pain, visual loss and dyschromatopsia. While ON is frequently acute, it can have long-lasting effects. Ophthalmological and neurological examinations include visual-evoked potentials and MRI to examine if ON is isolated or reflective of more extensive CNS involvement that may be predictive of MS and NMO. More recently, optical coherence tomography (OCT) has been developed to measure the thickness of the retinal tissues, specifically the nerve fibre layer that is thinner due to neurodegeneration, and retinal ganglion cell damage in the eye with ON (Lidster and Baker, 2012).

While the visual functions affected in ON usually spontaneously recover within 2–3 weeks, therapeutic approaches are necessary to aid recovery. They include the use of steroids and immune-modulatory therapies to prevent further disability following ON. However, there is still no consensus as to the dosage and length of treatment with corticosteroids in ON. Given the strong association of ON and MS, many approaches used to control MS (discussed further in this chapter) are also used to control ON.

Clinically isolated syndrome

The term ‘clinically isolated syndrome’ is used to describe an episode of neurological disturbance in which lesions in the CNS may either be monofocal or multifocal, but it does not include encephalopathy (except in brainstem syndromes) (Miller *et al.*, 2012). CIS includes transverse myelitis diagnosed by weakness and/or numbness of both legs that may involve the arms. The clinical neurological disease is frequently reflected by lesions of demyelination on MRI. Other clinical presentations of CIS include optic neuritis (as discussed here), and brainstem, cerebellar and/or hemispheric dysfunction can also be visualized by MRI studies.

Neuromyelitis optica

NMO is a severe inflammatory demyelinating disease that selectively affects the optic nerves and spinal cord (Wingerchuk, 2006). NMO is also known as Devic's disease, after Eugene Devic who described the first clinical presentation of optic neuritis with acute myelitis as a distinct syndrome (Devic, 1894). Only recently has NMO, first considered a rare variant of MS, been considered a separate disease with the distinction between MS and NMO based on the clinical as well as pathology and serological findings (Table 9.2). However, while antibodies to the water channel aquaporin-4 (AQP4) are much more frequent in NMO as compared to MS, these antibodies are not present in all NMO cases (Ratelade and Verkman, 2012). Regarding the epidemiology, NMO is 100 to 200 times less prevalent than MS, but both conditions share a female predominance, the most striking being 10:1 for NMO in Japan. The prevalence of NMO is 1–2/10^5, and although the disease affects all ethnicities, the prevalence differs geographically.

Clinical features NMO is characterized by acute severe episodes of ON, as described here, as well as by spinal cord symptoms that range from mild sensory disturbances to complete transverse myelitis with paraplegia or tetraplegia, sensory impairments and bladder and bowel dysfunction. In addition, one-third of patients also develop brainstem lesions causing diplopia, poor

Table 9.2 Distinguishing characteristics of NMO and multiple sclerosis

	Neuromyelitis optica	Multiple sclerosis
Demography		
Male-to-female ratio	1:9	3:7
Median age at onset	41 (30–50)	32 (28–38)
Caucasian	76%	95%
Clinical features		
Bilateral optic neuritis	67%	41%
Severe attack-related weakness	71%	14%
Imaging and CSF		
Initial MRI brain normal	77%	47%
MRI spinal cord lesion ≥3 segments	98%	15%
CSF OCB or raised IgG index	17%	67%
NMO-IgG	73%	9%
Pathology		
Leukocyte infiltrates	Neutrophils and eosinophils > T and B lymphocytes	T and B lymphocytes > neutrophils and eosinophils
AQP4	Loss in all lesions	Up-regulation in active lesions
GFAP	Loss in most lesions	Increased staining

Table 9.3 Diagnostic criteria for neuromyelitis optica

Criterion	Clinical manifestation
Absolute	Optic neuritis
	Acute myelitis
Supportive	Evidence of contiguous spinal cord lesion that consists of three or more segments in length on MRI
	Negativity for the diagnostic criteria for multiple sclerosis on brain MRI scans conducted at onset
	NMO immunoglobulin G seropositivity
Limited forms of NMO	Idiopathic single or recurrent events of longitudinally extensive transverse myelitis (≥3 vertebral segment spinal cord MRI lesion) (LETM)
	Bilateral simultaneous or recurrent optic neuritis (BRON)
	Asian optic-spinal MS (OSMS)
	Optic neuritis or longitudinally extensive myelitis associated with systemic autoimmune disease
	Optic neuritis or myelitis associated with 'specific' NMO brain lesions (hypothalamic, periventricular and brainstem)

appetite or prolonged hiccup. In most cases, there is rapid development of recurrent lesions, with only partial recoveries, which may ultimately lead to death from respiratory failure (González *et al.*, 2013). A related spectrum of disorders includes recurrent ON, relapsing transverse myelitis (TM) and some cases of encephalitis (Kitley *et al.*, 2012) (Table 9.3).

Aquaporin-4 In the CNS, AQP4 is the main water channel located at the border of the blood–brain barrier (BBB). It is expressed by foot processes of astrocytes in the glia limitans and in the ependyma, and regulates water movement in the brain. AQP4 is also expressed in several peripheral tissues, including kidney collecting ducts, skeletal muscle, gastric parietal cells, tracheal epithelial cells, airway epithelium and exocrine gland epithelium. AQP4 is composed of a short isoform (M23) that forms large orthogonal arrays of particles, and a long isoform (M1) that does not form orthogonal arrays by itself but can associate with M23 to form relatively small orthogonal arrays (Rossi *et al.*, 2012).

The importance of AQP4 in NMO and spectrum disorders first became apparent by the finding by Lennon and colleagues, who reported the presence of antibodies in NMO patients which recognized the water channel AQP4 (Lennon *et al.*, 2004). These antibodies, also referred to as NMO-IgG, bind to the extracellular epitopes of AQP4. They are predominantly IgG_1 and can initiate complement deposition. Several diagnostic assays are available to examine patients for the presence of AQP4 antibodies. These include a

fluorescence immunoprecipitation assay and a cell-based assay. These assays have a sensitivity of 76–80% and a specificity of 100%. These assays detect serum autoantibodies to AQP4 in only 80% of the patients, indicating that 20% of patients are seronegative (Jarius *et al.*, 2012). AQP4-IgG-positive NMO patients differ clinically and epidemiologically from seronegative patients (Kitley *et al.*, 2012).

(Jarius *et al.*, 2012). Differences are that AQP4-IgG-positive disease is predominantly seen in women, has a more frequent association with coexisting autoimmunity, causes more severe clinical attacks, shows higher spinal cord lesion load and causes more frequent relapse. However, it could be that the present assays are not sensitive enough to detect every seropositive patient. Another possibility is that some of the seronegative patients have antibodies that recognize other antigens such as myelin–oligodendrocyte glycoprotein in the CNS.

Pathogenesis of NMO The characteristic histological features of NMO include loss of AQP4 and glial fibrillary acidic protein (GFAP) expression as a direct effect of antibody-mediated damage and, thus, loss of astrocytes. Associated with the NMO-Ig are vasculocentric depositions of activated complement components, inflammatory cell infiltrates and demyelination (Parratt and Prineas, 2010). The mechanisms of damage observed in NMO patients are studied in mice in which NMO-like lesions can be induced following intracerebral injection of NMO-IgG and human complement. In this model, astrocyte injury is observed, followed by inflammation, cytokine release, extensive demyelination and neuronal death. AQP4-deficient mice do not manifest significant peripheral abnormalities, except for a very mild impairment in maximal urinary concentrating ability, probably reflecting redundancy in the system. In animals, T cells are not required for the formation of lesions, although T cells are necessary for the development of NMO-IgG-producing plasma cells.

Pathological studies reveal that infiltrating leukocytes seen in NMO lesions are different from those seen in other demyelinating diseases of the CNS, such as MS. The CNS lesions in NMO typically contain neutrophils, eosinophils and macrophages, with relatively few T lymphocytes. NMO lesions also contain neutrophil elastase, implicating degranulating perivascular neutrophils in the disease. A further finding is the abundant presence of granulocytes in the CSF of NMO patients.

While the mouse models do not completely reproduce the human disease, they are useful to develop therapeutic strategies. For example, lesions in an NMO mouse model are greatly reduced after intracerebral injection of the neutrophil protease inhibitors sivelestat and cathepsin G inhibitor I, or by intraperitoneal injection of sivelestat alone. These data implicate a central role for neutrophils in the pathogenesis of early NMO lesions, and suggest

that neutrophil protease inhibitors such as sivelestat may be helpful in NMO therapy (Saadoun *et al.*, 2012).

Therapies Aside from the experimental therapies discussed in this chapter, many therapies available for treatment in other demyelinating diseases are in use in NMO patients. Several treatment approaches for NMO patients exist, as shown in Figure 9.1. The first-line therapies include steroids for acute relapses, followed by therapies to block or remove the potentially pathogenic antibodies and B cells. One approach involves the use of Rituximab, a monoclonal antibody therapy directed to CD20 on B cells, which is also in use in MS patients (Barun and Bar-Or, 2012).

Multiple sclerosis

MS is the most common cause of neurological disability in young adults leading to inflammation, myelin loss and axonal damage in the CNS. Although the disease was first described by Charcot in 1865, reports of people presenting with MS-like symptoms can be dated back to the 14th century (Kipp *et al.*, 2012b). The incidence and prevalence of MS vary geographically, being higher in temperate climates where around 1 in 1000 people develop the disease. In some regions, such as the Orkney Islands, 402 cases have been documented per 100,000, with as many as 1 in 170 women affected (Visser *et al.*, 2012). Factors accounting for this differential distribution include genetic and environmental factors, infection and exposure to vitamin D, as well as socioeconomic factors. In general, the incidence of MS is also higher in females, although the incidence changes depending on the type of MS (i.e. primary progressive MS is higher in males). The incidence also depends on the month of birth, with a higher incidence in people born in May and a lower incidence in those born in November in the northern hemisphere; this is reversed in the southern hemisphere. In addition, the ratio of females to males is gradually increasing depending on the geographical region (Ramagopalan *et al.*, 2010).

Most patients develop a relapsing-remitting clinical course in which the damage in the CNS affects one or more sites in the CNS. With time, the disease develops into a secondary progressive form of MS in which progressive neurological disease becomes irreversible. In 10–20% of patients, the disease is progressive from the onset. The majority of patients develop their first signs between the ages of 20 and 40 years, although the disease is also seen in children.

Pathology Pathological studies of the CNS from MS cases are mainly focussed on studies of post-mortem tissues obtained from patients with long-standing disease. In rare cases, biopsy material can be taken from patients with suspected tumours termed 'tumifactive MS'. Radiological and pathological

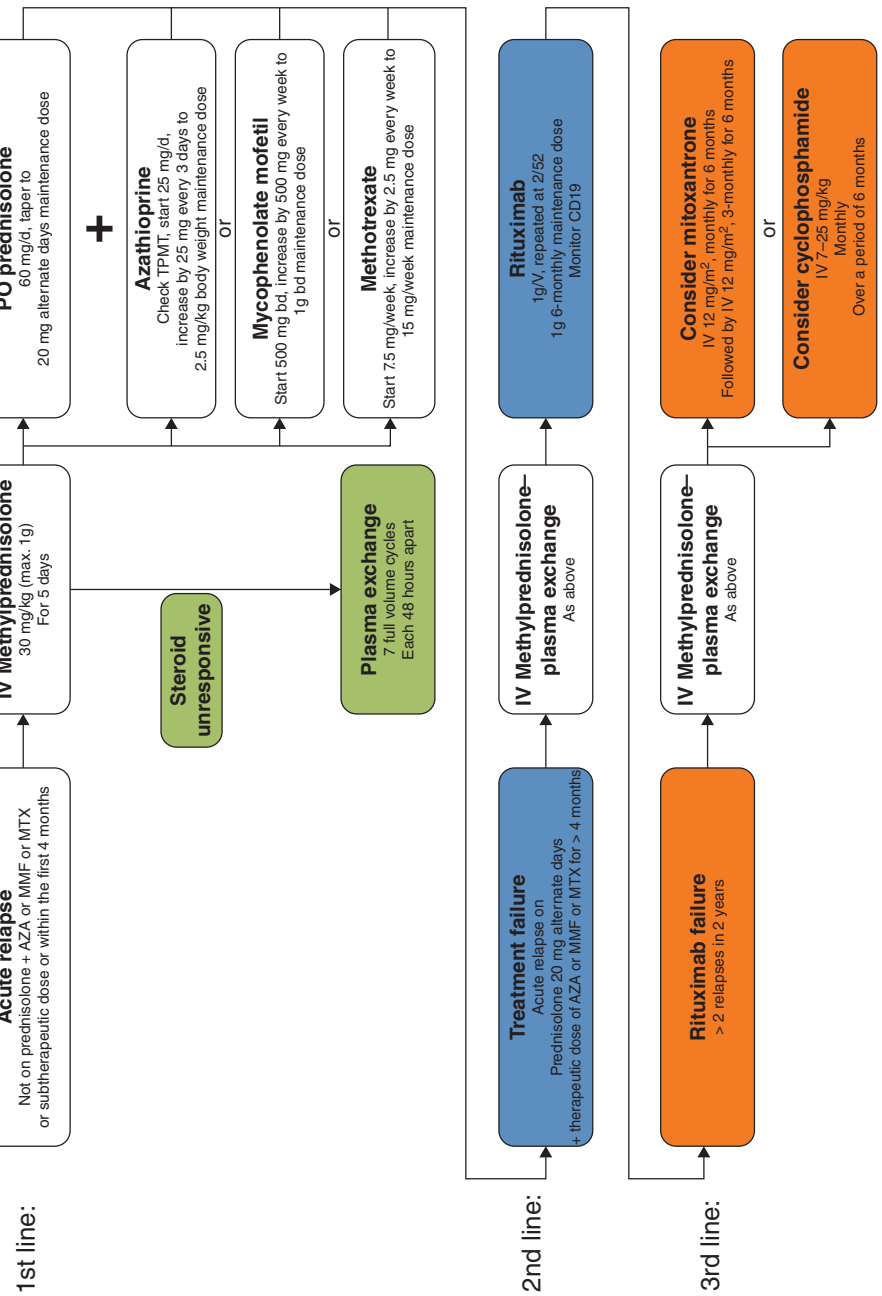

Figure 9.1 Treatment regimens for neuromyelitis optica.

studies have shown that new MS lesions develop continuously during the disease process and, thus, that early stages of lesion formation can also be studied even after many years of disease (van der Valk and Amor, 2009). The role of the immune system is reflected by a high expression of immune mediators in MS lesions, and the presence of activated cells of both the innate and adaptive immune systems. While frequently referred to as a T cell–mediated autoimmune disease, activated macrophages and microglia outnumber T and B cells in lesions. In addition, expression of MHC class II expression on microglia is observed in the normal-appearing white matter as well as on macrophages and microglia associated with the active and chronic active lesions. It has been suggested that white matter MS lesions begin in normal-appearing white matter as small clusters of activated microglia that are associated with oligodendrocyte stress (van Noort *et al.*, 2010). These clusters, termed 'pre-active lesions', may or may not develop into active lesions, which are characterized by a dense collection of myelin-laden macrophages or microglia, otherwise known as foam cells because of their appearance (Figure 9.2). As the lesion develops, the centre becomes hypocellular and demyelinated, and surrounded by a rim of foam cells, ultimately forming a chronic active lesion. Finally, the lesion dies out and the demyelinated region is repopulated by hypertrophic astrocytes. These form the scar tissue that gives MS its name. MS lesions are also observed in the spinal cord, optic nerve, cortex and deep grey-matter regions, including the hippocampus and hypothalamus (van der Valk and Amor, 2009; Kipp *et al.*, 2012a).

As already noted by Charcot, several subtypes of MS are recognized based on clinical manifestations. These include Marburg's fulminant, or acute MS, first described by Otto Marburg in 1909. Patients present with a rapid development, show rapid deterioration and eventually develop a persistent coma and tetraparesis within several months after the first symptoms. Unfortunately, patients with Marburg's MS do not respond well to treatments. Pathological studies show destructive lesions, and in addition to macrophage infiltration, myelin damage and acute axonal injury, regions of necrosis are observed. In another rapidly progressive form, Balo's concentric sclerosis, the pathology is characterized by concentric demyelination. This pattern of concentric gadolinium-enhancing hyperintense rings is observed in imaging studies, thus distinguishing this form from Marburg's disease. Yet another form of MS is termed Schilder's diffuse sclerosis, which manifests as a rapid decline in mental functions. It is more frequently observed in children. The pathology is typically characterized by bilateral, large hemispheric demyelinating lesions. Tumifactive MS is typified by large, rapidly expanding lesions suggestive of a tumour, hence the name. However, rather than a tumour, the lesions are rapidly expanding demyelinating lesions. The pathology of tumifactive MS is reminiscent of that of acute MS, in which foamy lipid-laden macrophages and activated microglia are observed alongside areas of myelin loss.

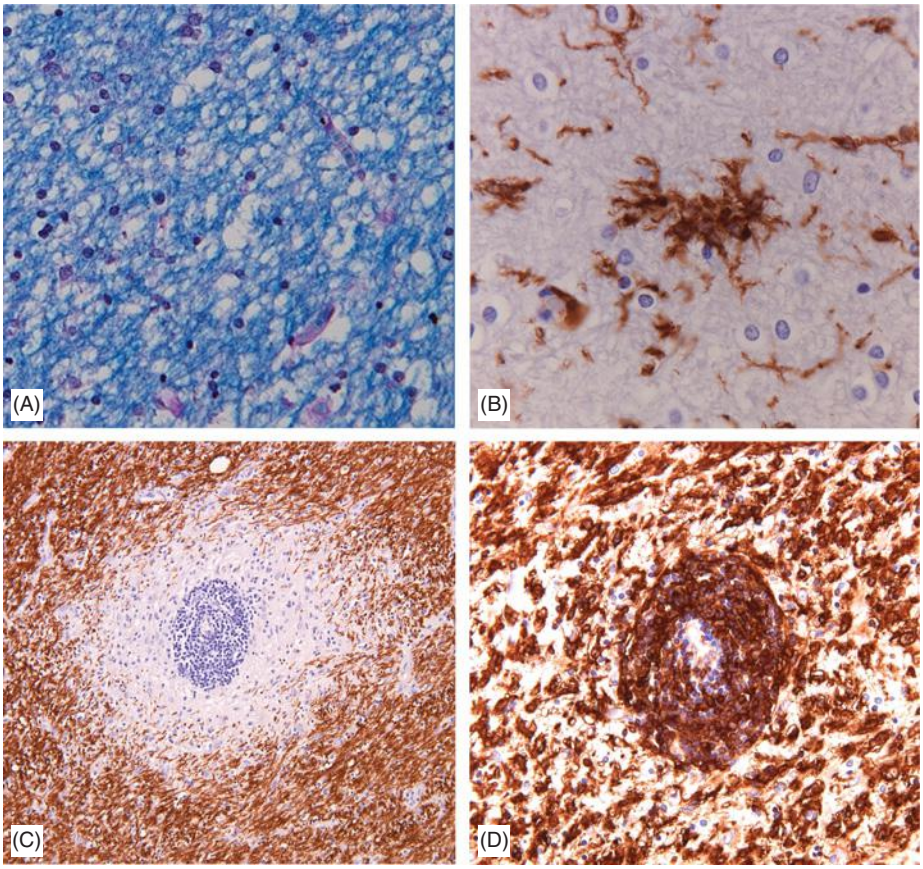

Figure 9.2 Pathology of multiple sclerosis. (A) Normal appearing white matter stained with luxol fast blue contains (B) clusters of activated microglia expressing MHC class II molecules that form pre-active MS lesions. (C) Active demyelinated MS lesions are frequently centred around blood vessels where cells of the adaptive immune system and foam cells are present. PLP staining (brown) shows region of myelin loss. (D) In active MS lesions, many cells express MHC class II molecules.

Pathogenesis To explain MS, it is widely assumed that following a viral infection, myelin-reactive T cells are triggered in the periphery and enter the CNS to subsequently cause inflammatory damage to myelin and axons. As a key element in these assumptions, anti-myelin T cell responses in MS patients should differ from those in people who do not develop MS. However, it is gradually emerging that peripheral anti-myelin, and indeed anti-neuronal immune responses, in MS patients are no different from that in controls (van Noort *et al.*, 1995, 2010; Huizinga *et al.*, 2009). However, cells of the immune system, especially $CD4^+$ and $CD8^+$ T cells and B cells, are clearly involved in MS. T cells are present within MS lesions, and preventing them from crossing

the BBB is effective in partially controlling the disease. This is exemplified by therapeutic approaches targeting VLA4 or blocking activated cells from leaving the lymph nodes (Table 9.4). More recently, attention has focussed on the role of B cells. Again, peripheral anti-myelin antibody responses in MS do not differ from those in healthy controls (van Noort *et al.*, 2010). B cells are present in MS lesions, including those in the meninges, where ectopic follicles have been suggested to be associated with grey matter lesions (Lassmann *et al.*, 2011). Nevertheless, oligoclonal immunoglobulins in the CSF are a diagnostic feature of MS and are present in over 95% people at diagnosis. While it is still unclear what antigens these antibodies recognize, it is thought that these oligoclonal bands are a result of clonal activation of B cells within the CNS.

Another clue to the pathogenesis has been offered by imaging studies in which changes in the normal-appearing white matter were seen to precede the development of gadolinium-enhancing demyelinating lesions. These changes reflect clusters of activated microglia, which we term 'pre-active lesions', strongly suggestive of an endogenous trigger of the innate immune response within the CNS. Detailed pathological studies of these pre-active MS lesions have revealed that the clusters of activated microglia are associated with stressed oligodendrocytes that express the heat shock protein alpha B crystallin. In animal models, the presence of alpha B crystallin has been shown to be protective during neuroinflammation. Alpha B crystallin knockout mice develop more severe experimental autoimmune encephalomyelitis (EAE), while administration of alpha B crystallin suppresses clinical signs (Ousman *et al.*, 2007). More recent *in vitro* studies support this issue in revealing that alpha B crystallin activates human microglia towards an immune-regulatory phenotype (van Noort *et al.*, 2013). How such innate immune activation ultimately allows for the formation of actively demyelinating lesions remains to be established. It has been suggested that production of interferon gamma by locally recruited memory T cells could promote such a transition (van Noort *et al.*, 2011, 2012).

Therapies The notion that the immune system is heavily involved in the pathogenesis of MS has led to the development of immune-suppressive and immune-modulatory approaches to treat the disease (Perumal and Khan, 2012; Saidha *et al.*, 2012; Yadav and Bourdette, 2012). To date, several immunosuppressive therapies have been approved, while others are still in development (Table 9.4). The side effects of such approaches, however, such as the more frequent occurrence of opportunistic infections or loss of control over latent infections, remain a concern.

Animal models of inflammatory demyelinating diseases

Although the primary cause of many inflammatory demyelinating diseases of the CNS is unknown, it is widely considered likely that viruses are

Table 9.4 Therapies for multiple sclerosis

Drug	Administration	Mechanisms of action	Adverse events	Efficacy – ARR	Status
Interferon beta (IFNβ) IFNβ1a Avonex® Rebif® IFNβ1b Betaferon® Betaseron® Extavia®	 30 μg IM weekly 22–44 μg SC 3× week 250 μg SC every other day 250 μg SC every other day	Not completely understood. Plays a role in several immunomodulatory pathways, including inhibition of T cell activation and proliferation, down-regulation of MHC-II expression and stimulation of anti-inflammatory cytokine expression pattern	Injection site reactions, flu-like symptoms, leukopenia, anaemia, liver enzyme elevation and neutralizing antibodies directed to INFβ abolishing the efficacy of the drug	27–50% reduction versus placebo CTs: Pivotal phase III PRISMS phase III	FDA approved 1996 2002 1993 2009
Glatiramer acetate (GA) Copaxone®	20 mg SC daily	Exact MOA not known. GA is thought to compete with MHC binding of myelin antigens and presentation to T cells. GA suggested to induce immunoregulatory response.	Injection site reactions and an immediate systemic reaction post injection	28% reduction versus placebo CT: CONFIRM	FDA approved 1996
Nataluzimab Tysabri®	300 mg IV every 4 weeks	Humanized mAb against VLA-4, thus blocks lymphocyte migration into the CNS	PML, infusion-related hypersensitivity, infections and headache	68% reduction versus placebo CT: AFFRIM	FDA approved 2004, reapproved 2006

(*continued*)

Table 9.4 (*Continued*)

Drug	Administration	Mechanisms of action	Adverse events	Efficacy – ARR	Status
Mitoxantrone Novantrone®	12 mg/m^2 IV every 3 months; restricted cumulative lifetime dose < 140 mg/m^2	Reduces B cell, T cell and macrophage proliferation by interfering with DNA repair through inhibition of topoisomerase II	Cardiotoxicity, therapy-related acute leukaemia, nausea, vomiting, alopecia, infections, amenorrhea and infertility	65% reduction versus placebo	FDA approved 2000
Fingolimod Gilenya®	0.5 mg oral daily	Acts as a sphingosine analogue by binding to sphingosine-1-phosphate receptor (S1P); blocks migration from secondary lymphoid tissue, thus decreasing cells in periphery; may have a neuroprotective role by interaction with neural cells expressing S1P receptors	Headache, nasopharyngitis, fatigue, infections, bradycardia, atrioventricular conduction block, hypertension, macular oedema, lymphopenia and liver enzyme elevation	55% reduction versus placebo CT: FREEDOMS	FDA approved 2010
Teriflunomide Aubagio®	7 or 14 mg oral daily	Activated lymphocytes depend on *de novo* pyrimidine synthesis. This is inhibited by teriflunomide resulting in suppression of immune cell proliferation.	Gastrointestinal symptoms, alopecia, increased levels of liver enzymes, skin rashes, weight loss, infections and hypertension	31% reduction versus placebo CT: TEMSO	FDA approved 2012

Cladribine	0.875 mg/kg/course; 2 courses 1 month apart, and in the second year another 2 courses	Purine nucleoside analogue, which preferentially accumulates into the DNA of lymphocytes where it induces apoptosis, leading to a dose-dependent depletion of lymphocytes	Lymphocytopenia, neutropenia, thrombocytopenia, pancytopenia, dermatomal herpes zoster reactivation, risk of infections and possible secondary malignancies	57% reduction versus placebo CT: CLARITY	Observational long-term safety (PREMIERE)
BG12	240 mg oral twice a day	Active component is dimethyl fumarate. Precise MOA is not known. Fumarate activates the nuclear factor E2-related factor 2 transcriptional pathway, which plays an important role in the oxidative stress response. It affects both neuronal and immune cells by changing their enzyme and cytokine expression.	Flushing, headaches, gastrointestinal symptoms, dose-related liver enzyme elevation, lymphopenia and eosinophilia	53% reduction versus placebo CT: DEFINE	Phase III study

(*continued*)

Table 9.4 (*Continued*)

Drug	Administration	Mechanisms of action	Adverse events	Efficacy – ARR	Status
Laquinimod	0.6 mg oral daily	Not fully understood; anti-inflammatory properties secondary to down-regulation of MHC-II and a shift from the Th1 to Th2 T cell profile; an increased production of BDNF may explain the neuroprotective role.	Arthralgia, elevated liver enzymes and reactivation of herpes simplex and varicella zoster viruses	23% reduction versus placebo CT: ALLEGRO	Phase III study
Alemtuzumab Campath®	12 mg IV daily for 5 d in year 1 and for 3 d in year 2. Consequent annual courses are based on the clinical disease activity.	Humanized mAb to CD52, expressed on the surface of mature leucocytes. Binding to CD52 leads to complement-mediated lysis, causing a rapid depletion of the target cells.	Other autoimmune disease (thyroid autoimmunity and idiopathic thrombocytopenic purpura), headache, rash, nausea, vomiting, hypertension, shortness of breath, fever, infections and cytokine-mediated transient worsening of pre-existing neurological deficits	55% reduction versus INFβ Study: CARE MS	Phase III study FDA approved for lymphocytic leukaemia (2007)

Daclizumab Zenapax®	150 mg SC every 4 weeks, except 2nd, which is at 2 weeks	Humanized mAb directed against CD25, the α-subunit of the IL2 receptor. CD25 is crucial for T-cell proliferation and activation. The clinical benefit has been linked to a down-regulation of adaptive T cell responses and an expansion of immunoregulatory CD56 NK cells.	Rash, infections, fever and fatigue lymphadenopathy, elevation in liver enzymes	50% reduction versus placebo; preliminary data from SELECT study	Phase II/III study FDA approved for the prophylaxis of acute organ rejection (1997)
Rituximab Retuxan®	1000 mg IV 2 weeks apart	Chimeric mAb against the CD20 on pre–B cells and B cells. Induces a transient depletion of B cells, but also has an indirect effect on macrophage and T cell responses.	Infusion-related reactions, infections and PML	No significant reduction versus placebo Trial: HERMES	Phase III study FDA approved for non-Hodgkin lymphoma (1997)
Ocrelizumab	300 mg IV on days 1 and 15 in a cycle, of 24 weeks	Humanized CD20 mAb recognizes a different CD20 epitope than Rituximab; depletes B cells primarily through antibody-dependent cellular cytotoxicity; also effect on T cell–mediated responses.	Infusion reactions	80% reduction versus placebo	Phase III

IM, intramuscular; SC, subcutaneous; IV, intravenous; MOA, mechanism of action; mAb, monoclonal antibody; VLA4, very late activating antigen-4; IL, interleukin; PML, progressive multifocal encephalopathy; ARR, annualized relapse rate; CT, clinical trial; FDA, US Food and Drug Administration; BDNF, brain-derived neurotrophic factor; IFN, interferon; MHC-II, major histocompatibility complex class II; ARR, annualized relapse rate.

implicated in triggering autoimmune responses directed to myelin. Thus, models to study demyelination and remyelination involve experimental viral infections in addition to experimental induction of autoimmunity or toxin-induced damage (Table 9.5). Viral models commonly used to study ADEM are discussed elsewhere (see Chapter 7; Amor *et al.*, 2010; van der Star *et al.*, 2012). Autoimmune models used to study these diseases rely on immunization with CNS antigens in a strong adjuvant, or adoptive transfer of CNS-specific T cells, either alone or in combination with antigen-specific immunoglobulins (Amor *et al.*, 2005). The use of humanized mice or transgenic mice expressing specific T cell receptors allows more detailed study of the role of specific components of the immune system (Ellmerich *et al.*, 2004). The nature of the antigen used for immunization in active models co-determines the nature of the experimental disease that is induced (Bettelli *et al.*, 2006). For example, antibodies to AQP4 are commonly used to mimic NMO, whereas antibodies to myelin oligodendrocyte glycoprotein (MOG) are used to evoke myelin damage similar to that found in MS. Given that the models commonly used to study MS also have features that mimic those of other demyelinating diseases of the CNS, we first discuss the models of MS.

Multiple sclerosis EAE is the most common model that attempts to recapitulate pathogenic features of MS (Amor *et al.*, 2005; Kipp *et al.*, 2012a). Susceptible animals immunized with myelin or neuronal antigens (Huizinga *et al.*, 2007) together with adjuvant develop monophasic neurological disease, from which the animals either recover (acute EAE) or go on to develop chronic disease without recovery. In contrast, some susceptible strains develop chronic-relapsing neurological episodes and progressive disability – features that better resemble relapsing-remitting MS (Baker *et al.*, 1990; Hampton *et al.*, 2008; Table 9.5). EAE can be induced following isolation of myelin-specific T cells, *in vitro* activation and adoptive transfer to naïve recipients. Adoptive transfer has revealed that both CD4+ and CD8+ T cells can be pathogenic (van der Star *et al.*, 2012).

In contrast to the autoimmune models, studies focussing on demyelination and remyelination in the absence of an adaptive immune response are better studied in toxin-induced demyelination models, for example those using cuprizone and lysolecithin (Kipp *et al.*, 2012a). As an alternative to the autoimmune model, or to study the role of viruses in the induction of immune-mediated demyelination, several virus infections of animals have been established. These have particular relevance to the question of how viruses can initiate an autoimmune disease.

Because the data collected in animal models frequently form the basis for the development of new therapeutic approaches for MS, it is crucial to follow well-defined ethical guidelines for performing studies in animals. These are discussed in more detail in Baker *et al.* (2011) and Amor and Baker (2012).

Table 9.5 Animal models of CNS demyelinating diseases

Disease	Animal model	Species	Mode of induction	Disease characteristics	References
Optic neuritis	EAE, Devic's mouse	Mice, rhesus monkeys, transgenic mice	Immunization with optic nerve tissues or OSP	Inflammation and demyelination of optic nerve; alterations in fast axonal transport of proteins	O'Neill *et al.* (1998) Bajramovic *et al.* (2008) Genain and Hauser (1996)
NMO	Experimental NMO	Mice, rats	Transfer of NMO-Ig or i.c. injection; immunization with AQP4 protein; AQP4 antibody plus NK cells	Many EAE models develop optic neuritis and spinal cord lesions and rarely affect brain. Astrocyte damage with little myelin damage.	O'Neill *et al.* (1998) Bettelli *et al.* (2006) Pohl *et al.* (2011) Ratelade *et al.* (2012)
MS	EAE models	Mice, rats, marmosets rhesus monkey	Immunization of myelin or neuronal antigens in adjuvant	Clinical neurological disease; Can be acute, chronic, relapsing-remitting or secondary progressive	Hampton *et al.* (2008), Kipp *et al.* (2012a), Van der Star *et al.* (2012), Amor *et al.* (2005)

OSP, oligodendrocyte specific protein; EAE, experimental autoimmune encephalomyelitis.

Optic neuritis Optic neuritis is frequently observed in EAE, and to specifically target T cells to the optic nerve, optic nerve tissues were used to immunize mice (O'Neill *et al.*, 1998). Inflammation and cell death in the optic nerve, with subsequent damage to the retinal ganglion cells, are thought to correlate with visual dysfunction observed in mice. ON in EAE is observed as early as 9 days after immunization, and is rapidly followed by myelin damage which is also present in the relapsing phase of disease. In this model, the ON is associated with a decrease in the fast axonal transport of proteins from the retina to the optic nerve. More recently, several models making use of transgenic mice which specifically express the T cell receptor for MOG have been described to induce disease in the optic nerve and spinal cord (Bettina *et al.*, 2008), although these phenomena actually occur in many mouse models of EAE.

Neuromyelitis optica In a way similar to EAE, models for NMO rely on immunization of susceptible animals with AQP4 in order to generate pathogenic antibodies to AQP4, the target of antibody responses in patients with NMO. Experimental models have been described that make use of a double transgenic myelin-specific B and T cell mouse. In addition, NMO-like disease has been induced by passive transfer of human anti-AQP4 antibodies into mice with EAE, or by intrathecal administration of such antibodies to naïve mice. Transfer of antibodies along with complement to rats with a damaged BBB induces astrocyte damage. In addition, NMO-Ig transferred along with natural killer cells also induces NMO lesions in mice without prior myelin loss, indicating a co-pathogenic role of immunoglobulin and NK cells (Ratelade *et al.*, 2012).

Similar to the studies in EAE models, attempts were made to immunize mice with AQP4 protein and peptides in rats. These studies show that in addition to the antibody, also T cells to AQP4 can be pathogenic in rats (Pohl *et al.*, 2011).

Conclusion

Demyelinating diseases of the CNS are frequently associated with inflammatory responses. In many cases, the pathogenic role of immune responses is demonstrable either by *in vitro* and *in vivo* approaches examining the pathogenicity of antibodies that are present in patients, or by the finding that immune modulation is beneficial in disease. In this chapter, we have reviewed human demyelinating disorders and discussed therapeutic approaches to such conditions. Many approaches that involve broad immune suppression leave the patient at an increased risk of infection. In some cases, these may even be fatal, as observed in MS patients who develop progressive multifocal leucoencephalopathy. Clearly, the development of more specific therapeutic approaches remains an important challenge.

Conflict of interest

J.M. van Noort holds equity in Delta Crystallon BV. The other authors declare no conflict of interests.

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10

Other Autoimmune Disorders: Systemic Lupus Erythematosus, Primary Sjögren's Syndrome, Gluten-related Neurological Dysfunction and Paraneoplastic Neurological Syndromes

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Systemic lupus erythematosus

Introduction

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease of unknown aetiology with diverse clinical manifestations. The diversity of clinical presentations means that the diagnosis of SLE can be challenging. As such, patients may present to different medical specialties depending on their primary clinical complaint. This diversity is also reflected by the fact that the diagnosis of SLE relies on the presence of four or more of 11 criteria (Table 10.1), a mixture of clinical findings and laboratory abnormalities. The 11 criteria were

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Table 10.1 The 11 SLE diagnostic criteria devised in 1982*

1. Malar rash: facial erythema over cheeks; referred to as 'butterfly rash'
2. Discoid rash: erythematus scaly raised patches anywhere over the body
3. Serositis: usually manifesting with pleurisy or pericarditis
4. Oral ulcers
5. Arthritis, usually non-erosive, affecting two or more joints
6. Skin photosensitivity
7. Haematological disorder such as haemolytic anaemia, leucopenia or thrombocytopenia
8. Renal involvement manifesting with proteinuria or urine cellular casts
9. Antinuclear antibody positive
10. The presence of other autoantibodies such as anti-dsDNA, anti-Sm, and false positive serological tests for syphilis
11. Neurological disorders

*For the diagnosis of SLE, at least four criteria are required.

originally devised in 1982 (Tan *et al.*, 1982) to facilitate research into SLE, and these were then revised in 1997 (Hochberg, 1997). One of the criteria for diagnosis of SLE includes central nervous system (CNS) involvement.

In practice, patients clinically suspected of having SLE are usually tested for antinuclear antibody (ANA) and antibodies to double-stranded DNA (dsDNA) as the first approach in making a diagnosis. This is perhaps the reason why the Systemic Lupus Erythematosus International Collaborating Clinics group revised and validated the criteria in 2012 (Petri *et al.*, 2012). Based on this revision, a patient can be diagnosed with SLE if they have kidney biopsy demonstrating lupus nephritis with positive ANA and dsDNA antibodies or if the patient satisfies four of the criteria (Table 10.1), assuming that these include at least one clinical and one immunological criteria (positive for ANA or anti-dsDNA antibodies).

Epidemiology

The prevalence of SLE is influenced by race and ethnicity with the highest rates reported amongst black and Hispanic populations in the United States (ranging between 40 and 100 per 100,000). The highest prevalences in the world have been reported in Italy, in Spain and amongst the Afro-Caribbean population in the United Kingdom (Danchenko *et al.*, 2006). However, environmental trigger factors are equally important, as highlighted by the fact that SLE is rarely reported amongst the black population currently living in Africa. The female-to-male ratio is 11:1 during childbearing years, and the onset is usually after puberty (Manzi, 2001).

Neurological manifestations

The American College of Rheumatology published a nomenclature system for neuropsychiatric manifestations of SLE in an attempt to standardize and

promote acceptance of the neuropsychiatric features of SLE (ACR Committee, 1999). This classification contained 19 neuropsychiatric conditions, including aseptic meningitis, cerebrovascular disease, demyelinating syndrome, headache, movement disorder (e.g. chorea), myelopathy, seizure disorder, cognitive dysfunction, mood disorder and psychosis for CNS involvement. Peripheral nervous system involvement includes acute inflammatory demyelinating polyradiculopathy, autonomic disorder, mononeuropathy multiplex, myasthenia gravis, cranial neuropathy, plexopathy and polyneuropathy (axonal sensorimotor neuropathy). Such classification has primarily been used for research purposes but may be less clinically helpful given that some of these 'conditions' represent symptoms (e.g. headache), others are based on case reports and are rare, and some are simply associations as a result of more than one autoimmune disease coexisting or coincidental in individuals, rather than aetiologically linked to SLE. For the purposes of this chapter, the neurological manifestations are divided into those affecting the CNS, including psychiatric manifestations; those affecting the peripheral nervous system; and those autoimmune neurological diseases that can be seen in SLE, probably as a result of more than one autoimmune disease coexisting in individuals. The prevalence of neuropsychiatric manifestations varies depending on what the spectrum includes; figures between 14 and 80% have been reported (Muscal and Brey, 2010).

CNS manifestations

Encephalopathy The term 'encephalopathy' has been used to describe diverse manifestations of CNS involvement in SLE, ranging from CNS vasculitis (usually presenting acutely with stroke-like episodes) to a slowly progressive CNS involvement, characterized by cognitive decline, depression, seizures, delirium and, rarely, psychosis.

One of the commonest neurological complaints in patients with SLE is headache, a symptom that has been reported in up to 57% of patients. Headache is, however, a common complaint in the general population. A meta-analysis of all controlled studies on SLE and headache (eight in total) that included studies that reported the type of headache according to the International Headache Society classification, demonstrated that both migraine and tension-type headaches were present, but that the prevalence was not significantly different from that of the control populations studied. No particular pathogenic mechanism of headache in SLE was identified, and there was no association between the presence of headache and disease activity (Mitsikostas *et al.*, 2004). Headache can be a feature of other CNS diseases affecting SLE patients, such as CNS vasculitis.

Vasculitis of the CNS can be seen rarely in SLE and usually presents with headache, seizures, confusion and focal neurological deficits in the context of stroke-like episodes. Angiographic studies demonstrate typical arterial changes (irregularity of the arterial wall) in keeping with vasculitis,

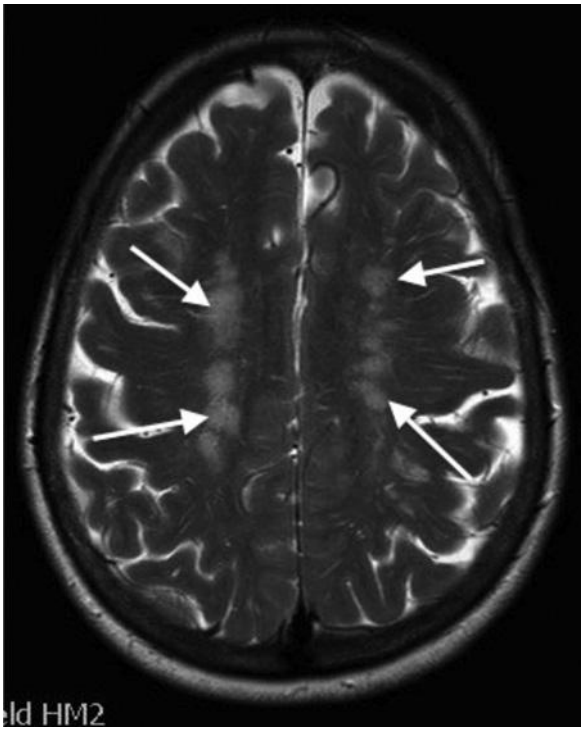

Figure 10.1 MR imaging of the brain of a patient with antiphospholipid antibodies showing the characteristic 'ischaemic'-looking abnormalities (arrows) primarily affecting the white matter.

whilst magnetic resonance imaging (MRI) may demonstrate the presence of ischaemic changes affecting multiple vascular territories. This entity is different from what is seen in the context of anti-cardiolipin antibodies (often present in patients with SLE) where brain imaging demonstrates small patchy hyperintensities often attributed to cerebrovascular ischaemia (Figure 10.1). An MRI study of an SLE population demonstrated a prevalence of such lesions in up to 50% of patients. However, such abnormalities did not correlate with the presence of clinical CNS involvement, and the prevalence was the same in patients with SLE without neurological symptoms (Gonzalez-Crespo *et al.*, 1995).

Another study used voxel-based morphometry to assess the regional grey matter volume in patients with SLE with and without neuropsychiatric manifestations. The study demonstrated reduction in the grey matter volume indicating atrophy affecting both groups of SLE patients, again unrelated to the clinical evidence of neuropsychiatric features. It therefore appears that a subclinical neurodegenerative and neuroinflammatory process affects a large number of patients with SLE (Cagnoli *et al.*, 2012).

CNS involvement in SLE is therefore common (probably as high as 50%) when assessed by imaging studies. In some cases, particularly those demonstrating increased white matter hyperintensities, CNS involvement may be related to the presence of anticardiolipin (aCL) antibodies and the prothrombotic tendencies associated with such antibodies. Such white matter involvement can be associated with cognitive deficits, and such deficits can be exacerbated by the presence of depression which is common in SLE.

Chorea Chorea and ballismus (an extreme form of chorea) have been described in SLE and can be seen in about 1% of all SLE cases. Chorea as a presenting feature of SLE is even rarer. In a series of 51 cases of SLE, patients who developed chorea did so at the early stages of the disease, the chorea was transient and symmetrical and in 50% of cases it was associated with additional neurological manifestations (Bruyn and Padberg, 1984). Whilst chorea in SLE was initially thought to be secondary to ischaemic damage affecting the basal ganglia, more recent evidence suggests that this is not the case, given that the majority of cases have no imaging evidence of such ischaemia and that the chorea can improve with immunosuppressive treatment (Galanaud *et al.*, 2000). Patients with SLE and chorea almost always have circulating anticardiolipin antibodies. The author has encountered a patient presenting with symmetrical ballismus and chorea, as a result of which the diagnosis of SLE was made. The patient had normal imaging and responded to steroids (prednisolone), but the chorea returned following reduction of steroid treatment. The patient responded to the introduction of mycophenolate, an immunosuppressant. These observations support an immunological rather than ischaemic aetiology for the chorea in SLE.

Other neurological manifestations and associations Cerebral venous sinus thrombosis can be seen in SLE patients, usually as a result of the prothrombotic tendency in the context of anti-cardiolipin antibodies. It usually presents with headache and papilloedema and is treated with anticoagulants. There are case reports describing associations of SLE with diverse neurological and psychiatric diseases, including transverse myelitis, optic neuritis, cerebellar ataxia, multiple sclerosis (MS), idiopathic intracranial hypertension, anxiety, psychosis and others. There are some neurological diseases that seem to be associated with SLE, presumably on the basis of the coexistence of more than one autoimmune disease. However, some of these autoimmune diseases are over represented amongst patients with SLE and include myasthenia gravis, polymyositis and neuromyotonia.

Pathophysiology of neurological manifestations SLE is characterized by multi-organ microvascular inflammation and damage involving autoantibody production, immune complex formation and deposition and possibly

intrathecal production of pro-inflammatory cytokines. SLE is therefore, above all, a disease of vessels. This explains the diversity of clinical presentations, including the involvement of the nervous system. Post-mortem studies in patients with CNS disease reveal a range of abnormalities that include, multifocal micro-infarcts as well as large infarcts, ischaemic demyelination and sometimes patchy demyelination reminiscent of MS. The microvasculopathy is suspected to arise from activation of complement and is probably the commonest histopathological brain finding in patients with SLE (Belmont *et al.*, 1996). Many of the clinical manifestations are mediated by circulating immune complexes or are due to the direct effects of antibodies on cell surface antigens. Immune complex deposition may lead to complement activation and further localized inflammation. Blood–brain barrier damage is an important factor in the context of neurological manifestations of SLE. The two main candidate mechanisms for such damage invoke microthrombii in cerebral vessels, leading to ischaemia or immune-mediated activation of the endothelium leading to local cytokine production (Abbott *et al.*, 2003).

Antibodies against dsDNA, ribosomal-P proteins and N-methyl-D-aspartate (NMDA) receptor have been found in the cerebrospinal fluid (CSF) of patients with SLE and CNS involvement (Fragoso-Loyo *et al.*, 2009). The same study examined the presence of IgG ANA, anti-dsDNA, anti-ribosomal-P, aCL, anti-beta-2 glycoprotein and anti-NMDA receptor antibodies as well as cytokines and chemokines in the serum and CSF of patients with SLE with neuropsychiatric symptoms. These assays were repeated 6 months later. There were no differences in the presence and levels of these antibodies, chemokines and cytokines between those patients with SLE and neurological involvement and those without. In both groups, the level of cytokines and chemokines decreased after 6 months, but this reached statistical significance in only the neurology group. The study did not identify a specific marker for neuropsychiatric manifestations.

Up to 30% of patients with SLE have abnormal clotting times as a result of the presence of anti-phospholipid antibodies (also known as aCL antibodies and lupus anticoagulant). These antibodies (usually of IgG and IgM class) can predispose patients to recurrent miscarriage, thrombocytopenia, and venous and arterial thrombosis and can be linked to other neurological manifestations (e.g. chorea). In a series of 15 patients with SLE who had the highest aCL antibody titre amongst patients with SLE, six had a history of venous thrombosis, five had cerebral infarcts, five had thrombocytopenia and two each had pulmonary hypertension and multiple abortions (Harris *et al.*, 1983).

The presence of repeatedly positive aCL antibodies in patients with SLE has also been associated with cognitive dysfunction (Muscal and Brey, 2010). A similar association has been observed in patients with SLE who have anti-glutamate receptor antibodies. The mechanism of such an association remains obscure, particularly given that such patients may have entirely normal brain imaging.

Primary Sjögren's syndrome

Introduction

Primary Sjögren's syndrome (PSS) is one of the commonest autoimmune diseases. It is characterized by lymphocytic infiltration of the exocrine glands leading to enlargement of the glands and clinically manifesting with dry mouth (xerostomia) and dry eyes (xerophthalmia). Secondary Sjögren's syndrome occurs in association with other connective tissue diseases such as rheumatoid arthritis, SLE and scleroderma. PSS can be associated with other organ involvement, including lungs (pneumonitis), renal involvement, pancreatitis, myositis and occasionally lymphoma (Mori *et al.*, 2005). The criteria for diagnosis of PSS have been the subject of a number of workshops, most recently in 2012 at a meeting of the Sjögren's International Collaborative Clinical Alliance Research Group. The diagnostic criteria were approved by the American College of Rheumatology (Shiboski *et al.*, 2012). The diagnosis relies on the presence of two out of three of the following: (i) positive serum antibodies known to be associated with PSS (anti-Ro and anti-La), (ii) demonstration of xerophthalmia using a special ocular-staining score and (iii) labial salivary gland biopsy showing focal lymphocytic sialadenitis.

Epidemiology

PSS affects up to 4% of the adult population, a figure that makes it one of the three most common autoimmune diseases. In PSS there is a female-to-male ratio of 9:1. The onset of the disease is usually in the fourth or fifth decade of life, but it can affect younger individuals. Point prevalence studies, however, have shown that PSS is approximately seven times higher in the elderly population aged 71–74 years when compared to individuals aged 40–44 years (Haugen *et al.*, 2008). PSS is associated with an increased risk of non-Hodgkin's lymphoma. A recent study from Norway demonstrated that the risk of Hodgkin's lymphoma in patients with PSS is increased nine times when compared to that in the general population (Johnsen *et al.*, 2012).

Neurological manifestations

The interest in neurological manifestations of PSS started in the 1980s following the publication of a series of papers by a group of researchers based at John Hopkins Hospital, Baltimore, United States (Alexander *et al.*, 1981). This same group came up with a figure of prevalence for neurological involvement of 20%. Prevalence figures for CNS involvement in PSS remains a controversial issue, reflected by the very wide range reported in different studies (from 1 to 100%). Possible contributory factors to the controversy include the type of diagnostic criteria used for PSS, the inclusion of patients with

secondary SS (secondary SS can be seen in the context of other connective tissue diseases such as SLE, rheumatoid arthritis etc.), the inclusion of neurological diagnoses that have no aetiological link to PSS, geographical variations and referral bias (Soliotis *et al.*, 2004). As PSS is a common disease often associated with other autoimmune diseases, the coexistence of PSS with common autoimmune neurological diseases such as MS has to also be considered. The concept that PSS may mimic MS was first put forward by the same Baltimore group in 1986 (Alexander *et al.*, 1986). The authors described a range of neurological signs in patients with PSS, including optic neuritis, intranuclear ophthalmoplegia, cerebellar ataxia and pyramidal weakness. In some cases, the neurological involvement followed a relapsing-remitting course, a pattern that is typical of MS. Subsequent studies, however, failed to identify an increased prevalence of PSS amongst patients with relapsing-remitting MS. The overall conclusion of such investigations and publications was that, rarely, PSS can be associated with MS-like features. Such features may follow a progressive course with sequential multifocal brain involvement, including spinal cord inflammation in the form of transverse myelitis (Figure 10.2) and optic nerve inflammation in the form of optic neuritis. It is rare for PSS patients to have brain MRI findings that are indistinguishable from MS unless the two diseases coexist.

CNS vasculitis can complicate PSS in the same way that it can be seen in the context of any connective tissue disease. The diagnosis is based on brain biopsy following typical angiographic changes on cerebral angiogram. PSS patients with CNS vasculitis present like those with any other CNS vasculitis with an encephalopathy, which is often associated with seizures and focal neurological deficits. Patients respond to immunosuppression with steroids and cyclophosphamide.

Widespread myoclonus can be a very prominent feature in PSS and responds to the use of clonazepam and other anticonvulsants. The aetiology of this remains obscure.

By far, the most common and better characterized form of peripheral nerve involvement in PSS is that of sensory ganglionopathy. This is a form of asymmetrical, purely sensory peripheral nerve involvement that affects the dorsal root ganglia. It is often associated with sensory ataxia and can often be the presenting feature of PSS. In a series of 92 patients with PSS-associated neuropathy, 93% were diagnosed with PSS after neuropathic symptoms appeared (Mori *et al.*, 2005). The commonest form of peripheral neuropathy was sensory neuronopathy (59%), with mononeuropathy multiplex being the second but much less common type (12%). Sensory ganglionopathy in PSS is slowly progressive but ultimately disabling because of the severe sensory ataxia. There are no published large treatment trials, and because of the nature of the slow progression, such patients are usually under observation without any active treatment being considered. Immunotherapies have been used in small uncontrolled and retrospective cases using intravenous

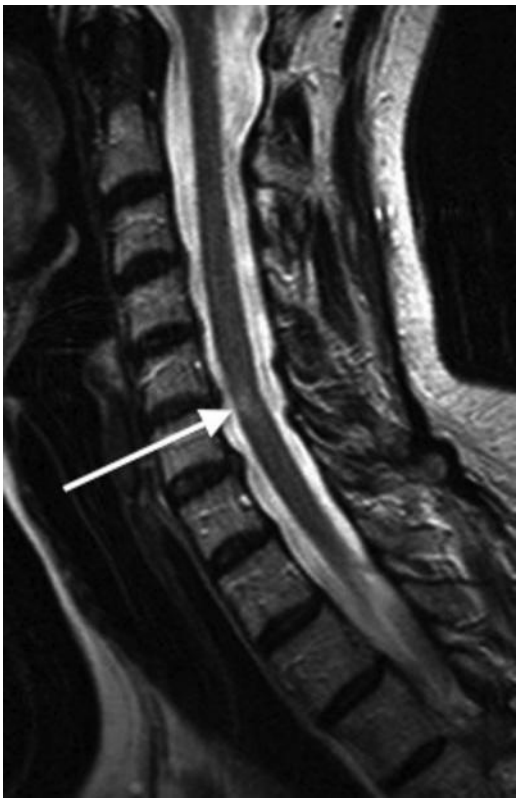

Figure 10.2 Inflammatory cord lesion (arrow) in a patient with myelopathy due to primary Sjögren's syndrome.

immunoglobulins, steroids and cyclophosphamide. One retrospective study found that up to 18% of patients with PSS and sensory ganglionopathy improved with the use of steroids (Mori *et al.*, 2005).

Pathophysiology of neurological manifestations

The common presence of secondary SS in patients with SLE led to the suggestion that the two diseases may share common pathogenetic features. SLE can be considered as a disease with several clinical subgroups each characterized by particular autoantibodies. These two diseases share such autoantibodies (e.g. anti-Ro and anti-La antibodies), and some clinicians consider PSS as a subset of SLE, characterized by homing receptors that allow lymphocytic infiltrates into particular sites such as the lacrimal and salivary glands (Fox and Liu, 2006). A potential role for anti-Ro antibodies in the pathogenesis of neurological involvement comes from *in vitro* studies where serum from patients with PSS containing anti-Ro antibodies was shown to stain the

cytoplasm and cell membranes of endothelial cells derived from umbilical vein and from brain tissue (Alexander *et al.*, 1994). Sensory ganglion cell destruction associated with lymphocytic infiltration has been seen in cases of PSS and sensory ganglionopathy on dorsal root ganglion biopsy. PM examination findings included diminution of sensory ganglion neurones as well as loss of nerve fibres in the dorsal columns of the spinal cord. Mild lymphocytic infiltration was also noted. The overall picture was suggestive of a ganglioneuritis affecting the sensory neurones (Mori *et al.*, 2005). Sensory ganglionopathy is also a common manifestation of other autoimmune-mediated disorders such as paraneoplastic neurological syndromes, particularly those associated with anti-Hu antibodies (discussed further in this chapter) and gluten-related neurological dysfunction.

Another pathophysiological mechanism seen in the context of PSS is that of small vessel vasculitis that can less commonly complicate PSS but almost certainly accounts for some of the neurological manifestations, in particular mononeuropathy multiplex and CNS vasculitis.

Gluten-related neurological dysfunction

Introduction

'Gluten-related disorders' (GRDs) is a term that encompasses a spectrum of systemic autoimmune diseases with diverse manifestations. GRDs are characterized by abnormal immunological responsiveness to ingested gluten (found in wheat, rye and barley) in genetically susceptible individuals. Coeliac disease (CD) or gluten-sensitive enteropathy is only one of a number of GRDs. CD is the best characterized disorder within the GRD spectrum. It classically presents with gastrointestinal symptoms (abdominal pain, weight loss, bloating and evidence of malabsorption), and a biopsy of the small bowel typically shows a triad of villous atrophy, crypt hyperplasia and increased intraepithelial lymphocytes. Whilst the small bowel has historically been considered the target organ in this disease entity, over the last two decades it has become apparent that sensitivity to gluten can manifest with extra-intestinal involvement. Extra-intestinal manifestations include dermatitis herpetiformis (DH) and neurological dysfunction. In the "Epidemiology" subsection below, the spectrum of neurological manifestations in GRDs is reviewed, recent advances in their diagnosis are discussed and possible pathophysiological mechanisms are explored.

Epidemiology

The prevalence of CD in the healthy population has been shown to be at least 1% in both European and North American studies (Sanders *et al.*, 2003). The prevalence of the neurological manifestations is more difficult to establish

for a number of reasons: firstly, it depends on who assesses the patient (neurologist vs. gastroenterologist) and to what extent investigations (e.g. brain imaging or neurophysiological assessments) are undertaken. Secondly, the mode of presentation is important (i.e. some patients may present purely with neurological symptoms, whilst others with gastrointestinal symptoms). If systematic enquiry (by the gastroenterologist) of possible neurological symptoms is not undertaken, it is probable that any neurological involvement will be missed. Finally, there is some evidence of geographical variations of the neurological manifestations between different countries. Some estimates of prevalence can be made from patient populations attending specialist clinics, although caution must be exercised in extrapolating these as they are inevitably affected by referral bias. Data collected from dedicated CD and gluten-related neurological dysfunction clinics in Sheffield, United Kingdom, suggest that for every seven patients diagnosed with CD as a result of their gastrointestinal symptoms by a gastroenterologist, there are two patients diagnosed with CD due to their neurological presentation (Hadjivassiliou *et al.*, 2010b). This is likely to be an underestimate because this ratio does not take into account those patients with neurological manifestations due to GRDs who do not have an enteropathy (i.e. approximately two-thirds of patients presenting with neurological dysfunction). Preliminary results from a prospective study on patients presenting to gastrointestinal clinics in Sheffield, who are newly diagnosed with CD, suggest that up to 40% have neurological involvement (unpublished personal observation).

Neurological manifestations

Gluten ataxia Gluten ataxia (GA) was originally defined as idiopathic sporadic ataxia in the presence of circulating anti-gliadin antibodies (AGAs) of IgG or IgA type (Hadjivassiliou *et al.*, 2003b). This original definition was based on the serological tests available at the time. In a series of 1000 patients with progressive ataxia evaluated over a period of 15 years in Sheffield, United Kingdom, there were 167 out of 805 patients with sporadic ataxia who had serological evidence of GRD. Therefore, gluten ataxia had a prevalence of 21% amongst sporadic ataxias but was as high as 43% amongst idiopathic sporadic ataxias.

GA usually presents with pure cerebellar ataxia or rarely ataxia in combination with myoclonus, palatal tremor (Kheder *et al.*, 2012), opsoclonus (Deconinck *et al.*, 2006) or chorea (Pereira *et al.*, 2004). GA is usually of insidious onset with a mean age at onset of 53 years. Rarely the ataxia can be rapidly progressive, mimicking paraneoplastic cerebellar degeneration. Gaze-evoked nystagmus and other ocular signs of cerebellar dysfunction are seen in up to 80% of cases. All patients have gait ataxia, and the majority have limb ataxia. Less than 10% of patients with GA will have any gastrointestinal symptoms, and only a third will have evidence of enteropathy on biopsy.

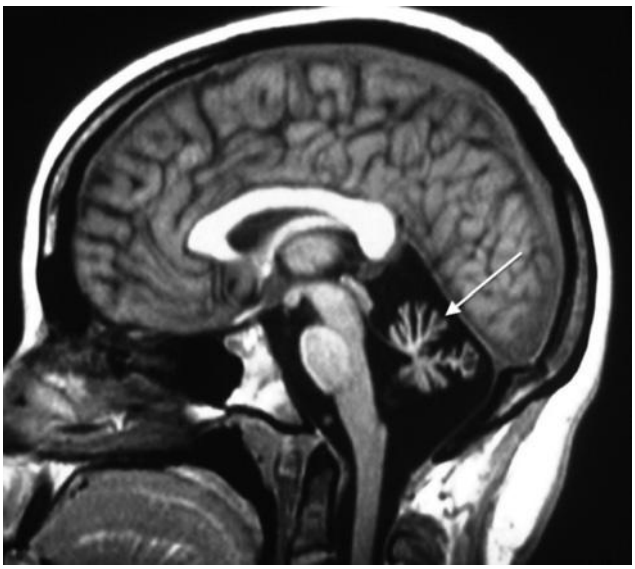

Figure 10.3 Cerebellar atrophy (arrow) on sagittal magnetic resonance imaging of a patient with gluten ataxia.

Up to 60% of patients with GA have evidence of cerebellar atrophy on MRI (Figure 10.3). Investigation of the metabolic status of the cerebellum in 15 patients with GA and 10 controls using proton MR spectroscopy demonstrated significant abnormalities in patients with GA when compared to healthy controls, suggesting that cerebellar neuronal physiology in these patients is abnormal (Wilkinson *et al.*, 2005).

There is emerging evidence that brain imaging in patients with newly diagnosed CD is often abnormal. This is based on the use of MRI with additional spectroscopy of the cerebellum as well as voxel-based morphometry and volumetric analysis of the cerebellum.

Response to treatment with a gluten-free diet depends on the duration of the ataxia prior to the diagnosis of GRD. Loss of Purkinje cells in the cerebellum, the end result of prolonged gluten exposure in patients with GA, is irreversible, and early diagnosis and treatment are more likely to result in improvement or stabilization of the ataxia. Whilst the benefits of a gluten-free diet in the treatment of patients with CD and DH have long been established, there are very few studies of the effect of a gluten-free diet on the neurological manifestations. Most reports primarily concern patients with established CD who then develop neurological symptoms. Such studies suggest variable but overall favourable responsiveness to a gluten-free diet. A small, uncontrolled study looked at the use of intravenous immunoglobulins in the treatment of four patients with GA without enteropathy (Bürk *et al.*, 2001). All patients improved. Only one systematic study of the effect of a gluten-free diet on

a cohort of patients presenting with ataxia, with or without an enteropathy, has been published (Hadjivassiliou *et al.*, 2003a). This study also reported serological evidence of elimination of the AGA as a confirmation of strict adherence to the diet. Forty-three patients with GA were enrolled. Twenty-six patients adhered strictly to the gluten-free diet, had serological evidence of elimination of antibodies and comprised the treatment group. Fourteen patients refused the diet and comprised the control group. Patient and control groups were matched at baseline for all variables (age, duration of ataxia etc.). There was no significant difference in the baseline performance for each ataxia test between the two groups. However, there was significant improvement in performance in test scores and in the subjective global clinical impression scale in the treatment group when compared to the control group. The improvement was apparent even after excluding patients with an enteropathy. The study concluded that a gluten-free diet can be an effective treatment for GA.

Gluten neuropathy Up to 23% of patients with established CD on a gluten-free diet have neurophysiological evidence of peripheral neuropathy (Luostarinen *et al.*, 2003). A large population based study of over 84,000 subjects in Sweden found that polyneuropathy had a significant association with CD (Ludvigsson *et al.*, 2007). In a UK based study, 34% of patients with idiopathic sporadic sensorimotor axonal neuropathy were found to have circulating AGA (Hadjivassiliou *et al.*, 2006). Gluten neuropathy is defined as otherwise idiopathic sporadic neuropathy with serological evidence of GRD. The commonest types are symmetrical sensorimotor axonal neuropathy and sensory ganglionopathy (Hadjivassiliou *et al.*, 2010a). Gluten neuropathy takes the form of a slowly progressive disease with a mean age at onset of 55 years (range 24 to 77) and a mean duration of 9 years (range 1 to 33). A third of the patients will have evidence of enteropathy on biopsy, but the presence or absence of enteropathy does not predetermine the effect of a gluten-free diet, which has been shown to be beneficial (Hadjivassiliou *et al.*, 2006a).

Pathological data suggest an inflammatory aetiology as indicated by perivascular lymphocytic infiltration within the dorsal root ganglia. Strict adherence to a gluten free diet may result in stabilisation or even improvement of the neuropathy irrespective of the presence of enteropathy (Hadjivassiliou *et al.*, 2010a).

Gluten encephalopathy Headache is a common feature in patients with CD. A report of a series of 10 patients with GRD and headache who in addition had CNS white matter abnormalities on MRI scan was published in 2001 (Hadjivassiliou *et al.*, 2001). The term ‘gluten encephalopathy’ was suggested to describe this entity. The headaches are usually episodic, often resembling migraines but sometimes taking the form of a chronic daily headache that is

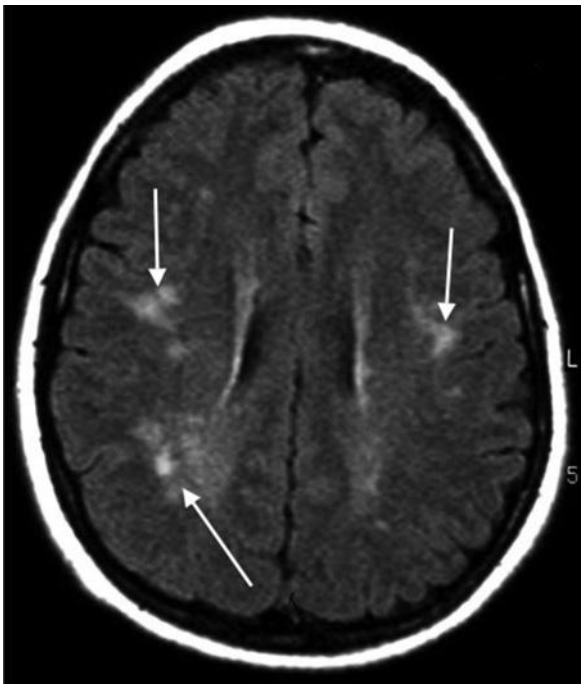

Figure 10.4 A patient with gluten encephalopathy presenting with severe disabling headaches. The headaches improved markedly following the introduction of a gluten-free diet. The arrows show patchy hyperintensities primarily affecting the white matter.

unresponsive to conventional treatments. In severe cases, the headaches may be associated with focal neurological deficits and characteristically resolve with the introduction of a gluten-free diet. The white matter abnormalities on MRI are usually diffuse and rarely focal, and they do not resolve but usually stabilize following a gluten-free diet (Figure 10.4). Their distribution is more suggestive of a vascular rather than a demyelinating aetiology. Using positron emission tomography (PET) brain imaging, a study looking at regional cerebral perfusion demonstrated that 73% of patients with CD not on a gluten-free diet had at least one hypoperfused brain region as compared to 7% in healthy controls and in patients with CD on a gluten-free diet (Addolorato *et al.*, 2004).

Over the last 15 years, we have encountered 80 patients with gluten encephalopathy, a figure that includes the initial 10 patients reported in the 2001 series. Gluten encephalopathy does not always occur in isolation, and such patients will often have additional neurological features such as ataxia and cognitive deficits. A study from the Mayo Clinic emphasized the significant cognitive deficits encountered in 13 such patients (Hu *et al.*, 2006). The observed improvement of the headaches and arrest of progression in the

MRI brain abnormalities suggest a causal link with gluten ingestion. Gluten encephalopathy represents a spectrum of clinical presentations ranging from episodic headaches at one end to severe debilitating headaches associated with focal neurological deficits and abnormal white matter on MRI at the other. The common feature is the improvement of the headache following the introduction of a gluten-free diet.

Myoclonic ataxia (hyperexcitable brain and refractory CD) A form of ataxia associated with myoclonus and CD was first described in 1986 (Lu *et al.*, 1986). It was then shown that the myoclonus was of cortical origin despite the presence of cerebellar atrophy (Bhatia *et al.*, 1995). In a series of over 500 patients with neurological manifestations of GRD, the author has encountered eight patients with what appears to be disabling myoclonus that starts focally and spreads to affect other parts of the body (including the face). All patients had evidence of enteropathy on biopsy and of interest is the fact that the enteropathy is that of refractory CD. This condition appears to be progressive, with the use of immunosuppressive drugs providing limited benefit. Such immunosuppression may result in improvement of enteropathy and the ataxia, but the myoclonus remains extremely disabling. The treatment of such patients thus remains problematic, but the limited evidence from this small series suggests that mycophenolate, an immunosuppressant, may be a useful therapeutic intervention for those patients who appear to progress neurologically despite a strict gluten-free diet.

Epilepsy A number of reports have suggested a link between epilepsy and CD. There is a particular type of focal epilepsy associated with occipital calcifications that appears to have a strong link with CD (Gobbi *et al.*, 1992). This entity is common in Italy but rare in other countries. It tends to affect young patients (mean age 16 years), and in the majority, the seizures are resistant to anti-epileptic drugs. The pathogenesis of the cerebral calcifications remains unclear. An autopsy study showed the depositions consisted of both calcium and silica. The epilepsy appears to respond to a gluten-free diet, even if the patient had proven resistant to numerous anticonvulsants previously (Johnson *et al.*, 2012).

Whilst studies examining the prevalence of CD amongst patients with epilepsy have suggested a prevalence of 1.2–2.3%, larger recent studies failed to demonstrate such an increased prevalence (Ranua *et al.*, 2005). However, most studies on the subject suffer from the same methodological problem of grouping patients with epilepsy as if epilepsy is a homogeneous disorder. The only study that attempted to look at the prevalence of GRDs in well-characterized subgroups of patients with epilepsy found a significant association between the presence of AGA and temporal lobe epilepsy with hippocampal sclerosis (Paltola *et al.*, 2009).

Myelopathy Clinical evidence of a myelopathy in the absence of vitamin and other deficiencies (particularly copper) can be a rare neurological manifestation of CD. It is usually associated with normal imaging of the spinal cord. However, there have been some case reports of patients with neuromyelitis optica (NMO) (see Chapter 9) and GRDs who have antibodies to aquaporin-4 (Jacob *et al.*, 2005). Such patients clearly had abnormal MRI of the spinal cord, but the diagnosis of CD was made only at the time of their neurological presentation. NMO and CD share the same human leukocyte antigen (HLA) genetic susceptibility (HLA DQ2). There are very limited data on the effects of diet on the likelihood of relapse of the disease, particularly given the fact that most patients with NMO require long-term immunosuppressive medication. In cases of myelopathy outside the context of NMO, a gluten-free diet is often beneficial and tends to stabilize the myelopathy.

Pathophysiology of neurological manifestations

There is now comprehensive evidence that the neurological manifestations of gluten-related disorders are immune mediated. Post-mortem examination from patients with gluten ataxia demonstrates patchy loss of Purkinje cells throughout the cerebellar cortex, with diffuse cell infiltration, mainly T lymphocytes within the cerebellar white matter as well as marked perivascular cuffing with inflammatory cells (Hadjivassiliou *et al.*, 1998). There is evidence to suggest that there is antibody cross-reactivity between antigenic epitopes on Purkinje cells and gluten proteins. Serum from patients with GA, and from patients with CD but no neurological symptoms, displays cross-reactivity with epitopes on Purkinje cells of both human and rat cerebellum (Figure 10.5). This reactivity can also be seen using polyclonal AGA, and the reactivity is eliminated by absorption with crude gliadin (Hadjivassiliou *et al.*, 2002). When using sera from patients with GA, there is evidence of additional antibodies targeting Purkinje cell epitopes since elimination of AGA alone is not sufficient to eliminate such reactivity. Furthermore, a recent study showed that HLA DR3 DQ2 mice do not develop ataxia in the presence of a high titre of AGA (Tarlac *et al.*, 2012). There is evidence that additional antibodies may be causing such reactivity, including antibodies against one or more transglutaminase isoenzymes (TG2, TG3 and TG6).

TG2 belongs to a family of enzymes that covalently cross-link or modify proteins by forming an isopeptide bond between a peptide-bound glutamine residue and a primary amine. However, in some instances TG2 may react with water in preference over an amine, leading to the deamidation of glutamine residues. Gluten proteins, the immunological trigger of GRD, are glutamine-rich donor substrates amenable to deamidation. Activation of TG2 and deamidation of gluten peptides appear to be central to CD development and are now well understood at a molecular level. However, events leading to the formation of autoantibodies against TG2 remain unclear. Questions

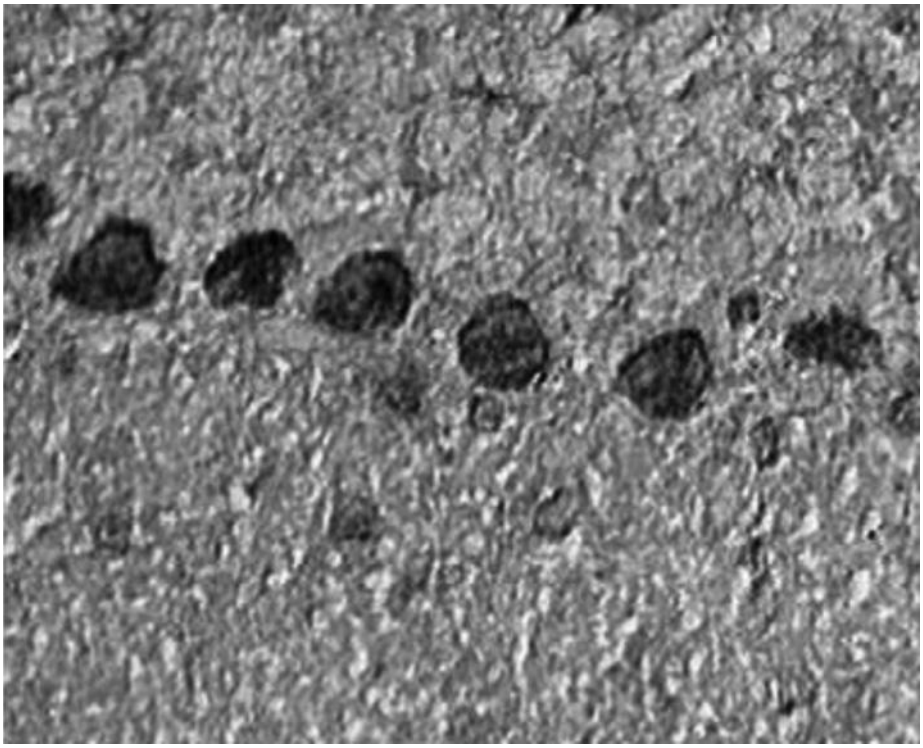

Figure 10.5 Rat Purkinje cell staining using serum from a patient with gluten ataxia. Both anti-gliadin and anti-transglutaminase antibodies have been shown to react with Purkinje cells.

also remain as to the contribution of these autoantibodies to organ-specific deficits. Anti-TG2 antibodies have been shown to be deposited in the small bowel mucosa of patients with GRDs even in the absence of enteropathy. Furthermore, such immunoglobulin deposits have been found in extra-intestinal sites, such as muscle and liver (Korponay-Szabo *et al.*, 2004) and around blood vessels in the CNS (Hadjivassiliou *et al.*, 2006b). The deposition of anti-TG2 antibodies was most pronounced in the cerebellum, pons and medulla. This finding suggests that such autoantibodies could play a role in the pathogenesis of the whole spectrum of manifestations seen in GRDs. However, it is not clear whether these antibodies are derived from the circulation or if their production is mediated within target organs after stimulation of B cells by gut-primed gliadin-reactive $CD4^+$ T cells.

Variations in the specificity of antibodies produced in individual patients could explain the wide spectrum of manifestations. Whilst TG2 has been shown to be the autoantigen in CD, the epidermal transglutaminase TG3 has been shown to be the autoantigen in the skin manifestation of GRD, dermatitis herpetiformis (Sárdy *et al.*, 2002). More recently, antibodies against

TG6, a primarily brain expressed transglutaminase, have been shown to be present in patients with GA (Hadjivassiliou *et al.*, 2008). In GA and dermatitis herpetiformis, IgA deposits of TG6 and TG3 respectively accumulate in the ablumenal side of blood vessels. This could indicate either that the deposits originate from immune complexes formed elsewhere, and are accumulating as a consequence of enhanced vascular leakage, or that TG6 and TG3 are derived from perivascular infiltrating inflammatory cells preceding deposit formation. Perivascular cuffing with lymphocytes is a common finding in brain tissue from patients with GA but is also seen in peripheral nerve in patients with gluten neuropathy. In most cases, sera reactive with more than one TG isoenzyme are detected and there is evidence that distinct antibody populations are responsible for the reactivity, rather than this being a result of cross-reactivity with different TG isozymes. This makes shared epitopes less likely to be the cause for immune responses to other TGs and points to the possibility that TG isozymes other than TG2 can be the primary antigen in GRDs.

IgA deposition in brain vessels and the pathological finding of perivascular cuffing with inflammatory cells may indicate that vasculature-centred inflammation may compromise the blood–brain barrier, allowing exposure of the CNS to pathogenic antibodies, and may thus be the trigger of nervous system involvement. Indeed, TG2 is expressed by smooth muscle and endothelial cells in non-inflamed brain, it is an abundant component of the blood–brain barrier and autoantibody binding could initiate an inflammatory response. Anti-TG2 and other autoantibodies (e.g. AGA) may directly cause selective neuronal degeneration. It is possible that neuronal degeneration is a consequence of the anti-TG antibody spectrum (i.e. it occurs in those patients with antibodies reactive with a neuronal TG). IgG class antibodies have been shown to be present in only 60% of CD patients, whereas in GA patients positive for anti-TG, the prevalence was 90%. It could be that the development and deposition of antibodies are an epiphenomena rather than being pathogenic. One method to demonstrate the pathological effect of an antibody is the passive transfer of the disease through antibody injection into a naïve animal. Using a mouse model it has been shown that serum from patients with GA, as well as clonal monovalent anti-TG immunoglobulins derived using phage display, cause ataxia when injected intraventricularly in mice (Boscolo *et al.*, 2010). The fact that not only Ig fractions but also monospecific single-chain variable fragments (scFvs) mediate functional deficits shows that there is no requirement for complement activation or for the engagement of Fc receptors on cells in the brain. These data therefore provide evidence that anti-TG immunoglobulins (derived from patients) compromise neuronal function in selected areas of the brain, once exposed to the CNS. While these data implicate anti-TG antibodies in ataxia, they do not explain the spectrum of distinct neurological deficits seen in patients with GRD.

To fully understand the immunological insults resulting from gluten ingestion, the research emphasis should perhaps shift towards the study of extra-intestinal manifestations. In addition, there is an urgent need for the early identification of those patients who are at risk of irreversible complications (e.g. gluten ataxia). To that effect, new diagnostic tools are now becoming available (e.g. measurement of antibodies against TG6) which may help in the reliable identification of those patients with neurological presentations (Hadjivassiliou *et al.*, 2013a). Up to 40% of patients presenting to the gastroenterologist who are ultimately diagnosed with CD also have antibodies against TG6 in addition to antibodies against TG2. This subgroup of patients with classic CD presentation, may well be the ones susceptible to the development of neurological dysfunction if they continue to consume gluten, although this remains to be shown in longitudinal studies. The presence of gastrointestinal symptoms, however, appears to offer a major advantage to this group, as it substantially increases their chances of being diagnosed with and treated for CD, whereas the diagnosis of those patients presenting purely with extra-intestinal manifestations may be delayed.

Paraneoplastic neurological syndromes

Introduction

Paraneoplastic neurological syndromes (PNS) are a group of immune-mediated neurological disorders triggered by cancer which is often occult. In the last 20 years or so, the discovery of specific antibodies that are present in the serum of patients with such syndromes resulted in better identification and clinical characterization of PNS (Table 10.2). Such syndromes are divided into classic and non-classic, on the basis of the strength of their association with cancer. Classic PNS comprises subacute or acute cerebellar ataxia, limbic encephalitis, ospsoclonus-myoclonus, encephalomyelitis, Lambert–Eaton myasthenic syndrome, sensory neuronopathy, dermatomyositis and, rarely, intestinal pseudo-obstruction.

Table 10.2 Paraneoplastic neurological syndromes

Condition	Tumour	Target antigens
Paraneoplastic cerebellar degeneration	Ovarian, breast SCLC, lymphomas	Yo, Hu, Ri
Limbic encephalitis	SCLC, germ cell tumours	VGKC, Ma
Opsoclonus-myoclonus	Breast, SCLC	Ri
Lambert–Eaton syndrome	SCLC, thymoma	VGCC
NMDA-receptor encephalitis	Teratoma (in women)	NMDAR1
Sensory ganglionopathy	SCLC	Hu

SCLC, small cell lung cancer; VGKC, voltage-gated potassium channels; VGCC, voltage-gated calcium channels; NMDA, N-methyl-D-aspartate.

Epidemiology

PNS are rare neurological disorders. Based on laboratory data from serological screening of patients suspected of having PNS, only 0.9% of patients had paraneoplastic antibodies. By contrast, in a more specialized centre with a particular interest in both clinical and serological characterization of patients suspected of having PNS, 25% were positive for such antibodies (Dalmau and Rosenfeld, 2008). Not all patients with PNS have such antibodies, however. There are certain types of cancer that are more commonly associated with PNS, such as small-cell lung cancer. Up to 5% of patients with this type of cancer develop PNS (Elrington *et al.*, 1991). The figure is even higher in patients with thymoma (15–20%) (Rosenfeld and Dalmau, 2010).

Neurological manifestations

Paraneoplastic cerebellar degeneration (PCD) This usually presents in an acute or subacute manner but is characterized by a rapid progression, unlike any other disorder seen in the context of progressive cerebellar diseases. The patient quickly becomes disabled and wheelchair bound. Prominent cerebellar signs take the form of very slurred speech and gait and truncal ataxia, which is extremely disabling. Initial brain imaging tends to be normal despite the severity of the clinical signs. MR spectroscopy of the cerebellum, however, reveals a severely reduced N acetyl aspartate–creatinine (NAA–Cr) ratio implying reduced cellular metabolic activity (Hadjivassiliou *et al.*, 2013b). Such presentation is so typical of PCD that management should be that of a neurological emergency in terms of searching for cancer. Common malignancies that can cause PCD in women include ovarian and breast cancer, often associated with anti-Yo antibody and lymphomas and small-cell lung cancer in both sexes. A whole-body PET scan has to be used if initial imaging does not identify any obvious malignancy. Whole-body PET scans have been shown to improve the diagnostic yield of malignancy in patients clinically suspected of having PNS (Hadjivassiliou *et al.*, 2009). If the malignancy is treatable, treatment (e.g. oophorectomy and mastectomy) has to be given urgently to avoid permanent severe neurological disability.

Limbic encephalitis The patient often presents sub-acutely with new onset of seizures, short-term memory loss and behavioural changes that tend to be progressive. In severe cases, the patient may develop severe encephalopathy requiring treatment in an intensive care setup. Limbic encephalitis can be primary autoimmune with identical presentation to that seen in the context of PNS. The differential diagnosis also includes CNS herpes simplex encephalitis. MRI confirms the involvement of the mesial temporal lobes, usually bilaterally (Figure 10.6). Limbic encephalitis is often associated with small-cell lung carcinoma and positive anti-Hu antibodies. This type of cancer has a poor

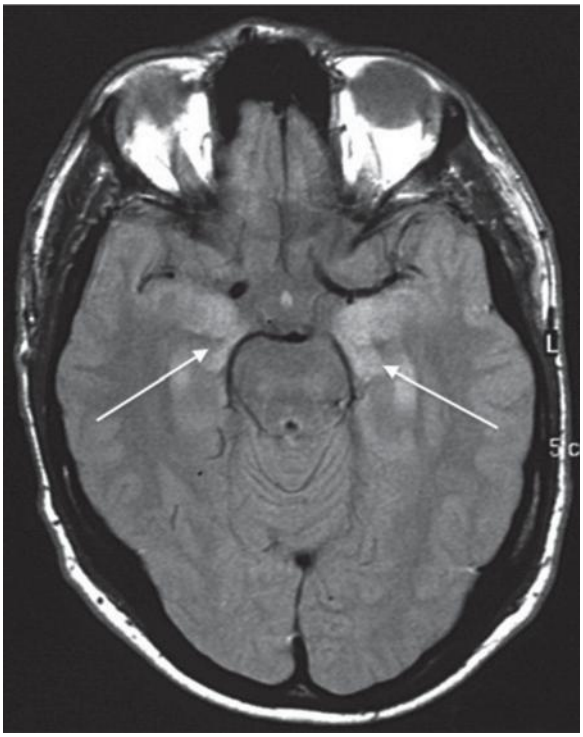

Figure 10.6 Magnetic resonance imaging of a patient with paraneoplastic limbic encephalitis showing increased signal in the limbic system bilaterally (arrows).

prognosis, and such patients often remain significantly disabled by their illness, although symptomatic treatment can be administered such as anticonvulsants to control seizures. Autoimmune limbic encephalitis is usually associated with antibodies against voltage-gated potassium, and patients respond to immunosuppression.

Lambert–Eaton myasthenic syndrome (LEMS) Whilst less common than the other PNS, LEMS has a very strong association with malignancy (up to 50% have small-cell lung cancer). It can be associated with voltage-gated calcium antibodies, and it involves the neuromuscular junction. It is characterized clinically by proximal weakness, potentiation of depressed tendon reflexes following sustained voluntary contraction and autonomic features.

Opsoclonus-myoclonus This is characterized by ocular involuntary multidirectional saccades (opsoclonus) with myoclonus, often with a degree of cerebellar ataxia. It can also be caused by infections and toxic-metabolic disorders, and can also be seen as a primary autoimmune disease. In children it is strongly associated with neuroblastoma (50% of cases). Most patients tend to

be antibody negative, but a small subset of adults with this condition may have anti-Ri antibodies. Treatment is removal of the malignancy. Immunosuppression is of limited value, particularly in adults with paraneoplastic opsoclonus-myoclonus.

Other paraneoplastic neurological syndromes Apart from the classic paraneoplastic syndromes listed in the introduction to this section, there are a number of other neurological conditions that are rarely associated with cancer. In some cases, a link to cancer may be considered coincidental unless the neurological problem is shown to improve following treatment of the cancer. Non-classic PNS include brainstem encephalitis, melanoma-associated retinopathy, stiff-person syndrome, autonomic neuropathy, necrotising myopathy and peripheral neuropathy, amongst others. More recently, a form of encephalitis characterized by acute behavioural and psychiatric disturbances often necessitating intensive therapy unit care has been identified and linked to the presence of anti-NMDA-receptor antibodies and ovarian teratomas (Dalmau *et al.*, 2008). This entity is unusual in that it is associated with non-cancerous growth (teratoma) and can at times go into remission without any specific treatment. It can sometimes follow a relapsing-remitting course. It is likely, however, to belong to the classic group of PNS.

Pathophysiology of PNS

The best evidence that PNS is immune mediated comes from the demonstration of anti-neuronal antibodies in both the serum and the CSF of patients with PNS. These antibodies react with neuronal proteins that are usually expressed by the tumour. Patients with PNS often have lymphocyte pleiocytosis in the CSF and oligoclonal bands detected by isoelectric focussing of CSF. The target antigen can either be exposed on the cell membrane or be intracellular. Some antibodies seem to have a direct pathogenic role in causing PNS. These antibodies usually react with cell surface antigens and are mostly found in syndromes involving the neuromuscular junction (e.g. LEMS) or the peripheral nerves. NMDA receptor antibodies associated with encephalitis and psychiatric symptoms may also be pathogenic. However, a pathogenic role of other paraneoplastic antibodies has not been proven as transfer of these antibodies into animal models failed to induce disease (Graus and Dalmau, 2010). Circumstantial evidence of T cell–mediated pathogenesis in these syndromes comes from studies on patients with anti-Hu and anti-Yo antibodies where antigen-specific T cells have been identified in both serum and CSF (Albert *et al.*, 2000). The same researchers reported a role for cytotoxic T cells in the autoimmune destruction of Purkinje cells in paraneoplastic cerebellar degeneration. T cells in the CSF were predominantly Th1 proinflammatory T lymphocytes. Further support for T cell–mediated mechanisms include the fact that it is difficult to treat these disorders with

immunosuppression directed against the humoral immune response and that there is evidence of extensive T cell infiltration in the CNS of patients with PNS.

Immunosuppressive treatment has been used in all of the PNS disorders but with limited benefit. The only effective treatment is removal of the underlying malignancy with either surgery or chemotherapy.

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11
Inflammation in the Pathogenesis of Depression

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Introduction

Over the last three decades, clinicians and scientists have explored the idea that dysfunction of the immune system could be a contributor to the pathophysiology of major depressive illness. In addition to studying the immune system as a contributor to psychopathology, the immune system has been studied with a view to identifying peripheral biomarkers that may predict the onset of depression and those patients who are likely to respond to treatment versus those patients who are likely to relapse, with an ultimate view to developing personalized medicine strategies for this disorder (Haroon *et al.*, 2012). More recently, investigators have directly examined the potential role of the brain's resident immune cells, microglia, in the pathogenesis of depression (Beumer *et al.*, 2012; Blank and Prinz, 2013). Such studies make use of immunohistochemical analysis of post-mortem brain, and also, more importantly, examine the living psychiatric patient using positron emission tomography (PET) scans. The use of PET assesses microglial activation using the binding of the ligand [^{11}C](R)-PK11195, a ligand for the peripheral benzodiazepine receptor (PBR) that is also known as translocator protein 18 kDa (TPSO) and is upregulated on activated microglia (Doorduin *et al.*, 2008; Beumer *et al.*, 2012).

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As depression is largely considered a brain disorder, the predictive value of changes in peripheral immune function as biomarkers is often questioned. Thus, comparing central and peripheral immune cell changes in patient populations will shed light on the usefulness of peripheral immune cells as biomarkers in this disorder.

A role for the immune system in depressive illness – what is the evidence?

Evidence has accumulated over the last two decades to suggest that activation of the innate immune response may be involved in the aetiology of depressive illness (Capuron and Dantzer, 2003; Raison *et al.*, 2006). Specifically, it has been suggested that excessive secretion of monocyte and/or T cell–derived inflammatory cytokines has the propensity to precipitate depression in susceptible individuals. In addition to *de novo* synthesis of cytokines within the brain produced primarily by resident microglia, cytokines secreted in the periphery can also impact brain function via a number of well-described mechanisms, including active transport, penetration through the blood–brain barrier and stimulation of the peripheral vagus sensory nerve (for a review, see Dantzer *et al.*, 2000). Consequently, cytokines produced by peripheral immune cells have the propensity to impact brain function and ultimately alter behaviour.

Support for a role of inflammatory cytokines in the aetiology of depressive illness stems from two major lines of evidence. Firstly, administration of exogenous cytokines to humans and animals can precipitate behavioural disturbances akin to depression (Raison *et al.*, 2005). Secondly, a body of data indicates that depressive illness is associated with a low-grade inflammatory response characterized by increased circulating concentrations of inflammatory cytokines and acute-phase proteins (Raison *et al.*, 2006; Simon *et al.*, 2007; Howren *et al.*, 2009; Hughes *et al.*, 2012). Whilst it has been recognized for many years that immune system activation can alter the mental state, it is only recently that the molecular mechanisms that could underlie such mood alterations have begun to be elucidated.

Immune system activation induces changes in mood

Administration of low-dose endotoxin to blinded healthy volunteers provoked symptoms of depression and anxiety and cognitive impairment in the hours following administration, and these mood changes were correlated with increased circulating concentrations of the cytokines interleukin 6 (IL6), tumour necrosis factor alpha (TNFα) and IL1 receptor antagonist (IL-1ra) (Reichenberg *et al.*, 2001). Similarly, typhoid vaccination induced negative changes in mood that were accompanied by and correlated with increased circulating concentrations of IL6 (Harrison *et al.*, 2009). Interestingly, vaccination-associated mood deterioration correlated with enhanced activity

within subgenual anterior cingulate cortex (sgACC) during emotional face processing assessed using functional magnetic resonance imaging (fMRI). The fact that inflammation-related deterioration in mood is associated with increased sgACC activity is of significance considering the large body of data implicating sgACC hyperactivity in the pathophysiology of depression, and as an antidepressant target (Drevets *et al.*, 2008).

Inflammatory stimuli such as the Toll-like receptor-4 (TLR4) agonist bacterial lipopolysaccharide (LPS), the TLR3 agonist and viral mimetic polyinosinic–polycytidylic acid (Poly I:C), or infection with Bacillus Calmette–Guérin (BCG), can provoke symptoms of anxiety and depression distinguishable from generalized sickness behaviour on a temporal profile in animal models (O'Connor *et al.*, 2008b, 2009; Gibney *et al.*, 2012). Specifically, it has been demonstrated in these animal models that anxiety and depressive-like behaviours are still evident when acute sickness has resolved. In the case of BCG and experimental autoimmune encephalomyelitis (EAE), the development of depressive behaviours lasts for several weeks and is associated with a persistent increase in circulating interferon gamma (IFNγ) and TNFα levels. Evidence supporting the role of cytokines in the brain responsible for depressive symptoms is reviewed elsewhere (Dunn *et al.*, 2005; Dantzer *et al.*, 2008). IL1β and TNFα are considered the main cytokines as there are numerous reports that systemic or central administration of these cytokines to laboratory rats and mice induces sickness behaviour in a dose- and time-dependent manner. It has been shown that IL1β can instigate anhedonia in laboratory rodents independent of effects on anorexia. Other cytokines too are considered to play a role, including IL6 which contributes to the expression of brain IL1β and TNFα and may play a role in LPS-induced hippocampal-mediated cognitive impairment (Sparkman *et al.*, 2006). In other reports IL2, but not IL1β or IL6, has been reported to provoke long-lasting anhedonic effects in mice and rats (reviewed by Dunn *et al.*, 2005).

By contrast to pro-inflammatory mediators, central administration of the anti-inflammatory cytokine IL10 attenuates the behavioural signs of sickness induced by centrally injected LPS (Bluthe *et al.*, 1999). Some growth factors such as insulin-like growth factor-1 (IGF1) also antagonize pro-inflammatory cytokines in the brain and attenuate sickness behaviour following central administration of LPS. The interplay between pro- and anti-inflammatory mediators and associated signalling is suggestive of a balance between cytokines in the regulation of the sickness and depressive-like response to immune stimuli. Furthermore, it is of interest that pro-inflammatory cytokines including TNFα and IL1β can provoke a reduction in IGF1 sensitivity and promote resistance to this growth factor (reviewed by O'Connor *et al.*, 2008a).

Increased expression of IL1β and TNFα observed in the circumventricular organs and blood–brain barrier is associated with the initial wave of LPS-induced sickness. The secondary wave of depression-like behaviour,

which occurs 12 to 24 h later, may be associated with increased expression of cytokines within parenchymal regions. Mapping of neuronal activation in response to LPS administration has been carried out, and the peak of sickness behaviour is associated with increased expression of the cellular activation marker and immediate early gene product, c-fos, in the paraventricular nucleus and the bed nucleus of the stria terminalis (BNST), areas involved in endocrine and autonomic components of LPS-induced sickness. By contrast, immunostaining for FosB and its truncated splice variant δFosB, both of which have a longer half-life than c-fos and accumulate during repeated or long-lasting stimulation, are increased in several hypothalamic nuclei and extend to the amygdala and hippocampus. Such activation patterns point towards the involvement of these structures in cytokine-induced depression (reviewed by Dantzer *et al.*, 2008).

Exogenous cytokine immunotherapy as a trigger for depressive illness

By far, the largest body of data relating to the ability of cytokines to induce changes in mood stems from studies where cytokine immunotherapy for viral hepatitis or various forms of cancer provokes severe psychological disturbances, including depression. Numerous studies have reported that previously psychiatrically healthy individuals treated with high doses of exogenous cytokine IL2 or IFNα develop depressive-like symptoms such as depressed mood, increased somatic symptoms, stress reactions and cognitive impairment (Capuron *et al.*, 2003, 2004; Valentine and Meyers, 2005). Literature on animal studies supports the view that peripherally administered IFNα can directly impact brain function, in that a robust induction of IFNα-inducible genes was observed in the CNS following systemic administration of IFNα to mice (Wang *et al.*, 2008).

It is not entirely clear why only a proportion of individuals, typically less than 50%, that receive cytokine immunotherapy become depressed. However, it has been suggested that psychological stress may sensitize to the neurochemical and behavioural actions of cytokines, and therefore individuals with an anxious or stress-prone phenotype may be more susceptible to developing psychiatric sequelae in response to cytokine administration. In support of this idea, it was reported that patients with a higher cortisol response (stress response) to the initial injection of IFNα displayed a greater propensity to develop depression following treatment (Capuron *et al.*, 2003). Further support for this notion stems from a pre-clinical study demonstrating that mice exposed to psychosocial stress showed exaggerated central monoamine changes, hypothalamic–pituitary–adrenal (HPA) axis reactivity and sickness behaviour to IFNα treatment (Anisman *et al.*, 2007).

When one considers the literature on cytokines as a trigger for depressive illness, it is indisputable that treatment with IL2 or IFNα can induce

depression; however, one must remember that the doses of cytokines administered to patients in these studies far exceed physiological concentrations. This is a factor that should be considered when implicating elevated endogenous cytokine secretion as a causal factor in the development of depressive symptoms. Thus, one cannot simply regard the psychiatric and biological sequelae that occur following exogenous administration of cytokines such as IFNα or IL2 as being akin to the biology of idiopathic depression where no obvious exogenous agent is driving changes in the psychiatric state.

Biochemical mechanisms implicated in mediating the depressive effects of cytokines

Depressive symptoms appear to be due to the biochemical changes induced by cytokine treatment rather than psychological reactions to the illness for which the agents are being administered. For instance, evidence indicates that treatment with IFNα or IL2 induces metabolism of the essential amino acid tryptophan to kynurenine, which has the potential to limit tryptophan availability for serotonin synthesis (Capuron *et al.*, 2002; Widner *et al.*, 2002). It has been demonstrated that peripheral administration of IFNα to patients increases kynurenine concentrations in the plasma and also in the cerebrospinal fluid (CSF) (Raison *et al.*, 2010). Furthermore, it was observed that the extent of tryptophan depletion in patients treated with IFNα was correlated with the incidence of depression (Capuron *et al.*, 2002; Raison *et al.*, 2010). However, despite the original hypothesis proposing that inflammatory cytokines could deplete tryptophan availability for serotonin synthesis, both clinical (Raison *et al.*, 2010) and pre-clinical studies in animal models (O'Connor *et al.*, 2009; Gibney *et al.*, 2012) do not support this hypothesis. A number of studies have consistently reported increased (as opposed to decreased) tryptophan availability in the CNS following administration of LPS or other inflammogens. Moreover, there is no evidence of central serotonin depletion following administration of LPS or inflammatory cytokines to animals; in fact, an increase in serotonin release and metabolism coupled with increased activity of the rate-limiting enzyme for serotonin biosynthesis, tryptophan hydroxylase, have been observed in rats following a systemic inflammatory challenge with LPS (Nolan *et al.*, 2000; Dunn *et al.*, 2005). Clinical studies to date also argue against a kynurenine pathway–mediated depletion of serotonin synthesis in depressed patients (Wichers *et al.*, 2005; Hughes *et al.*, 2012). Of note is the fact that kynurenine itself has depressogenic effects in the forced-swimming and tail suspension tests, and it has therefore been proposed that kynurenine, or more likely one of its neuroactive pathway metabolites, mediates depressogenic behaviour in these animal models (O'Connor *et al.*, 2009). Similarly, a role for neuroactive kynurenine metabolites has been implicated as mediators of IFNα-induced depression in humans (Wichers *et al.*, 2005; Raison *et al.*, 2010). It has been

suggested that the action of kynurenine metabolites on the glutamatergic system may be involved in producing depressive symptomatology (Müller and Schwarz, 2007).

Some studies have demonstrated that inflammatory cytokines, including IL1β, TNFα and IFNα, increase expression of the serotonin transporter (SERT) and serotonin re-uptake *in vitro* (Tsao *et al.*, 2008), and that a systemic inflammatory challenge with bacterial LPS increases SERT expression in rodent brain (Zhu *et al.*, 2010). A single systemic injection of Poly I:C induces a persistent increase in IFNα expression in the CNS accompanied by increased SERT expression and reduced extracellular serotonin as quantified by *in vivo* microdialysis in the prefrontal cortex of rats (Katafuchi *et al.*, 2005). Raised inflammatory cytokine expression is correlated with increased SERT expression on circulating leukocytes of depressed patients, and increased IFNα and SERT expression in depressed patients is restored to normal following chronic treatment with the selective serotonin reuptake inhibitor and antidepressant fluoxetine (Tsao *et al.*, 2006). Moreover, we have recently demonstrated that activation of the BV2 mouse microglial cell line with LPS increases SERT mRNA expression in these cells. These findings suggest that central microglia may play a role in sequestering serotonin, particularly under inflammatory conditions. This finding has all the more significance as we have also demonstrated that microglia express monoamine oxidase A (MAO_A), the enzyme responsible for the metabolism of serotonin (Fagan *et al.*, 2013).

Increased serum anti-serotonin antibody titres (Schott *et al.*, 2003; Maes *et al.*, 2012) and serum antibodies against the serotonin 5-HT_{1A} receptor (Tanaka *et al.*, 2003) have been reported in depressed patients. The presence of such antibodies was associated with features of immunological activation indicated by increased plasma TNFα and IL1 concentrations. Autoimmune reactions to serotonin may play a role in the pathophysiology of depression as significant association between autoimmune activity to serotonin and the number of previous depressive episodes has been reported (Maes *et al.*, 2012).

In addition to the serotonergic system as a biological target of cytokines, studies also indicate that inflammation can negatively impact brain-derived neurotrophic factor (BDNF). Specifically, a systemic inflammatory challenge with bacterial LPS reduces expression of BDNF in rat brain (Guan and Fang, 2006), and intra-hippocampal LPS administration has been shown to inhibit expression of BDNF and also its receptor, Trk_B (Tanaka *et al.*, 2006). More recently, we demonstrated that systemic treatment with Poly I:C inhibited expression of both BDNF and Trk_B in hippocampus and cortex and that this occurred following a robust expression of the inflammatory cytokines IL1β, TNFα and IL6 in these brain regions (Gibney *et al.*, 2012). IL1β is thought to be one of the main cytokines to compromise the BDNF pathway. Administration of IL1β has been shown to reduce hippocampal BDNF mRNA expression, and IL1ra has been shown to reverse stress-related reductions in BDNF (Barrientos *et al.*, 2003). Alterations in BDNF expression and function

are central to the 'neurotrophin hypothesis of depression', which suggests that reduced BDNF can lead to reduced neuronal protection and neurogenesis, and ultimately to the development of depressive symptoms (Martinowich *et al.*, 2007). These are important findings considering the role that BDNF plays in driving neurogenesis, a process implicated in the pathogenesis of depression and in the therapeutic response to antidepressants.

Increased incidence of depression in patients with inflammatory disorders

Assessments of various psychological parameters that accompany the onset or recovery from infection consistently report depression as a psychological disturbance (Dantzer *et al.*, 2008). Indeed, infection with Borna disease virus has been suggested as a risk for depressive illness in humans (Bode and Ludwig, 2003). There is also evidence that co-morbidity exists between a number of inflammatory disease states and depressive illness. For instance, depression and anxiety disorders represent a significant co-morbidity with inflammatory bowel disease (IBD) which adversely affects the quality of life of patients. In addition, individuals with IBD experience rates of depression that are triple those of the general population (Graff *et al.*, 2009). Moreover, considering the emerging literature on the brain–gut axis, it is likely that stress-related psychiatric disorders such as anxiety and depression could exacerbate the severity of or slow recovery from the clinical symptoms of IBD (Hollander, 2003). Systemic lupus erythematous (SLE) is a progressive autoimmune disorder and is associated with chronic stimulation of various components of the immune system. Compared to control subjects, tryptophan was decreased and kynurenine was significantly increased in patients with SLE. The study also indicates that tryptophan depletion may be associated with neurologic and psychiatric disturbances in patients suffering from SLE (Widner *et al.*, 1999; and see Chapter 10). Clinically significant depression can affect up to 50% of patients with multiple sclerosis over the course of their lifetime. In particular, an association between depression and structural brain abnormalities, including those derived from diffusion tensor imaging, was noted. Results from randomized controlled trials of antidepressant medication, cognitive behaviour therapy and mindfulness therapy reveal that depression in these patients can be successfully treated (Feinstein, 2011). Depression is 2–3 times more common in patients with rheumatoid arthritis when compared to the general population, and the degree of depression is associated with the level of pain experienced (Walker *et al.*, 2011). It may be the case with immune-mediated inflammatory disease that depressive symptoms relate to factors such as changes in quality of life and the experience of pain and distress associated with the disease. Evidence is growing, however, for cytokines as a trigger for changes in affect as opposed to a consequence of incapacitation associated with the primary medical condition (reviewed by Gibney and Drexhage, 2013).

Evidence for activation of the immune system in depressed patients

Innate immune system activation

Depression is associated with increased circulating concentrations of pro-inflammatory cytokines, soluble cytokine receptors, chemokines and acute-phase proteins. Moreover, in the cases of IL6, IL1, TNFα, soluble IL2 receptor (sIL2R) and C-reactive protein (CRP), original findings have been supported by recent meta-analyses (Howren *et al.*, 2009; Dowlati *et al.*, 2010; Liu *et al.*, 2012). An interesting feature of a study by Simon and co-workers (2007) is that serum concentrations of anti-inflammatory cytokines such as IL10 and IL4 were elevated in addition to pro-inflammatory cytokines, a feature that is shared with many of the studies from Maes and colleagues, who reported increased concentrations of anti-inflammatory cytokines such as IL1ra in depressive patients in parallel with increased concentrations of pro-inflammatory cytokines (Maes *et al.*, 1997). Another interesting feature of a study by Simen and co-workers (2006) is that, despite a robust inflammatory profile observed in depressed patients, concentrations of the pro-inflammatory cytokine TNFα were not significantly altered between controls and depressives. This was somewhat surprising give the role that TNFα has in inducing a variety of molecules in the cytokine network, and also given the attention that TNFα has received as a potential mediator of depressive symptomatology and in response to antidepressant treatment (Simen *et al.*, 2006; Powell *et al.*, 2012; Raison *et al.*, 2013). Similarly, in a recent study we failed to observe a significant increase in circulating TNFα concentrations in a group of treatment-resistant depressed patients, and whilst we observed a significant increase in plasma IL6 and IFNγ in depressives relative to controls, these increases were very modest (Hughes *et al.*, 2012) compared to those reported in other studies (Simon *et al.*, 2007; Cizza *et al.*, 2008).

It should be noted that whilst statistically significant increased cytokine concentrations are consistently observed in the serum or plasma of depressed patients, the magnitude of the changes observed between patients and control subjects are very small, and therefore one has to question the ability of such small increases to impact brain function. Similarly, while the increase in CRP in depressed patients is statistically significant, it generally falls in the normal range (below 6 mg/L) and would not indicate the presence of overt inflammation *per se* (Hughes *et al.*, 2012). Nonetheless, recent studies indicate that even mildly elevated IL6 and CRP concentrations independently predict the subsequent development of depression over a decade or more, even in individuals with no history of depression at the time of sampling (Gimeno *et al.*, 2009; Pasco *et al.*, 2010). Whilst further study is required to provide a mechanistic basis for these findings, it is noteworthy that we have recently observed that elevated circulating IL6 concentrations are associated with reduced hippocampal volumes in major depressive disorder (Frodl *et al.*,

2012). These data may suggest that elevated IL6 has the potential to impact hippocampal neuroplasticity.

Adaptive immune system activation in depressed patients

A high incidence of depression is evident in individuals with autoimmune diseases, including multiple sclerosis, SLE and RA where inflammatory cytokines are overexpressed (reviewed by Gibney and Drexhage, 2013), pointing to an association and a role for the adaptive immune system in depression. Reduced circulating T cell numbers and proliferation of peripheral blood mononuclear cells (PBMCs) in response to T cell mitogens has been reported in depressed patients. Moreover, depressed patients are reported to show fewer resting $CD3^+/CD25^-$ T cells with significantly more $CD20^+/CD5^+$ B cells when compared to healthy controls. Studies employing flow cytometry analysis have revealed that depressed patients have an increased number and percentage of T cells bearing activation markers, such as CD25 and HLA-DR, indicating the presence of acquired immunological activation in these patients (Irwin and Miller, 2007; Maes, 2011). The mechanisms of T cell alterations in depression are proposed to involve apoptosis, tryptophan depletion and changes in glucocorticoid and adrenergic receptor sensitivity (Miller, 2010).

By measuring stimulated cytokine production from PBMCs or diluted whole blood, investigators have examined the functional status of cytokine-producing cells in depressed patients. Mixed results have been observed using this approach. Seidel *et al.* (1995) reported a significant increase in mitogen-stimulated IFNγ and sIL2R production from PBMC cultures and elevated serum APP concentrations in depressed patients, which were maximal during the acute phase of the illness, and returned to control levels over a 6-week hospitalization period during which time a concomitant decrease in depression (HAMD) scores was apparent. However, in contrast to the studies conducted by Maes and colleagues (1993, 1995) that reported significant elevations in stimulated IL1β and IL6 production from PBMCs in depressed patients, the study by Seidel and co-workers (1995) reported only a slight but non-significant increase in mitogen-stimulated PBMC production of these cytokines in depressed patients. In stark contrast to the findings already outlined, Weizman and co-workers (1994) reported that IL1β, IL2 and IL3 production from mitogen-stimulated PBMC cultures was significantly reduced in depressed patients, when compared to age- and sex-matched controls.

Whilst the studies reporting increased stimulated cytokine production tally with the findings of increased plasma cytokines in depressed patients, the studies reporting reduced cytokine responses to immune stimulants tally with reports of reduced proliferative responses to immune stimulants (mitogens) which were published in the 1980s as one of the first pieces of evidence of an aberrant immune system in depression. It has been proposed that the hyporesponsiveness of PBMCs may be accounted for by the increase in circulating cytokines which suppress the ability of cells to respond to stimulation

(see Maes *et al.*, 2012) and that the inflammatory response may be related to impaired function of lymphocytes in depression through direct effects of cytokines on signalling through T cells (Blume *et al.*, 2012; Haroon *et al.*, 2012).

Evidence for inflammatory cytokine production in the CNS of depressed patients

Levine and co-workers (1999) reported increased CSF IL1β concentrations in a group of depressives versus controls and reported a positive correlation between IL1β concentrations and the severity of depression. A more recent study reported increased CSF concentrations of IL6 in depressed patients. Patients who performed violent suicide attempts displayed the highest IL6 concentrations. IL6 and TNFα correlated significantly with the serotonergic metabolite 5-hydroxyindole acetic acid (5-HIAA) and the dopaminergic metabolite homovanillic acid (HVA) in CSF, but not the noradrenergic metabolite methoxyhydroxyphenylglycol (MHPG). Cytokine levels in plasma and CSF were not associated, and patients with increased blood–brain barrier permeability did not exhibit elevated cytokine levels. Thus, a role for CSF IL6 has been proposed in the symptomatology of suicidal behaviour, possibly through mechanisms involving alterations of dopamine and serotonin metabolism (Lindqvist *et al.*, 2009). In a separate investigation, CSF levels of IL1, IL6 and TNFα were reported to be significantly correlated with depression severity in support of the presence of central inflammatory activation in depressed patients (Martinez *et al.* 2012).

Increased expression of IFNα and its receptor IFNα/βR1 were observed in post-mortem dorsolateral prefrontal cortex tissue from major depressives relative to a group of matched controls (Kang *et al.*, 2007). This finding is of significance considering that interferons have been associated with major depressive disorder and in particular with depression associated with multiple sclerosis. Increased expression of HLA-DR has been reported in the hippocampus and prefrontal cortex of depressed patients. More recently, increased quinolinic acid, a tryptophan metabolite and end product of the kynurenine pathway, has been detected in ramified microglia within sub-regions of the anterior cingulate cortex of severely depressed patients (Steiner *et al.*, 2011; see also Beumer *et al.*, 2012).

Glial loss and alterations in density have been reported in patients with depression and bipolar affective disorder in the dorsolateral prefrontal cortex, the amygdala and the orbitofrontal cortex as well as the subgenual and supergenual regions of the anterior cingulate cortex. An increase in the glial density of the CA subfields of the hippocampus and the granule layer of the dentate gyrus has also been observed and attributed to an increase in packing density that may be associated with a loss of glial processes, implying glial disconnection from neurons. Specifically, immunoreactivity and expression of

glial fibrillary acidic protein (GFAP), a cytoskeletal marker for reactive astrocytes, are reported to be reduced in the dorsolateral prefrontal cortex, cingulated cortex, orbitofrontal cortex and hippocampus from post-mortem brains of depressed patients (reviewed by Miguel-Hidalgo *et al.*, 2010). Astrocytic dysfunction has, in turn, been proposed to lead to reduced glutamate clearance and higher extracellular levels of glutamate (reviewed by Kugaya and Sanacora, 2005). Links between inflammation and microglial activation coupled to astrocytic disruption and glutamatergic dysfunction are currently not well understood, but one such possibility may relate to tryptophan metabolism and regulation of the kynurenine pathway where the enzyme kynurenine aminotransferase, expressed in astrocytes, leads to the formation of the glutamate N-methyl-D-aspartic acid (NMDA) receptor antagonist kynurenic acid, whereas microglia are responsible for the production of the excitotoxin quinolinic acid. Therefore, activation of microglia and reduction in the activity of astrocytic function may lead to an imbalance in the ratio of quinolinic acid to kynurenic acid, leading to dysregulated glutamatergic transmission (Steiner *et al.*, 2011).

Stress as a trigger for activating the immune system in depressed patients

Alterations in immune response due to stress-related adaptations in glucocorticoid and beta-adrenergic receptor expression and sensitivity are commonly referred to as receptor desensitization, which involves reductions in receptor expression and function that remain for a period following stressor termination (reviewed by Pace and Miller, 2009). As glucocorticoid receptors mediate tonic anti-inflammatory effects, down-regulation of these receptors in response to chronic stress and persistent HPA axis activation could promote the development of an immuno-enhanced and pro-inflammatory state. In addition, pro-inflammatory cytokines may promote the expression of the beta isoform of the glucocorticoid receptor that is inactive but still able to bind its ligand. Such mechanisms promote resistance to glucocorticoids, and this may account for cytokine-induced dysregulation of the HPA axis on account of the reduced ability of glucocorticoids to feed back to the hypothalamus. In tandem, depressed patients show elevated levels of corticotropin-releasing hormone (CRH), a hypothalamic neuropeptide under the regulation of glucocorticoids that drives HPA axis activation and plays an important central role in behavioural, endocrine and immune responses to stress (Irwin and Miller, 2007).

Similarly, chronic stress-related activation of the sympatho–adrenal–medullary (SAM) axis with continuous exposure to catecholamines leads to a reduced beta-adrenergic receptor response by a process of receptor phosphorylation, internalization and down-regulation leading to a possible diminished

negative feedback response to catecholamines on immune cells. For instance, forced exercise, which can be considered to provoke robust activation of the SAM axis and to be a stressor for laboratory animals, provokes a reduction in β_2-adrenoceptor expression in peritoneal macrophages associated with an increase in IL12 production for 24 h following termination of the regime (Itoh *et al.*, 2004). While this is indicative of a diminished feedback response to catecholamines on immune cells, other reports reveal an increase in catecholamine reactivity in mice subjected to chronic mild stress (CMS). Normally, catecholamines exert a β_2-adrenoceptor-mediated inhibitory effect on mitogen-induced T cell proliferation and a stimulatory effect on B cell proliferation in response to selective B lymphocyte mitogens. Lymphocytes from mice subjected to CMS have an increased response to catecholamine-mediated inhibition or enhancement of proliferation in T and B cells, respectively, coupled with an increase in β_2-adrenoceptor density and responsiveness (Edgar *et al.*, 2003). In this model, chronic stress is associated with increased sympathetic influence on the immune response, indicative of an alternative mechanism through which stress can alter immunity.

Central administration of CRH induces a robust decline in innate immune responses by acting within the CNS to co-ordinate changes in peripheral immunity, for example, T cell response to antigen and reduced NK cell activity (Irwin and Miller, 2007). Centrally mediated actions of CRH are supported by the facts that the immune effects of CRH can be antagonized by benzodiazepines which alter the perception of stress and that CRH-mediated changes in immune responsiveness are linked to CRH receptors in the amygdala rather than the pituitary–adrenal axis. Repeated administration of CRH leads to a down-regulation of CRH receptors in the amygdala which is associated with reduced CRH-mediated immune responses. Moreover, immunoneutralization of CRH in the brain antagonized the immunosuppressive effects (decline in NK cell activity) of foot shock, demonstrating that brain CRH was involved in the regulation of immune responses in the periphery. The nonselective beta-blocker propanolol and the β_2 adrenoceptor antagonist butoxamine blocked CRH-induced immune suppression, highlighting the functional significance of noradrenergic innervation of the spleen in mediating CRH effects on immunity. A model of CNS stress circuitry activation involving CRH activation and ultimately sympathetic nervous system outflow are proposed to link the brain to immune responses to stress in this instance (Irwin and Miller, 2007).

Cytokine expression in the CNS – a key mediator of stress-induced behavioural, HPA axis and neurotrophin-related changes

Stress protocols in animals, such as mild foot shock and social isolation, have been shown to induce expression of the pro-inflammatory cytokine IL1β in

the CNS. This is supported by the finding that a severe regimen of psychological stress increases TNFα concentrations and nitric oxide synthase (iNOS) expression in the CNS via NF-κB activation (Madrigal *et al.*, 2002). More recently, stress-susceptible mice were found to develop elevated expression of TNFα and SERT and increased numbers of microglial cells in the prefrontal cortex and indoleamine-2,3-dioxygenase (IDO) in the raphe nucleus when compared to stress-resilient mice (Couch *et al.*, 2013). Thus, susceptibility to stress is associated with a unique molecular profile when compared to resilient animals, providing a rationale for exploring anti-inflammatory targeted therapy for stress-induced depression-related behaviour. In this regard, in a chronic stress model of depression in rats, Krügel and co-workers (2013) reported that blocking TNFα with the human TNFα receptor p75-Fc fusion protein etanercept induced antidepressant effects. This was consistent with observations in patients with psoriasis who showed improvements in symptoms of depression which were weakly correlated with objective measures of skin clearance or joint pain (Tyring *et al.*, 2006).

Exposure to CMS provokes depression-related behaviours which are mimicked by exogenous subcutaneous administration of IL1β delivered via osmotic mini-pump for 4 weeks. Mice with IL1β receptor knockout or brain-restricted overexpression of IL1ra do not display CMS-induced behaviour, adrenocortical activation or reduced hippocampal neurogenesis, indicating that brain IL1β mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis (Goshen *et al.*, 2008). Recent preclinical evidence indicates that IFNα treatment, which provokes depression symptoms in humans, reduced BDNF expression in the hippocampus via the induction of IL1β expression (Kaneko *et al.*, 2006). Thus, other pro-inflammatory cytokines may converge upon IL1β as the cardinal cytokine in mediating stress-related changes of relevance to depression. Reduced BDNF coupled with increased glucocorticoid production may result in neuronal atrophy, particularly in the hippocampus. In this regard, it has been reported that prolonged IFNα treatment reduced the density of serotonergic and noradrenergic axons in a number of limbic brain structures, including the amygdala, prefrontal cortex and hippocampus (Ishikawa *et al.*, 2007), but whether these effects are due to reduced trophic support or neurotoxicity has not been determined.

Chronic stress and depression have been consistently linked with the increased peripheral production of IL6 which in turn can promote glucocorticoid resistance (Pace and Miller, 2009). In this regard, it has been reported that IL6 knockout mice exhibit resistance to stress-induced development of depression-like behaviours (Chourbaji *et al.*, 2006).

Reduced hippocampal glucocorticoid receptor expression following exposure to chronic stress in animal models has been reported by numerous groups to date (Kitraki *et al.*, 1999; Quan *et al.*, 2003). The implications of a stress-related down-regulation of glucocorticoid receptors in the hippocampus for

the activity, regulation and proliferation of resident microglia comprise a subject of considerable current interest. Recent evidence from animal studies demonstrates that psychological stress can increase activation of microglial cells in the CNS and prime them for cytokine production in response to a subsequent inflammatory challenge. For instance, Frank and co-workers (2007) observed increased expression of the microglial marker CD11b in the CNS in response to foot shock stress in rats, and Sugama *et al.* (2007) reported that stress induces morphological activation of microglia in mouse brain. Moreover, a role for microglial activation in stressor-induced increases in brain IL1β concentrations was confirmed, in that pre-treatment with the microglial inhibitor minocycline completely blocked the ability of foot shock stress to increase brain IL1β concentrations (Blandino *et al.*, 2006).

Repeated social defeat stress in mice is associated with enhanced activation and reactivity of microglia, which are indicated by the presence of inflammatory markers on the surface of microglia (CD14, CD86 and TLR4) and increased de-ramification of microglia in the medial amygdala, prefrontal cortex and hippocampus. Increased expression of IL1β following exposure to chronic social stress is associated with reduced levels of glucocorticoid-responsive genes (glucocorticoid-induced leucine zipper (GILZ)) and the glucocorticoid receptor co-chaperone FK-506 binding protein-51 (FKBP51), which are indicative of GR insensitivity and impaired regulatory feedback on microglia. Moreover the stress-dependent changes in microglia are prevented by administration of the beta-adrenergic receptor antagonist propanolol (Wohleb *et al.*, 2011). Taken together, reports to date suggest that increased activity through the SAM axis coupled with reduced sensitivity to the inhibitory effects of glucocorticoids may operate during chronic stress to contribute to chronic activation of the inflammatory response system and central microglial activation leading to depression at least in some patients where inflammation is evident.

Antidepressants have anti-inflammatory actions

A recent meta-analysis of studies on the effect of antidepressant treatments on serum levels of inflammatory cytokines reported that antidepressant treatment reduced levels of IL1β and possibly IL6, especially following treatment with serotonin reuptake inhibitors (Hannestad *et al.*, 2011). In addition to the effects of antidepressants on circulating inflammatory cytokines, existing monoamine-based antidepressants have been shown to be efficacious at treating depression induced by IFNα and IL2 immunotherapy in humans (Schramm *et al.*, 2000; Musselman *et al.*, 2001; Kraus *et al.*, 2002).

Antidepressants suppress mitogen-stimulated IFNγ production in human blood (Diamond *et al.* 2006). We have demonstrated that antidepressants with selectivity for either serotonin (fluoxetine and clomipramine) or noradrenaline reuptake (reboxetine and desipramine) share this property.

Trimipramine is an atypical tricyclic antidepressant lacking affinity for monoamine transporters, yet it is still a clinically efficacious antidepressant agent. Observations that trimipramine suppressed IFNγ production and T cell proliferation indicate that these immunomodulatory actions of antidepressants are most likely unrelated to inhibition of monoamine reuptake. In addition, the fact that second-generation antidepressants elicited similar actions on cytokine production to the first-generation tricyclic compounds suggests that the immunomodulatory actions of the compounds tested are not related to well-established antagonistic actions of tricyclic antidepressants at histamine, muscarinic or adrenergic receptors.

The ability of antidepressants to suppress IFNγ production is particularly significant, considering that IFNγ is the most potent of the pro-inflammatory cytokines, in the induction of IDO and subsequent metabolism of tryptophan via the kynurenine pathway, possibly reducing tryptophan availability for serotonin synthesis (Widner *et al.*, 2002). Thus it is possible that by inhibiting IFNγ production, antidepressants could prevent low serotonin, and consequently prevent a depressive episode that occurs secondary to an inflammatory state.

In contrast to the ability of antidepressants to alter T cell cytokine production, antidepressants had little effect on monocyte-derived pro-inflammatory or anti-inflammatory cytokines (Diamond *et al.*, 2006). As IL12 is a potent inducer of IFNγ, the fact that antidepressants failed to suppress LPS-induced IL12 production is of particular interest, and indicates that the suppressive effect of antidepressants on IFNγ production previously observed following dual stimulation with LPS with phytohemagluttinin (PHA) (Maes *et al.*, 1999) occurs via a direct effect on T cells.

Our finding that *in vitro* exposure to antidepressants largely fails to alter LPS-induced cytokine production is consistent with a recent study where a number of antidepressants failed to suppress TNFα production from diluted whole blood (Kubera *et al.*, 2004), and is also consistent with an *in vivo* study in rats where treatment with imipramine and fluoxetine failed to alter LPS-induced IL1β or TNFα expression (Yirmiya *et al.*, 2001). However, our data are at variance with a previous study indicating that antidepressants suppress production of monocyte-derived pro-inflammatory cytokines in human PBMCs (Xia *et al.*, 1996), and are also at variance with earlier studies, where we observed that desipramine suppressed production of IL1β and TNFα, and increased production of IL10 following an *in vivo* LPS challenge in rats (Shen *et al.*, 1999; Connor *et al.*, 2000). The reason for the discrepancy between these studies is unclear but may be due to differences in methodological approach. We suggest that future studies in this field should examine *ex vivo* cytokine production following antidepressant administration to human volunteers to give a clearer picture of the impact of therapeutic doses of antidepressants on cytokine production. Whilst antidepressants largely failed to alter production of macrophage-derived pro-inflammatory cytokines *in vitro*, the suppressive

effect of antidepressants on IFNγ production has the potential to suppress macrophage-derived cytokines in an *in vivo* setting. Specifically, IFNγ primes monocytes and macrophages (including microglial cells) to produce IL1β and TNFα. Overall, the data generated to date indicate that IFNγ-producing Th_1 cells, as opposed to monocytes, are the major target of the immunomodulatory actions of antidepressants.

As therapeutic plasma concentrations of antidepressants are typically in the region of 1 μM or below (Maes *et al.*, 1999; Kubera *et al.*, 2004), the concentration range of 10–50 μM required to suppress IFNγ production is far in excess of therapeutic plasma concentrations. However, based on pharmacokinetic studies in animals, concentrations of antidepressants detected in organs such as the brain and the spleen were 10–20-fold higher than plasma concentrations (Uhr and Grauer, 2003). Thus, it is possible that immune cells residing in such compartments could be exposed to higher concentrations of antidepressants than are typically observed in the plasma.

Some components of sickness behaviour such as a decreased preference for sweet solutions and reduced social exploration are improved by some antidepressants (reviewed by Dunn *et al.*, 2005). The antidepressant response is observed most often with LPS, is less evident with IL1β and is more evident in rats than mice. The mechanisms by which antidepressants suppress LPS-induced sickness behaviour are currently unknown but may involve the suppression of LPS-induced expression or alteration of the response to pro-inflammatory cytokines in the brain. A number of studies have documented that serotonin enhancers have immunosuppressive and anti-inflammatory actions in a number of *in vivo* model systems. Treatment with the tricyclic antidepressants imipramine and clomipramine, both of which elevate synaptic serotonin, reduces the clinical signs of inflammation and the number of Th_1 cells secreting IFNγ in an animal model of Guillain–Barré syndrome (Zhu *et al.*, 1997). Further evidence supporting a role for central serotonin in producing an anti-inflammatory response is that intracerebroventricular administration of serotonin and its precursor, 5-hydroxytryptophan, suppresses peripheral inflammation in a rat model (Dumka *et al.*, 1996). Serotonin has been reported to have both immuno-enhancing and immunosuppressive properties, depending on the doses of serotonin employed, and on the immune parameters being studied (Stefulj *et al.*, 2001). Treatment with the tricyclic antidepressant and noradrenaline re-uptake inhibitor desipramine also promotes an anti-inflammatory phenotype *in vivo* as chronic treatment inhibits LPS-induced sickness behaviour in rats and suppresses and augments LPS-induced increases in circulating TNFα and IL10, respectively. By contrast, the serotonin re-uptake inhibitors paroxetine and venlafaxine failed to alter LPS-induced responses in this investigation (Shen *et al.*, 1999). In a more recent study, desipramine and fluoxetine were reported to reduce LPS-induced pro-inflammatory cytokine secretion in response to LPS in mice, and dramatically reduced mortality in response to a septic dose of LPS (Roumestan *et al.*, 2007).

Microglia as a target for the anti-inflammatory actions of antidepressants

Obuchowicz and colleagues (2006) reported that the tricyclic antidepressant amitriptyline and its major metabolite, nortriptyline, inhibit LPS-induced IL1β and TNFα release from both mixed glial cells and microglia, without altering mRNA expression for these two cytokines. Data from our laboratory demonstrate that *in vitro* exposure of mixed glial cells to the tricyclic antidepressant desipramine fails to alter either mRNA or protein expression of IL1β or TNFα (O'Sullivan *et al.*, 2009). However, we have demonstrated that desipramine inhibits LPS-induced IL1β and TNFα expression in rat cortex *in vivo*, and this is associated with reduced microglial activation indicated by reduced CD11b and CD40 expression (O'Sullivan *et al.*, 2009). We suggest that the ability of desipramine to suppress IL1β and TNFα expression *in vivo* is due to its ability to increase noradrenaline availability in the CNS, which can act to suppress microglial activation and pro-inflammatory cytokine production via activation of β_2-adenoceptors. This proposition is supported by the fact that administration of the highly selective noradrenaline reuptake inhibitor atomoxetine to rats also suppresses LPS-induced microglial activation and pro-inflammatory cytokine expression in the rat cortex, without having any direct suppressive effect on pro-inflammatory cytokine production from LPS-stimulated mixed glial cell cultures (O'Sullivan *et al.*, 2009, 2010). Thus, antidepressants that modulate central noradrenergic transmission can promote an anti-inflammatory phenotype which may be a useful property in their therapeutic effects, particularly where depression may be associated with inflammation. It was also reported that the tricyclic antidepressant imipramine, the SSRI fluvoxamine and the norepinephrine reuptake inhibitor reboxetine all reduced IFNγ-induced IL6 and NO production in a murine microglial cell line, via a mechanism mediated by cyclic adenosine monophosphate (cAMP) and protein kinase A (Hashioka *et al.*, 2007). However, these findings are at odds with a study reporting increased NO and IL6 production in a murine microglial cell line stimulated with the SSRI fluoxetine in the absence of any inflammatory stimulus (Ha *et al.*, 2006).

Considering recent pre-clinical evidence indicating that IFNα therapy reduces BDNF expression in the hippocampus via induction of IL1β expression (Kaneko *et al.*, 2006), it may well be the case that the depressive symptoms induced by IFNα are mediated by the induction of pro-inflammatory cytokines such as IL1β. In this regard, both IFNα and IL2 immunotherapy activate the cytokine network. Therefore, the ability of antidepressants to prevent or reverse the depressive symptoms induced by IL2 or IFNα therapy in humans may be due to a knock-on inhibition of the induction of other cytokines.

The anti-inflammatory effect of antidepressants appears to occur following acute exposure to the drugs both *in vitro* and *in vivo*, and this time course is not in line with the chronic treatment regimens that are required to elicit

antidepressant efficacy in the clinical setting. Thus, the precise contribution of the anti-inflammation actions of antidepressants to the therapeutic efficacy of these drugs remains to be determined.

Can non-pharmacological treatments for depression impact the inflammatory response?

Clinical improvement in patients with depression following electroconvulsive therapy (ECT) was accompanied by a decline in TNFα concentrations approaching those observed in healthy controls. Furthermore, such a decline in TNFα was not observed in the depressed patients not receiving ECT, who instead showed raised TNFα levels throughout the study period (Hestad *et al.*, 2003). It has been demonstrated that successful treatment of depression in a group of multiple sclerosis patients with either cognitive-behavioural therapy, group psychotherapy or the antidepressant sertraline was associated with a reduction in IFNγ production, and that there was no significant differential effect of treatment modality (Mohr *et al.* 2001). These data suggest that behavioural interventions can also ameliorate excessive cytokine production observed in depressed patients.

Anti-inflammatory actions may be independent of monoamine re-uptake inhibition

Antidepressants, including imipramine, amitriptyline, tianeptine and fluoxetine, influence the expression and activity of phosholipase A2 (PLA2) in plasma membranes of the rat brain cortex in a dose- and time-dependent fashion, following acute and chronic treatment, and it has been suggested accordingly that PLA2 may be a common target for drugs with different mechanisms of action. Chronic fluoxetine has been shown to up-regulate activity, protein and mRNA levels of cytosolic PLA2 in rat frontal cortex. More recently, arachidonic acid (AA) signalling including expression levels of PLA2 protein, and phosphorylation and AA turnover in rat brain was also reported to be increased following chronic imipramine treatment in rats (see Lee *et al.*, 2010). AA that is released by PLA_2 can be converted to eicosanoids or other active metabolites, which influence a number of brain processes, including signal transduction, transcription, neuronal activity, apoptosis, inflammation and blood flow.

Increased levels of cAMP in microglia brought about by the use of phosphodiesterase inhibitors have been shown to suppress both cytokine production and NO production, and in a recent report have been implicated in the anti-inflammatory actions of SSRIs in microglia (Tynan *et al.* 2012). This study incubated the BV2 microglia cell line with an adenylate cyclase inhibitor and five different SSRIs and found that this partially reversed the anti-inflammatory actions of these drugs.

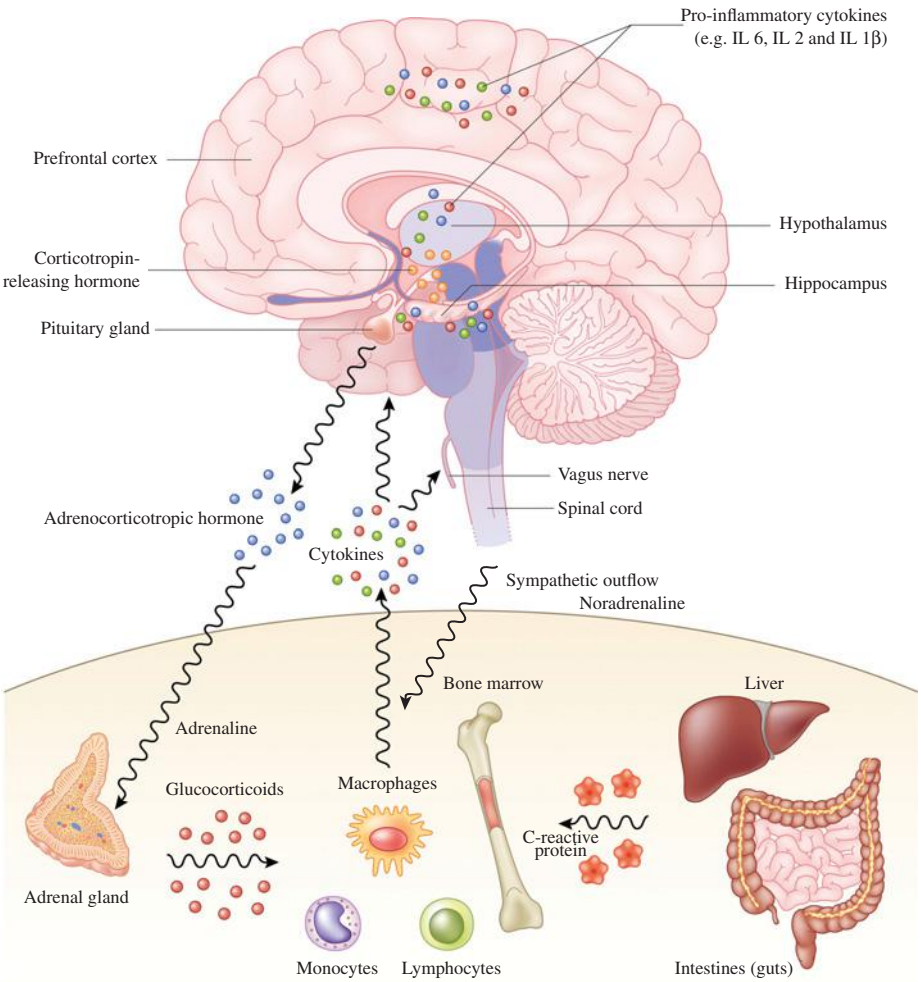

Figure 11.1 Cytokine effects on the brain.

Do anti-inflammatory therapies have antidepressant potential?

Studies in laboratory animals show that administration of specific cytokine antagonists such as IL1ra or anti-inflammatory cytokines such as IL10 directly into the brain can produce antidepressant-like effects (reviewed by Raison *et al.*, 2006). Very recent evidence indicates that anti-TNF therapy and cyclooxygenase-2 (COX2) inhibitors have antidepressant effects. Such evidence has been reviewed extensively (Dunn *et al.*, 2005; Miller *et al.*, 2009; Müller, 2010) and includes preliminary clinical studies on the efficacy of COX2 inhibitors (see Müller, 2010) and the TNFα antagonist infliximab

(Raison *et al.*, 2013) in depression. Infliximab produced antidepressant effects in treatment-resistant patients with a baseline inflammatory phenotype, as indicated by raised circulating high-sensitivity CRP concentrations (Raison *et al.*, 2013).

Conclusion

Changes in the immune system play a role in the pathogenesis of depression, and cytokines are involved in the behavioural, neurochemical and endocrine changes that characterize the disorder (Figure 11.1). Future studies will help clarify whether activation of the immune system is associated with subtypes of depression such that anti-inflammatory treatments may be employed as antidepressants. A greater understanding of the anti-inflammatory properties of existing antidepressants will guide clinicians when deciding upon appropriate treatment in cases of depression associated with inflammation that are difficult to treat.

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12
Immune Responses in the CNS in Epilepsy

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Introduction

Epilepsy is a brain disorder characterized by a predisposition to generate seizures and is frequently associated with cognitive and psychological sequelae. Seizures, the hallmarks of epilepsy, originate from synchronized aberrant firing of neuronal populations due to underlying hyper-excitability. Epilepsy occurs in about 1% of the general population; therefore, there are about 50 million people worldwide affected by this disease. Seizures can be caused by various aetiologies which include genetic factors, or they may be associated with focal damage to the brain. However, which factors actually trigger seizures remains unknown. Despite the availability of a wide range of antiepileptic drugs (AEDs) with predominant actions on voltage-dependent ion channels and classical neurotransmitter systems (Rogawski and Loscher, 2004), about one-third of affected individuals experience seizures which are not controlled by the available treatments. Therefore, there is an urgent medical need to develop new effective therapeutics. In addition, the available AEDs are predominantly symptomatic drugs that often do not interfere with the aetiopathogenic mechanisms underlying the onset of the disease or its progression (Pitkanen and Lukasiuk, 2011).

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Research for new therapies in epilepsy is reliant upon understanding the pivotal mechanisms of neuronal hyper-excitability. In a number of cases, this has led to the identification of immune or inflammatory processes in the epileptic brain in different forms of the disease, which may be involved in the pathogenesis of seizures.

Clinical and experimental findings suggest that the activation of both the innate and adaptive immune systems, and the associated inflammatory processes, are involved in many forms of epilepsy. A role for inflammatory molecules in the generation of seizures has long been suspected since it was shown that selected anti-inflammatory treatments can control seizures in active and difficult-to-treat paediatric epilepsies. These treatments, in particular steroids and the use of intravenous immunoglobulins (IvIg) and adrenocorticotropic hormone (ACTH), are used in specific syndromes which are refractory to conventional AEDs (e.g. infantile spasms, continuous spikes and waves in sleep, and Rasmussen encephalitis (RE)). In recent years, a small but significant number of epileptic disorders have been associated with the presence of specific autoantibodies to neuronal receptors, ion channels or their accessory proteins in serum and cerebrospinal fluid (CSF). A pathogenic role for some of these autoantibodies is suspected based on clinical response to immunotherapy and other experimental observations. Indeed, the recent description of faciobrachial dystonic seizures (FBDS) has linked a clinically distinctive epilepsy with antibodies directed against the voltage-gated potassium channel (VGKC) complex protein LGI1 (Irani *et al.*, 2008, 2011). Moreover, systemic and neurological autoimmune disorders have been associated with seizures or epilepsy in a small but significant number of cases (Palace and Lang, 2000; Vincent and Crino, 2011). These disorders are increasingly recognized and often treated successfully with immunotherapies. In addition, a careful assessment of inflammatory molecules and immune cells was performed in surgically resected brain tissue from patients with pharmaco-resistant epilepsies without an established or suspected autoimmune aetiology. In these common forms of epilepsy, the results revealed a significant component of the inflammatory response confined to resident brain cells (i.e. microglia, astrocytes and neurons) (Vezzani *et al.*, 2011a; Aronica *et al.*, 2012). Mechanistic insight into the role of these inflammatory molecules in the aetiopathogenesis of seizures demonstrated their prominent effects in inducing neuronal network hyper-excitability. First, these molecules can act as neuromodulators via stimulation of their cognate receptors expressed by neurons in diseased tissue. In addition, the effects of inflammatory molecules released by brain cells on blood–brain barrier (BBB) permeability function or astrocytic properties have been recently described to play important roles in tissue hyper-excitability (Vezzani *et al.*, 2013). Proof-of-concept pharmacological evidence in experimental models of temporal lobe epilepsy (TLE) has identified anticonvulsive properties of anti-inflammatory drugs already used for other clinical indications. These drugs may be considered for prospective

clinical trials for treating seizures not only in autoimmune forms but also in more common forms of epilepsy.

The aim of this chapter is to outline the recent discoveries in this field by combining pre-clinical and clinical evidence of activation of the immune system and its functions in epilepsy. Mechanisms underlying the immune system's effects in epileptic brain tissue will also be addressed. Finally, the current immunotherapies and the future challenges for new anti-inflammatory treatments in epilepsy will be discussed.

Innate immunity in epilepsy

Human epilepsy

As shown in animal models, the innate immune system may contribute directly to the induction of seizures (Vezzani *et al.*, 2011a). Like other organs, the human central nervous system (CNS) contains cells (e.g. microglia and astrocytes) which after activation can secrete a large range of soluble inflammatory mediators, including interleukins (IL), chemokines, prostaglandins and complement components (Vezzani *et al.*, 2011a). The activation of these cells and the concurrent production of inflammatory molecules have been shown not only in inflammatory and neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease (Link, 1998; McGeer and McGeer, 2002; Maccioni *et al.*, 2009; Fernandez *et al.*, 2010; Gandhi *et al.*, 2010), but also in various epileptic disorders (Crespel *et al.*, 2002; Boer *et al.*, 2006; Choi *et al.*, 2009; Wirenfeldt *et al.*, 2009) (Figures 12.1 and 12.2). Thus, it is likely that innate immunity plays a significant role in epilepsies. One of the predominant players investigated in epilepsy research is IL1β (Figures 12.1 and 12.2). In a variety of human epileptic disorders (TLE, focal cortical dysplasia (FCD) type IIb, glioneuronal tumours and tuberous sclerosis), a moderate to strong up-regulation of IL1β expression is seen in (dysplastic) neurons, microglial cells and astrocytes (Ravizza *et al.*, 2006, 2008; Iyer *et al.*, 2010; Aronica and Crino, 2011). A link between IL1β and seizures is supported by the positive correlation between IL1β levels in surgically resected brain specimens from patients and the frequency of their epileptic seizures (Ravizza *et al.*, 2006). The same positive correlation was found between the number of activated microglia, the frequency of seizures and the duration of epilepsy (Boer *et al.*, 2006) (Figure 12.2). Interestingly, in FCD type I patients with intractable seizures, characterized by cortical dyslamination in the absence of obvious neuropathology or aberrant cells, only low IL1β immunoreactivity in some microglial cells was found (Iyer *et al.*, 2010). In TLE, FCD type IIb and the glioneuronal tumours, the cells ((dysmorphic) neurons, balloon cells, microglia and astrocytes) that showed increased expression of IL1β also demonstrated up-regulation of its receptor IL1RI (Ravizza *et al.*, 2006, 2008; Iyer *et al.*, 2010). Notably, akin to experimental animals, the level of the anti-inflammatory molecule

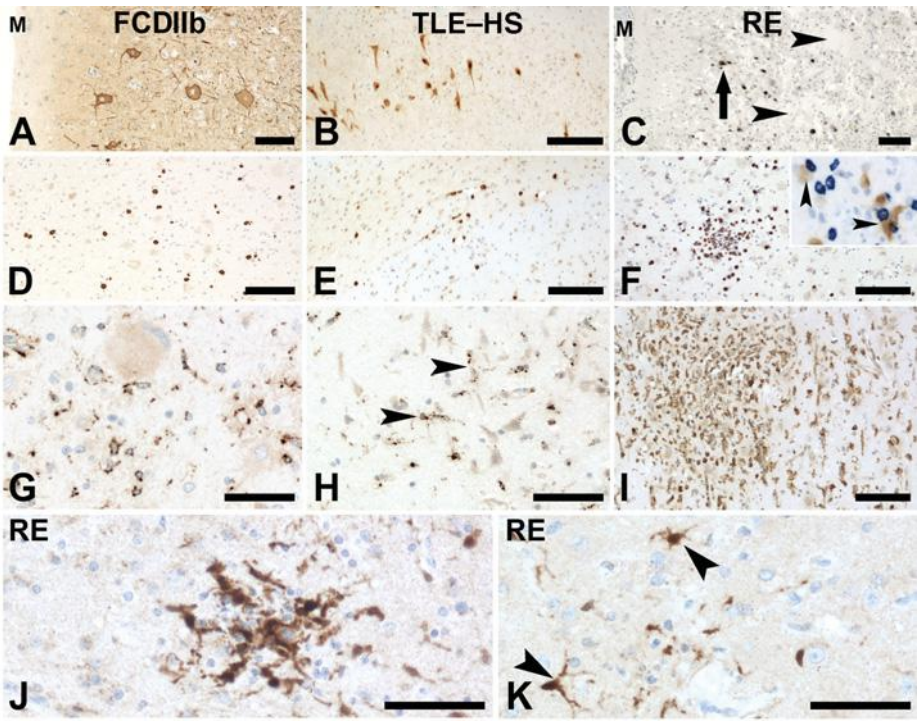

Figure 12.1 Neuropathology in different forms of human epilepsy. (A) Dystrophic neurons in the cortex of a focal cortical dysplasia (FCD) type IIb brain stained by antibodies against neurofilament (SMI32). M: meninges. Bar: 100 μm. (B) NeuN staining shows severe neuronal loss in the hippocampus of a temporal lobe epilepsy (TLE) with hippocampal sclerosis (HS) brain. Bar: 200 μm. (C) NeuN staining shows severe cortical degeneration with the presence of a few remaining neurons (arrow) in a Rasmussen encephalitis (RE) brain. Arrowheads indicate areas of vacuolization. M: meninges. Bar: 200 μm. (D–F) T lymphocytes in epilepsy. (D) Moderate numbers of CD3+ T cells in the cortex of FCD type IIb brain. Bar: 100 μm. (E) Moderate numbers of T cells in the hippocampus of a TLE–HS brain. Bar: 100 μm. (F) Infiltrate with CD3+ T cells in an RE brain. The inset shows the apposition of multiple cytotoxic (CD8+) T cells (blue) to neurons (brown: staining for NeuN). Bar: 100 μm. (G–I) Microglia activation in epilepsy. (G) Moderate up-regulation of CD68 in microglia in FCD type IIb brain. Bar: 50 μm. (H) Mild up-regulation of CD68 in microglia (arrowheads) is seen in a TLE–HS brain. Bar: 50 μm. Strong CD68 reactivity in microglia in an RE brain. Bar: 100 μm. (J and K) IL1β in an RE brain. (J) IL1β in a microglial nodule. Bar: 100 μm. (K) IL1β (arrowheads) in cells which probably are astrocytes. Bar: 100 μm.

IL1 receptor antagonist (IL1ra) was moderate as compared to that of IL1β (Ravizza *et al.*, 2006, 2008; Iyer *et al.*, 2010), suggesting that key mechanisms controlling the extent and duration of inflammation are inefficient in brain tissue. Besides IL1β, IL6 has also been implicated in epilepsy. After tonic-clonic seizures in humans, increased levels of IL6 can be found in CSF (Peltola *et al.*, 1998). Furthermore, elevated serum levels of IL6 are found in patients with refractory epilepsy (Hulkkonen *et al.*, 2004). Conversely, a number of studies

did not show differences in IL6 gene or protein expression in epileptic brains (van Gassen *et al.*, 2008; Kan *et al.*, 2012a,b). Thus, the role of IL6 in epilepsy requires further study.

Although most epilepsy research has focused on cytokines, other components of the innate immune system also have been investigated. Examples of these are endogenous ligands for the Toll-like receptors (TLRs) and receptors for advanced glycation end products (RAGE), such as high-mobility group box-1 (HMGB1). HMGB1 is a chromatin-bound factor that, following cell damage or neuronal hyper-excitability, translocates from the nucleus to the cytoplasm where it is released in the extracellular milieu and induces pro-inflammatory signals by stimulation of TLR4 or RAGE (Lotze and Tracey, 2005). In brain tissue from TLE or malformations of cortical development (MCD) patients, HMGB1–TLR4 signalling is up-regulated in resident brain cells, similar to observations in experimental models of epilepsy (Maroso *et al.*, 2010). In addition, in MCD patients, up-regulation of TLR2 and RAGE has been found (Zurolo *et al.*, 2011). A recent study performed with a multiplex immunoassay compared 39 immune modulators in TLE patients with (+) and without (−) hippocampal sclerosis (HS). The results showed up-regulation of 21 inflammatory mediators, including 10 cytokines and seven chemokines. Further analysis revealed that TLE+HS, TLE−HS and control groups could be distinguished based on unique expression patterns of cytokines and chemokines such as IL7, IL10, IL25 and CCL4, molecules which previously had not been implicated in TLE (Kan *et al.*, 2012a).

Also, complement factors are associated with seizure induction. In human TLE+HS, astroglial, microglial and neuronal up-regulation of complement factors C1q, C3c and C3d was observed, particularly within regions where neuronal cell loss occurs. The membrane attack protein complex (C5b–C9) was predominantly detected in activated microglial cells (Aronica *et al.*, 2007). Microarray data on cortical tubers from patients with tuberous sclerosis complex revealed strong up-regulation (7 to 30 times) of complement mRNA (C factor I, C1R, C1s, C3 and C4a) (Boer *et al.*, 2010). Also, in gangliomas, C1qa, C1qb, C1qc, C1r, C1s, C3, C4a and C7 showed more prominent expression than in control specimens.

Finally, a role for innate immunity also can occur through activation of cyclooxygenase-2 (COX2), an inducible pro-inflammatory enzyme required for the synthesis of prostaglandins. COX2 was found to be up-regulated in neurons and astrocytes in human TLE+HS (Desjardins *et al.*, 2003; Holtman *et al.*, 2010).

Experimental studies

Experimental animal models of epilepsy have helped to address the crucial question of whether the complex and sustained inflammatory response, described in resected brain tissue from patients with refractory focal epilepsy,

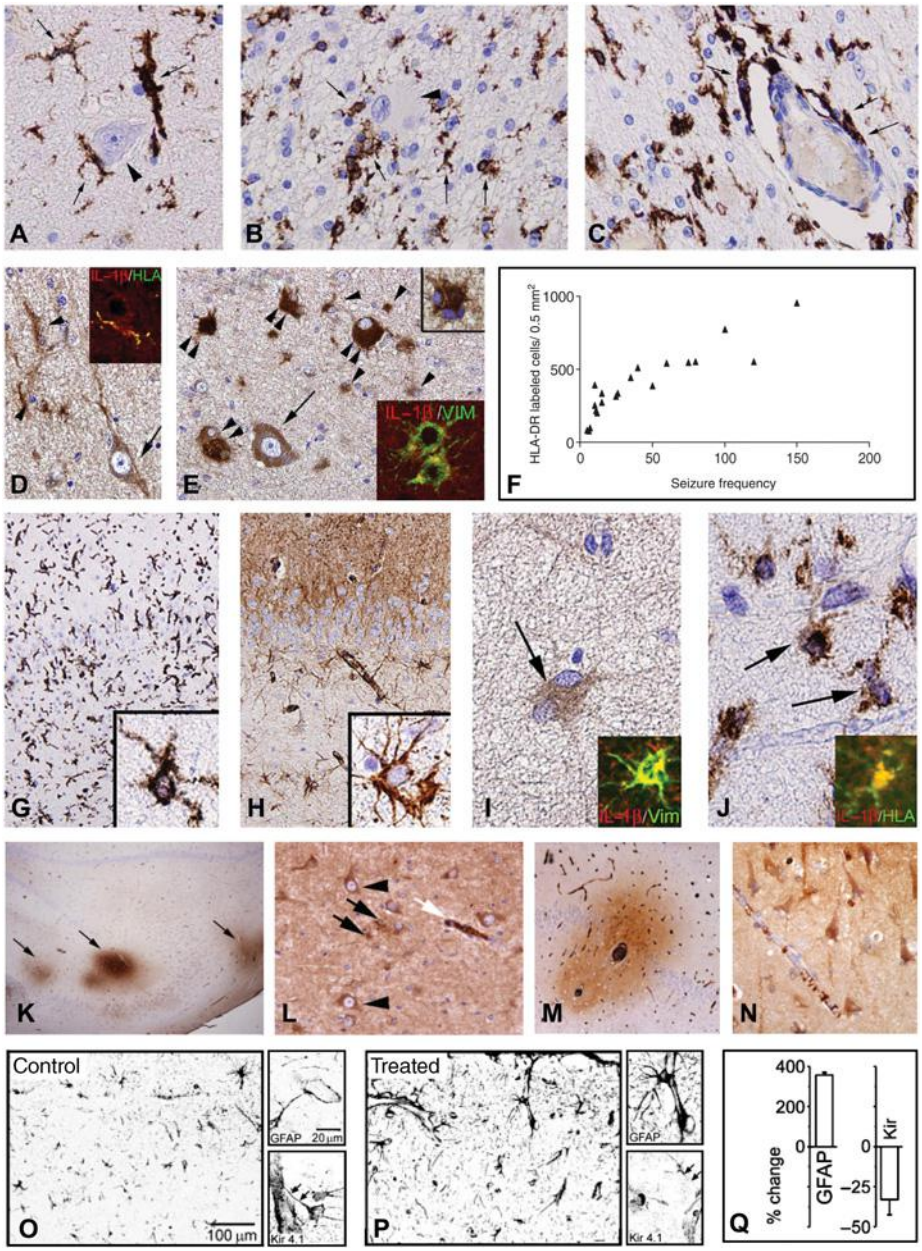

Figure 12.2 (*Continued*)

occurs as a cause of the underlying cellular pathology, as a consequence of seizures or both. In this context, experimental studies have demonstrated that recurrent seizures activate innate immunity in areas of seizure generation and generalization; in turn, the pre-existence of an inflammatory state in the brain induced by prototypical inflammatory molecules such as lipopolysaccaride or polyinosinic–polycytidylic acid (activating innate immunity via TLR4 and TLR3, respectively) can promote seizure activity and decrease seizure threshold (Riazi *et al.*, 2010). The experimental studies were also instrumental in showing that the inflammatory molecules described in human epilepsy tissue play an active role in seizure mechanisms.

Seizure-induced brain inflammation Brain inflammation that is rapidly induced (<30 min) by an experimental epileptogenic injury (e.g. status epilepticus, traumatic brain injury, stroke or infection) outlasts the primary insult by days, and it is inefficiently opposed by endogenous anti-inflammatory mechanisms (Aronica and Gorter 2007; Bartfai *et al.*, 2007; Vezzani *et al.*, 2011a). Following a convulsive challenge provoked by administration of chemoconvulsants, but also after a brain injury which predisposes to epilepsy development, cytokines, prostaglandins, complement cascade and other downstream inflammatory molecules are induced, together with their cognate receptors, in neurons and in activated glial cells, as well as in endothelial cells of the BBB.

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Figure 12.2 Panels A–E depict the distribution of microglia (A–D) and astrocytes (E) in focal cortical dysplasia and the production of IL1β in cellular elements within the dysplastic cortex (FCD type IIB) (D, E). Activated and ameboid HLA-DR-positive microglia (arrows) clustered around dysplastic neurons (arrowhead in A), balloon cells (arrows in B) and blood vessels (arrows in C) (adapted from Boer *et al.*, 2006). Panel D depicts IL1β-positive microglia (arrowheads; colocalization between HLA-DR and IL1β in inset) clustered around IL1β-positive neurons (arrow). Panel E shows IL1β immunoreactivity in large dysplastic neurons (single arrow), astrocytes (single arrowheads and insets) and balloon cells (double arrowheads) within the dysplastic cortex (adapted from Ravizza *et al.*, 2006). Panel F shows the significant positive correlation between HLA-DR cell counts and seizure frequency in patients (seizures/month) (adapted from Boer *et al.*, 2006). Panels G–J depict the distribution of microglia (G) and astrocytes (H) and their IL1β production in the hippocampus of TLE patients with HS. Panel G shows strong HLA-DR immunoreactivity in activated microglia (inset); panel H shows increased vimentin (vim) immunoreactivity in reactive astrocytes (inset). Panels I and J depict IL1β immunoreactivity (arrows) in microglia and astrocytes, respectively (adapted from Ravizza *et al.*, 2008). Panels K–N show serum protein extravasation in brain parenchyma in TLE with HS. Panel K depicts albumin immunoreactivity in the hippocampus around blood vessels (arrows); panel L shows albumin uptake in neurons (arrowheads) and in astrocytes (black arrows); white arrows depict albumin in a blood vessel (adapted from van Vliet *et al.*, 2007). Panels M and N depict IgG extravasation around a blood vessel and its uptake by neurons, respectively (adapted from Rigau *et al.*, 2007). Panels O–Q illustrate the reduction of Kir4.1 in activated GFAP-positive astrocytes in rat cortex exposed for 24 h to serum albumin (treated, P) versus control tissue (O) and their respective mRNA percentage changes compared to non-treated tissue (Q) (adapted from Ivens *et al.*, 2007).

These changes occur in the brain areas of focal injury, and in those affected by ongoing seizures. In the last decade, increasing knowledge of the mechanisms underlying seizures have shown that microglia and astrocytes significantly contribute to neuronal network dysfunctions (Fellin and Haydon 2005; Seifert *et al.*, 2006; Halassa *et al.*, 2007; Hanisch and Kettenmann, 2007).

Recently, the crucial role played by pro-inflammatory molecules synthesized and released by activated glial cells in the mechanisms of seizures has gained increasing recognition (Aronica *et al.*, 2012). It is now well established that, in addition to their involvement as effector molecules of immune activation following infections, pro-inflammatory molecules released by glia and neurons following various brain injuries may exert either direct or indirect neuromodulatory effects. The possibility that these molecules may contribute to aberrant neuronal excitability underlying seizure recurrence is a novel concept supported by clinical and experimental findings. The extent of brain inflammation in neurons and glia, as well as the activation of microglia in seizure-prone areas, positively correlates with the frequency of seizures and the severity of neuropathology both in animal models and in epilepsy patients (Boer *et al.*, 2006; Ravizza *et al.*, 2006; Iyer *et al.*, 2010), showing a strict association between these events. Inflammatory molecules released by brain resident cells during seizures or following a brain injury may activate target cells (e.g. neurons, glia or endothelial cells of the BBB) to produce pathological effects. In particular, specific inflammatory pathways such as those activated by IL1β, transforming growth factor beta (TGFβ) or the complement cascade contribute not only to the recurrence of seizures but also to the precipitation of the first seizure. This was shown by pharmacological intervention to facilitate or block these pathways with concomitant proconvulsive or anticonvulsive effects, respectively. Pharmacological evidence that blockade of specific pro-inflammatory pathways affords anticonvulsive activity was supported by evidence showing decreased intrinsic seizure susceptibility in animals with genetic inactivation of the same inflammatory pathways (Vezzani *et al.*, 2011a, 2013).

Pre-existing inflammation and seizure susceptibility Notably, experimental induction of a pro-inflammatory state in rat or mouse brain by activating TLR3 or TLR4 mediates a long-term increase in brain excitability, and favours seizure precipitation and excitotoxicity (Riazi *et al.*, 2010). This study supports the concept that brain inflammation *per se*, before the onset of seizures, may contribute to excitability changes in brain tissue that are pivotal for epilepsy development (Bartfai *et al.*, 2007; Pitkanen and Lukasiuk, 2011). Infection and fever, which are conditions associated with the activation of TLRs and rapid induction of pro-inflammatory molecules by cells of the innate immune system, are recognized precipitating factors of seizures (Singh *et al.*, 2008). Therefore, common molecules and signalling pathways activated by either pathogens or brain injuries might contribute via converging innate immune mechanisms to the development of a chronic hyper-excitable neuronal

network. In this context, the induction of IL1 receptor (IL1R)–TLR receptor signalling, which is pivotal for activation of innate immunity and inflammation following infections, is also induced by endogenous molecules released from injured cells (i.e. 'danger signals') such as HMGB1. This protein has proconvulsive properties (Maroso *et al.*, 2010; Vezzani *et al.*, 2011b). The activation of the IL1R–TLR signalling by IL1β and HMGB1 released following epileptogenic events occurs before seizure onset, but is also activated by recurrent seizures, thus establishing a pathologic vicious circle. Antagonism of the IL1–TLR pathway affords significant anticonvulsive activity and delays seizure onset in various animal models (Vezzani *et al.*, 2011b; Aronica *et al.*, 2012).

Blood–brain barrier and inflammation Opening of the BBB has been suggested to be a crucial player in the generation of neuronal hyper-excitability (Figure 12.2). A positive correlation between the extent of BBB opening and the number of spontaneous seizures was shown in epileptic rats (van Vliet *et al.*, 2007). Moreover, focal opening of the BBB in the rat neocortex resulted in the delayed development of paroxysmal hyper-synchronous activity (Seiffert *et al.*, 2004; Ivens *et al.*, 2007). This epileptogenic process was recapitulated by exposing the rat brain cortex to serum albumin. It was demonstrated that extravasation of serum albumin into the brain following local BBB breakdown activates a TGFβ receptor type 2 in astrocytes promoting local inflammation and astrocytic dysfunction (Cacheaux *et al.*, 2009). In particular, albumin provokes transcriptional down-regulation of Kir4.1 potassium channels and of the glutamate transporter (Seiffert *et al.*, 2004; Ivens *et al.*, 2007; David *et al.*, 2009; Friedman *et al.*, 2009) in astroctyes (Figure 12.2). The resulting higher extracellular K^+ and glutamate facilitate seizure precipitation and recurrence. Notably, inflammatory molecules produced and released by perivascular glia have a prominent role in BBB breakdown by provoking the down-regulation of tight junctions on microvascular endothelial cells (Morin-Brureau *et al.*, 2011; Librizzi *et al.*, 2012).

Anti-inflammatory drugs and epileptogenesis To address the role of specific inflammatory molecules in epilepsy development after a primary brain injury, non-steroidal anti-inflammatory drugs, immunosuppressant molecules, and drugs inhibiting glia activation were administered to experimental animals after induction of status epilepticus. These post-injury treatments decreased the recurrence of spontaneous seizures in epileptic rats and ameliorated the associated neuropathology without interfering with the onset of the disease (Ravizza *et al.*, 2011). Cognitive dysfunction and depression-like behaviours were also reduced in some instances (Mazarati *et al.*, 2010, 2011; Riazi *et al.*, 2010; Pineda *et al.*, 2012).

In summary, increasing experimental evidence suggests that pharmacological targeting of brain-borne inflammatory molecules may be a promising strategy for seizure control and possibly to modify the disease course after

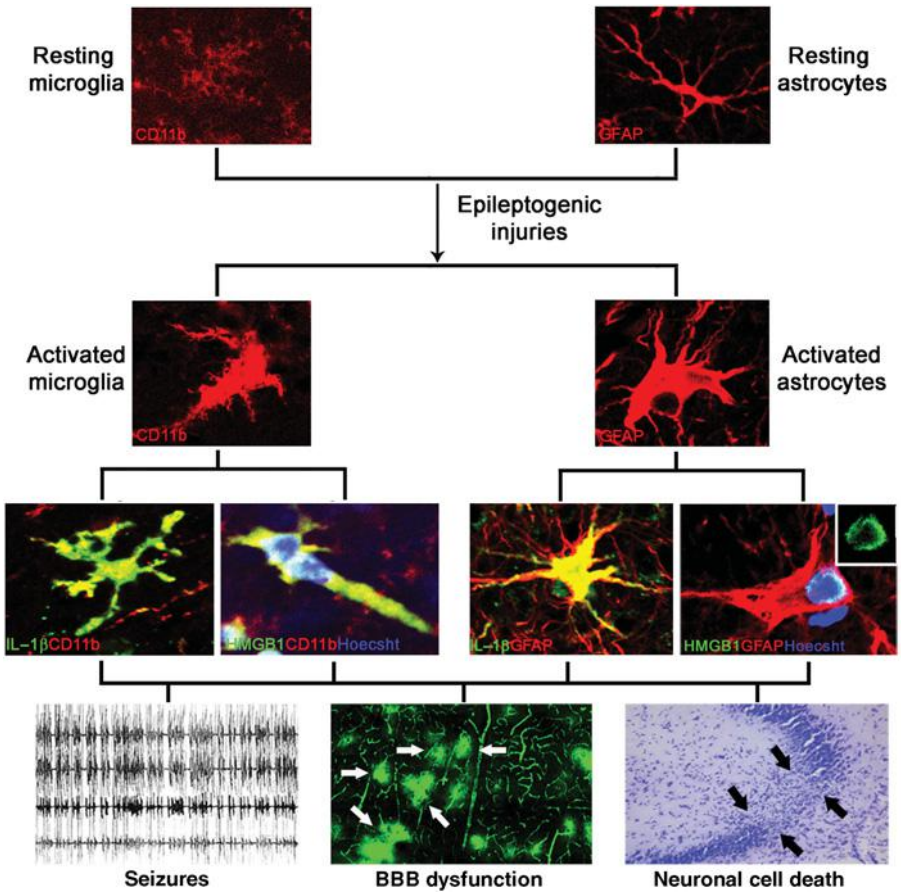

Figure 12.3 Functional consequences of the production of inflammatory mediators by microglia and astrocytes. Epileptogenic brain injuries activate glial cells which in turn release inflammatory mediators such as IL1β and HMGB1, triggering brain inflammation. This event leads to changes in brain physiology provoking neuronal hyper-excitability, blood–brain barrier dysfunction and cell damage that contribute to lower seizure threshold and trigger epileptogenesis.

a challenging event (Figure 12.3). Additional studies are required to further characterize the detrimental inflammatory signals induced by brain injury and seizures, and to understand which treatments given before epilepsy onset in individuals at risk could potentially arrest the disease progression.

Adaptive immunity in epilepsy

Human epilepsy

There are two major direct mechanisms by which the adaptive immune system can play a role in epilepsy or epileptic disorders: antibody-mediated

mechanisms and T cell cytotoxicity. Notably, immunohistochemical investigations of brain tissue from drug-resistant focal-onset epilepsies demonstrated albumin and IgG extravasation in the brain parenchyma, indicating BBB damage. These macromolecules have been found both extracellularly and within glial cells and neurons, suggesting a possible role in cell dysfunction and immune activation (Rigau *et al.*, 2007; van Vliet *et al.*, 2007; Ravizza *et al.*, 2008). Infiltration of immunoglobulins and T lymphocytes in the brain can be found in a variety of encephalitis, including Rasmussen's encephalitis (RE), a variety of viral encephalitis and a group of encephalitides which are defined by the presence of specific neural antibodies in serum and/or CSF. The latter, so-called antibody-associated encephalitis, is most commonly non-paraneoplastic but can also be paraneoplastic.

Rasmussen's encephalitis For a long time, it was thought that antibody-mediated mechanisms played an important role in Rasmussen's encephalitis (RE). RE can be modelled in rabbits following immunization with recombinant glutamate receptor type-3 (GluR3). Animals developed severe seizures and had inflammatory histopathological changes in their brains, similar to those found in patients with RE. Furthermore, antibodies to GluR3 were detected in the sera of some RE patients, and plasma exchange in one child significantly reduced the serum titres of GluR3 autoantibodies, decreased seizure frequency and improved neurologic function (Rogers *et al.*, 1994). However, the pathological significance of anti-GluR3 antibodies diminished when it was shown that these antibodies were only infrequently found in RE or intractable epilepsies (Wiendl *et al.*, 2001; Mantegazza *et al.*, 2002), and that few patients improved clinically after plasmapheresis (Andrews *et al.*, 1996; Granata *et al.*, 2003). Besides anti-GluR3 antibodies, antibodies against other antigens such as the alpha7 acetylcholine receptor or Munc-18-1 have been reported in RE sera (Watson *et al.*, 2004; Alvarez-Baron *et al.*, 2008). Again, these antibodies are found in only a few patients, and thus the pathophysiological relevance of these autoantibodies is questionable. Nevertheless, the finding of autoantibodies with multiple specificities in RE patients raises the question as to how these antibodies are generated in the first instance. In RE and other neurodegenerative disorders, a wide spectrum of anti-neural antibodies may result from a bystander or secondary immune response against antigens released during neurodegeneration in the CNS (Figure 12.1). Other studies of the immune response in brains of RE patients suggest that cytotoxic T lymphocytes play a role in RE. Most of the inflammatory T cells in the parenchyma are $CD8^+$, and around 10% of these cells are granzyme B positive (GrB^+) cytotoxic T cells. Such GrB^+ cytotoxic cells can be found in apposition to neurons and astrocytes, with polarization of the cytotoxic granules facing the target cell membrane (Bien *et al.*, 2002; Bauer *et al.*, 2007) (Figure 12.1). Furthermore, spectra typing of the T cells from brain lesions from RE patients shows that these cells expanded from discrete epitope-recognizing precursor T cells and

likely are specific for single brain antigens (Li *et al.*, 1997; Schwab *et al.*, 2009). MRI studies suggest that inflammatory activity in RE may occur outside of the epileptic network. Although still speculative, this evidence suggests that the epileptic activity may not be directly associated with the presence of infiltrating T cells (Hauf *et al.*, 2009). Furthermore, the immunosuppressive drug tacrolimus has a positive effect on conservation of motor and cognitive functions and on degeneration of brain tissue, but it shows no effect on seizure frequency (Bien *et al.*, 2004).

Viral encephalitis The second group of encephalitis is caused by infection of the CNS by viruses. Not all viral encephalitides lead to the induction of seizures. The type of seizures is dependent on the type of virus and the severity and location of inflammation. An example of such a viral encephalitis with prominent seizures is herpes simplex virus encephalitis (HSVE). Infection often occurs in the hippocampus and temporal lobes, which is probably the reason for the seizures. HSVE leads to a strong inflammatory response with infiltration of large numbers of cytotoxic T cells, and trials using steroids as adjuncts to antiviral therapy are on the horizon.

Antibody-associated encephalitis A third group of encephalitis with prominent seizures is the antibody-associated encephalitis. This condition often has a predilection for the limbic system (limbic encephalitis (LE)), although multiple areas of the CNS may often be involved. In recent years, it has become clear that the neurological features are associated with the antigenic specificity of the autoantibodies, rather than the paraneoplastic or non-paraneoplastic nature of the underlying disease. While there is strong clinical evidence, and emerging *in vitro* data, for the pathogenicity of these antibodies, the absence of definitive proof means that we will refer to these as antibody *associated* rather than antibody *induced*. The antibodies can be divided into those that are directed against intracellular targets (e.g. glutamic acid decarboxylase (GAD)65, amphiphysin, Hu, Yo and Ma2) and antibodies directed to surface antigens such as the VGKC complex, various glutamate receptors (NMDA or AMPA receptors (NMDARs and AMPARs, respectively) or metabotropic glutamate receptor subunits) and the GABA-B receptor (Table 12.1). It is believed, and supported by negative passive transfer experiments, that antibodies which target intracellular antigens themselves are not pathogenic because they cannot access the intracellular antigens within intact neurons (Vincent *et al.*, 1999). The situation appears to be different in the encephalitis with antibodies against surface receptors: we will focus on the VGKC complex and NMDA receptor antibodies as examples where a pathogenic role for these antibodies is likely.

VGKC complex antibodies VGKC complex-antibody encephalitis can be found in both paraneoplastic (Buckley *et al.*, 2001) and non-paraneoplastic

Table 12.1 Autoantibodies in seizure-associated diseases (Modified from Irani SR, Bien CG, Lang B (2011). Autoimmune Epilepsies. Current Opinion in Neurology 24, 146–153, with permission (Lippincott, Williams & Wilkins).)

Syndrome	Antibody	Patients with seizures (%)	Seizure details	Comment	Response to treatment	
					Antiepileptic drugs (AED)	Immunotherapy
VGKC complex antibody limbic encephalitis	VGKC complex (LGI1 > CASPR2)	58–90	Varied: generalized, complex partial, temporal and extra-temporal, simple motor all described	Seizures occur with or without cognitive problems	Partial response	Generally good
Faciobrachial dystonic seizures (FBDS)	VGKC complex (LGI1)	100	Very frequent, brief episodes of uni- or bilateral arm posturing with facial grimacing	Often progresses to amnesia which characterizes limbic encephalitis	Poor, often with marked side effects	If prompt, good response and may prevent progression to limbic encephalitis
NMDAR encephalitis	NMDAR (NR1 subunit)	72–100	Generalized or partial complex seizures	Can be associated with ovarian teratoma	Partial	Slow response; prolonged care needed
GAD-Ab limbic encephalitis	GAD65	100	Temporal lobe seizures with cognitive disturbances		Generally poor response, with few exceptions	Generally poor response, with few exceptions
AMPAR encephalitis	GluR1/2 (mostly GluR2)	40	Focal motor or generalized tonic-clonic seizures	Tumour associated in 70%		Treatment responsive, but relapses frequent
$GABA_BR$ encephalitis	GABA receptor (β1 subunit)	100	Often a temporal-lobe onset with secondary generalization; some progress to status epilepticus	50–80% of patients have an associated tumour, often with additional autoantibodies		Good response to immunotherapy and cancer removal (if found)

cases (Thieben *et al.*, 2004; Vincent *et al.*, 2004). It has recently been shown that these antibodies are predominantly directed to proteins which are associated in the VGKC complex rather than the VGKC molecule itself. Three different target proteins have so far been recognized: contactin-associated protein-like-2 (Caspr2) (Irani *et al.*, 2010a, 2012; Vincent and Irani, 2010); leucine-rich, glioma-inactivated-1 (LGI1) (Irani *et al.*, 2010a; Lai *et al.*, 2010); and contactin-2 (Irani *et al.*, 2010a). In most cases with encephalitis or seizures, the antibodies are directed against LGI1 (Irani *et al.*, 2010a; Lai *et al.*, 2010). Clinically, these patients present with memory loss, confusion, behavioural changes and often prominent seizures (Thieben *et al.*, 2004; Vincent *et al.*, 2004; Chan *et al.*, 2007). Most of these patients do not have an underlying tumour. A useful serum marker appears to be the presence of a hyponatraemia. MRI typically shows high signal in the temporal lobes, but an increasing number of patients are being diagnosed with normal imaging.

Importantly, steroids, I*v*Ig and plasmapheresis have been found to improve the neurological deficits. This suggests that these antibodies are directly pathogenic and responsible for the clinical presentation (Vincent *et al.*, 2004; Wong *et al.*, 2010). One piece of evidence in support of this argument is improvement after plasma exchange, although this procedure may also remove pathogenic non-IgG serum factors such as interleukins and complement factors. Furthermore, as patients are often administered multiple immunotherapies at the same time, the role of each individual immunotherapy is currently unclear. However, available evidence suggests that steroids appear to play a major role in the clinical improvements (Vincent *et al.*, 2004). Cyclophosphamide has also been used in a few patients, but its toxicity in comparison to steroids plus I*v*Ig or plasma exchange probably limits its more widespread administration (Wong *et al.*, 2010).

Most recently, a highly distinctive seizure semiology has been observed in a subset of these cases (Irani *et al.*, 2008, 2011; Barajas *et al.*, 2009). These seizures typically involve short episodes of dystonic arm posturing with facial grimacing and have been termed 'faciobrachial dystonic seizures' (FBDS) to emphasize the features which should prompt their clinical recognition. They are typically very frequent (a median of 50 per day) and are often exquisitely immunotherapy responsive. By contrast, FBDS tend to respond poorly to AEDs (Irani *et al.*, 2011). Interestingly, in around 75% of cases, FBDS appear to predate the onset of amnesia which characterizes the 'full-blown' encephalitis (Irani *et al.*, 2011). Therefore, FBDS are an immunotherapy-responsive prodrome to the VGKC complex antibody, LE, and it is possible that their treatment may prevent the onset of amnesia.

Pathological investigation of the cases with VGKC complex antibodies revealed neuronal degeneration in the hippocampus (Bien *et al.*, 2012) and moderate numbers of infiltrating T cells (Vincent *et al.*, 2004; Dunstan and Winer 2006; Park *et al.*, 2007; Khan *et al.*, 2009; Bien *et al.*, 2012). These degenerating hippocampi show the presence of human IgG as well as deposition of C9neo, the end complex of complement activation (Bien *et al.*, 2012). This

indicates that antibody-mediated complement activation may be responsible for neuronal cell death in these patients.

NMDAR antibodies The clinical syndrome of NMDAR antibody encephalitis is associated with antibodies against the NR1 subunit of the NMDAR (Dalmau *et al.*, 2008). Like VGKC complex antibody encephalitis, NMDAR antibody encephalitis can be both paraneoplastic as well as non-paraneoplastic in nature (Dalmau *et al.*, 2008; Irani *et al.*, 2010b). The syndrome was first described in young females often with an underlying ovarian teratoma (Dalmau *et al.*, 2011), although it is increasingly recognized in males and older females (Irani *et al.*, 2010b; Gabilondo *et al.*, 2011). Clinically, a prodromal stage with symptoms such as fever, nausea, vomiting or diarrhoea can occur (Iizuka *et al.*, 2008). After this, patients develop seizures, (partial) status epilepticus, short-term memory loss and, in addition, psychiatric symptoms such as anxiety, fear, mania and paranoia. Within weeks, this is followed by a dramatic movement disorder, decrease in consciousness and autonomic manifestations such as tachy- or bradycardia (Dalmau *et al.*, 2007, 2011). Remarkably, however, patients may, despite these severe clinical signs, recover significantly, often after prolonged courses of immunotherapy. An algorithm for immunotherapy administration to these patients has recently been suggested and involves the use of second-line treatments such as rituximab (anti-CD20 B cell–depleting antibody) and cyclophosphamide when first-line therapies, as used in VGKC complex antibody LE, have shown limited benefit (Dalmau *et al.*, 2011). In paraneoplastic patients, tumour resection plays an important role in clinical improvement (Dalmau *et al.*, 2008; Irani *et al.*, 2010b), and this surgical intervention may reduce the requirement for a second-line immunotherapy (Dalmau *et al.*, 2011).

The brains of NMDAR antibody–positive patients show few inflammatory cells (mostly T cells) (Tuzun *et al.*, 2009; Camdessanche *et al.*, 2011; Martinez-Hernandez *et al.*, 2011; Bien *et al.*, 2012). This syndrome therefore may be best referred to as an 'encephalopathy' rather than encephalitis. Moreover, MRI and pathological studies show that the neuronal loss in these patients in most cases is remarkably mild (Dalmau *et al.*, 2007; Iizuka *et al.*, 2010; Bien *et al.*, 2012). Few publications have analysed the pathogenicity of these antibodies. IgG binding to hippocampal neurons has been shown in the absence of the complement C9neo complex deposition (Tuzun *et al.*, 2009; Martinez-Hernandez *et al.*, 2011; Bien *et al.*, 2012). In the hippocampus, however, a decrease in NMDAR is found; thus, it is suggested that the NMDAR antibodies may cross-link and internalize the NMDAR receptors. This may lead to a reduction of surface-expressed NMDAR and to a state of reversible NMDAR hypofunction (Hughes *et al.*, 2010).

Paraneoplastic encephalitides Evidence for a role of cytotoxic T lymphocytes is best documented in paraneoplastic encephalitis (PE) cases with antibodies against intracellular antigens. In neuropathological studies of PE, with

anti-Hu, anti-Yo or anti-Ma antibodies, the presence of CD8-positive T cells in the CNS infiltrates has been shown (Graus *et al.*, 1990; Posner 1991; Jean *et al.*, 1994; Verschuuren *et al.*, 1996; Giometto *et al.*, 1997; Dalmau *et al.*, 1999; Bien *et al.*, 2012). In addition, in the vicinity of neurons, these T cells possess and release cytotoxic granules, suggesting that these T lymphocytes, like in RE (Bien *et al.*, 2002), play a role in the causation of neuronal cell death (Tanaka *et al.*, 1999; Bernal *et al.*, 2002; Blumenthal *et al.*, 2006; Bien *et al.*, 2012). Although these T cells are found targeting neurons, no studies have yet shown the target antigens of these cytotoxic T lymphocytes. Most likely, however, these T cells target the same protein to which the antibodies are generated. Evidence for this comes from studies which show that cytotoxic lymphocytes from a PE patient with anti-Yo antibodies could lyse Yo protein-expressing fibroblasts (Tanaka *et al.*, 1998).

Autoantibodies in idiopathic epilepsies Although autoantibodies are unlikely to be a major cause in idiopathic epilepsy, an increasing number of studies have detected autoantibodies in the sera of patients with idiopathic epilepsy. Serum autoantibodies to components of the VGKC complex have been detected in approximately 7% of large cohorts of unselected patients with epilepsy (McKnight *et al.*, 2005; Majoie *et al.*, 2006). However, the pathological relevance of these antibodies is yet to be determined, given that most of them do not show specificity for LGI1, Caspr2 or contactin-2 (Lang *et al.*, unpublished).

Additionally, high levels of antibodies to GAD are found in around 2% of patients with focal drug-resistant epilepsies. These antibodies have also been found in 6% of children with myoclonic epilepsy (Aykutlu *et al.*, 2005), and two recent reports described high titres in 2–6% of epilepsy patients, mainly with TLE (Errichiello *et al.*, 2009; Liimatainen *et al.*, 2009). In a much larger study, Malter *et al.* (2010) investigated a large cohort (n = 138) of recent-onset epilepsy patients, 53 of whom fulfilled the criteria for LE. Antibodies to VGKC complex antibodies were detected in 10 patients, and GAD antibodies in nine patients. The GAD antibody–positive patients were more resistant to immunotherapy and AED treatments than patients with antibodies to VGKC complex and so represent a non-paraneoplastic chronic form of the disorder which should be included in the differential diagnosis of TLE (Malter *et al.*, 2010). Immunotherapies offered to these patients have sometimes resulted in benefit (Giometto *et al.*, 1998) and are probably still worth pursuing, but their overall efficacy is often disappointing, particularly in comparison to patients with LGI1 antibodies.

Experimental studies

Pathological relevance of autoantibodies in epilepsy Autoantibodies to a range of neuronal channels, receptors and accessory proteins have been

described in NMDAR-associated LE, in FBDS and in patients in whom epilepsy is the primary diagnosis. In many of these disorders, the presence of the specific antibodies is now a diagnostic criterion. A pathogenic role for these antibodies is suggested by the clinical improvement of the patients on immunomodulatory therapy and seen in the inverse relationship between antibody titre and clinical status. However, the precise mechanism of antibody action is still largely unknown.

Antibodies to glutamate receptors A number of experimental studies have shown modulation of the target antigen by antibodies to NMDA receptors. Hughes *et al.* (2010) demonstrated that infusion of NMDAR antibodies into the rodent brain caused a down-regulation of NMDARs *in vivo*, whilst incubation of hippocampal neurones in CSF or purified NR1-IgG from patients with NMDAR encephalitis caused a significant time-dependent reduction in NMDAR density compared to the equivalent control. This effect was also dependent on the titre of the antibodies. Moreover, the application of NR1 antibodies caused a concomitant reduction in the levels, both surface and total, of the NR2A/B–NMDA receptor subunits. Specifically, this down-regulation appeared to dramatically reduce the synaptic localization of NMDARs on the hippocampal neurones. However, in this model system, the effect was reversible within 4 days after removal of the patient's antibodies. Other neuronal proteins such as PSD95 or GluR1 were unaffected. This effect is considered to be due to cross-linking and down-regulation solely of the NMDAR as Fab fragments alone could not reproduce the internalization (Hughes *et al.*, 2010). Direct effects of these antibodies on minimal evoked excitatory postsynaptic currents were also shown using whole-cell patch clamp studies on hippocampal neurones. Similar results were shown using sera and CSF from patients with antibodies to AMPARs. Application to cultured hippocampal neurones resulted in a significant reduction in the number and density of the AMPA receptors on the cell surface (Lai *et al.*, 2009).

Antibodies to VGKC complex Lalic *et al.* (2010) used whole-cell patch clamp recordings of visually identified CA3 pyramidal cells in rat hippocampal slices to investigate the action of anti–VGKC complex antibodies from an LE patient. Synaptic stimulation of CA3 neurons incubated in IgG from LE patients induced epileptiform activity, increased the tonic firing rate and strengthened the mossy fibre–evoked synaptic responses. Additionally, the failure of evoked excitatory postsynaptic currents (EPSCs) was significantly lower in the presence of the LE IgG compared to the control IgG. These results, which can be mimicked by incubation in α-dendrotoxin, an antagonist of the Shaker mouse VGKC channel, suggest that the antibodies increase the release probability and hence cell excitability in the CA3 neurones and, by down-regulating the VGKC complexes, produce prolongation of the synaptic events and hyper-excitability.

Recently, it was shown that cats too can develop an epileptic disorder characterized by orofacial seizures (Pakozdy *et al.*, 2011) associated with antibodies against the LGI1 component of the VGKC complex (Pakozdy *et al.*, 2013). Pathologically, these cats show severe hippocampal degeneration and the presence of C9neo complement deposition on neurons (Klang *et al.*, in preparation) almost identical to that seen in humans with VGKC complex antibody encephalitis (Bien *et al.*, 2012). This set of data also suggests that genetic or environmental factors are unlikely to be involved in this disorder.

Antibodies to GAD GAD is a cytoplasmic enzyme that is responsible for the synthesis of GABA. Low levels of antibodies are frequently found in patients who develop type 1 diabetes, but they are present at much higher levels in patients with a range of CNS disorders that include stiff-person syndrome, cerebellar ataxia, LE and TLE. The cytoplasmic location of GAD suggests that antibodies to GAD are unlikely to be pathogenic, but may represent a biomarker for an underlying immunological disorder. In patients with anti-GAD antibodies, the levels of serum antibodies inversely correlate with cortical GABA levels as measured by magnetic resonance spectroscopy (Stagg *et al.*, 2010). Direct pathogenic activity has been demonstrated electrophysiologically on cultured hippocampal neurons using patch clamp techniques. Application of sera from GAD-positive epilepsy patients onto cultured hippocampal neurons increases the frequency of the post-synaptic inhibitory potentials, resulting in neuronal inhibition. Although this evidence does not necessarily demonstrate a pathogenic role for the anti-GAD antibodies, it supports the hypothesis of pathologically active components being present in patient sera (Vianello *et al.*, 2008).

Role of leukocytes in seizure models There is evidence for the involvement of peripheral immune cells in a number of forms of epilepsy. In experimental models of epilepsy, it appears that such a contribution differs depending on the nature of the epileptogenic trigger. In particular, perivascular extravasation of CD4+ and CD8+ lymphocytes has been reported in the hippocampus after prolonged seizures (i.e. status epilepticus) induced in mice by systemic injection of pilocarpine (Fabene *et al.*, 2008), or intrahippocampal administration of kainic acid (Zattoni *et al.*, 2011), two convulsant drugs priming cholinergic or glutamatergic neurotransmission, respectively. This phenomenon may simply reflect passive cell extravasation due to leakage at the BBB, induced by injury or seizures. Alternatively, lymphocytes may be primed to pass into the perivascular space by parenchymal or vessel inflammation induced by cell injury or seizures. Up-regulation of adhesion molecules on endothelial cells of brain microvessels can be induced by cytokines released from perivascular astrocytes and microglia (Librizzi *et al.*, 2007, 2012), thus favouring interactions with complementary receptors on circulating leukocytes. Active brain extravasation of these cells may contribute to alteration of the BBB

permeability properties (Fabene *et al.*, 2008). The presence of circulating antigens that may activate T cells has not been demonstrated as yet. One might envisage that during brain injury and BBB breakdown, brain-borne molecules released by damaged or activated neurons or glial cells may drain out of the brain into the systemic circulation. If these molecules express molecular mimicry of pathogens (e.g. formylated peptides released from damaged mitochondria), they may activate adaptive immunity as occurs during an infection. Whatever the mechanism involved, the crucial question is whether this phenomenon has some relevance for tissue hyper-excitability or neuropathology. Two divergent sets of data have been presented in mouse models of TLE. In pilocarpine-treated mice, leukocytes (i.e. neurotrophils, macrophages and T cells) appear to play a detrimental role in the post-injury phase (i.e. epileptogenesis) because mice lacking key adhesion molecules or treated with anti-integrin antibodies develop a milder form of epilepsy characterized by less recurrent seizures and decreased neuropathology as compared to control epileptic mice (Fabene *et al.*, 2008). Differently, in intracerebral kainic acid–treated mice, macrophages and T cells play a protective role by preventing neurotrophils from entering the brain tissue and by delaying the onset and reducing the recurrence of spontaneous seizures (Zattoni *et al.*, 2011). For a correct interpretation of these discrepant results, it is important to consider that pilocarpine has a primary mode of action involving muscarinic receptors expressed by leukocytes, whose activation induces the release of IL1β into the bloodstream (Marchi *et al.*, 2009). This is a required step for inducing BBB damage and the subsequent entry of pilocarpine into the brain at pathologically relevant concentrations for inducing seizures, which otherwise would not be reached (Vezzani and Janigro, 2009). This epilepsy model, therefore, uses a peculiar mechanism reminiscent of a systemic infection favouring seizure precipitation, as opposed to intracerebral kainic acid which more closely represents a sterile type of brain injury leading to spontaneous seizures. Additionally, as in human TLE epilepsy tissue, the extent of T cell infiltrates in rodent brain is very limited and mostly restricted to the perivascular space (Fabene *et al.*, 2008; Ravizza *et al.*, 2008; Marchi *et al.*, 2010).

Conclusions

Clinical and experimental studies show evidence of the presence of activated inflammatory cells (microglia, astrocytes and leukocytes), related inflammatory molecules (cytokines, danger signals, complement factors and COX2) and their cognate receptors in brain tissue specimens from epilepsies of differing aetiologies, as well as soluble mediators of inflammation (e.g. cytokines) in the CSF or blood of some of the affected individuals. These findings, together with the identification of subsets of autoantibodies in some forms of epilepsy or seizure disorders, highlight a pathogenic role of both innate and adaptive immunity in epilepsy. These findings are substantiated by clinical reports that

in some cases of drug-refractory seizures, anti-inflammatory or immunosuppressive treatments have anticonvulsive efficacy.

Both the innate and adaptive immune systems contribute to the inflammatory cascade demonstrated in epilepsy, although their relative contribution varies depending on the underlying epilepsy aetiology. While activation of innate immunity, chiefly involving glial cells, is commonly observed in epilepsy brain tissue surgically resected for therapeutic reasons from drug-resistant epilepsies, the presence of activated T cells, or circulating autoantibodies, is restricted to more specific cases.

What causes the inflammation and which are the pivotal triggering mechanisms of chronic inflammation in epilepsy are still open questions. Experimental findings, however, suggest that brain inflammation can predispose the patient to seizures, and in turn seizure recurrence can perpetuate brain inflammation (Figure 12.3). In this regard, there is as yet no evidence that the adaptive immune system can predispose to seizures directly; however, indirect mechanisms can be envisaged via (i) activation of the innate immune system, (ii) cytotoxicity to neurons leading to neurodegeneration and (iii) induction of BBB permeability. On the contrary, there is experimental evidence that innate immunity is directly involved in the generation of seizures by factors such as IL1β, HMGB1 and TLR activation, or COX2 and components of the complement system. Activation of immunity and inflammation represents an endogenous homeostatic mechanism instrumental for correct tissue function and physiology. The challenge is, therefore, to understand the key molecular events by which inflammatory cells and related molecules can compromise brain physiology and promote seizure generation. Therefore, further studies are required to understand the role of specific inflammatory pathways and cell types in different forms of epilepsy. Animal models will be instrumental to address these issues and to provide proof-of-concept evidence of anticonvulsive or neuroprotective effects of novel anti-inflammatory treatment, for controlling pharmaco-resistant seizures, and possibly delay or arrest the progression of the disease in individuals at risk.

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13

Inflammatory Mediators and Dysfunction of the Neurovascular Unit following Ischaemia Reperfusion

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Stroke is a highly complex process, involving cerebrovascular and parenchymal tissues through the interaction of multiple mechanisms. In the acute stage, the processes are dominated by calcium-mediated cell demise, activation of lipolytic pathways and alterations in protein phosphorylation related to the depletion of adenosine triphosphate (ATP) stores with depression of glucose metabolism (Siesjo, 1992a,b). These events are modified by the generation of free radicals, specifically nitric oxide generation, protein oxidation and lipid peroxidation (Siesjo, 1992b; Siesjo and Katsura, 1992; Siesjo *et al*., 1995). At the cellular level, there is an activation of cell-signalling pathways and decreased endothelial cell matrix expression. Associated with this cascade of events are major changes related to the blood–brain barrier (BBB), basal lamina degradation, the detachment of astrocyte end feet and the expression of matrix proteases (Hamann *et al*., 1996; Haring *et al*., 1996; Wagner *et al*., 1997). The combination of pro-inflammatory cytokine and endogenous tissue factors is known to contribute to endothelial cell adhesion receptor activation of polymorphonuclear leukocytes and monocytes, and increased adhesion and

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transmigration of polymorphonuclear (PMN) leukocytes and platelet activation, accompanied by increasing leakage of the BBB (Giulian *et al.*, 1989; Hallenbeck, 1996). Established tissue injury results in oedema formation, cellular swelling and a focal no reflow within the ischaemic regions and ongoing increase in microvascular permeability with leakage.

Focal ischaemia and early mechanisms of injury

Following focal stroke, the extent of the brain injury is ultimately dependent on the critical thresholds of reduced cerebral blood flow (CBF), duration of the ischaemic insult, tissue temperature, blood glucose level and other physiological variables (Barber *et al.*, 2004). Flow is dramatically diminished but usually persists at a low level in the microvasculature. Some neurons are thought to be more vulnerable to hypoxia and decreased CBF than other neurons, such as the CA1 cells in the hippocampus; this is termed 'selective vulnerability'. With prolonged reductions in CBF below 10 ml per 100 g of brain tissue per minute, cellular transport mechanisms and neurotransmitter systems fail, potentially toxic neurotransmitters are released (Rothman and Olney, 1986), free radicals and lipid peroxides are formed which further injure the cells (Kontos *et al.*, 1979; McCord, 1985), and potentially neurotoxic platelet-activating factor (PAF) is released from neurons (Lindsberg *et al.*, 1991). At this threshold, lack of oxygen inhibits the mitochondrial metabolism and activates the inefficient anaerobic metabolism of glucose, causing a local rise in lactate production and so a fall in pH, leading to intra- and extracellular acidosis. The energy-dependent functions of cell membranes to maintain ion homeostasis become progressively impaired. Potassium ions leak out of cells into the extracellular space, Na^+ and water enter cells (cytotoxic oedema) and Ca^{2+} enters the cell, where it impairs mitochondrial function and compromises intracellular membranes to control subsequent ion fluxes, leading to further cytotoxicity. This degree of ischaemia represents a 'threshold for loss of cellular ion homeostasis'. Mechanisms that give rise to ischaemic cell death occur via three major mediators: unregulated increases of Ca^{2+} concentration intracellularly, tissue acidosis, and nitric oxide and free radical production. Repeated waves of spreading depression further compromise the reduction in CBF. Additionally, the ischaemic brain injury is modulated by inflammation, by the induction of immediate early genes and later by apoptotic mechanisms (Dirnagl *et al.*, 1999).

Calcium ion homeostasis

Extracellular Ca^{2+} concentrations are several thousand times greater than its intracellular concentrations, and the mechanisms that control this gradient are energy dependent. Calcium enters the cell predominantly through two types of channels: voltage-controlled and receptor-operated channels. There

are several agonists that increase permeability to Ca^{2+} when bound to specific receptors, namely, excitatory amino acids (glutamate, nucleotides and cyclic nucleotides) (Siesjo, 1992a,b). The export of Ca^{2+} from neurons into the extracellular environment occurs via processes which are linked directly or indirectly to the utilization of energy (Kontos *et al.*, 1979; Lindsberg *et al.*, 1991). In the former category is the ATP-dependent calcium pump, and in the second is the Na^{+}–Ca^{++} exchanger that utilizes energy stored in the sodium electrochemical gradient. The exchanger can operate in either direction so that when the Na^{+} gradient falls, it can import rather than export Ca^{2+}. Substantial amounts of Ca^{2+} are stored within intracellular organelles, mainly the mitochondria and endoplasmic reticulum. Release of Ca^{2+} from mitochondria requires energy, which is either Na^{+} dependent or independent. The accumulation of Ca^{2+} in the endoplasmic reticulum requires energy and is mediated by an ATP-consuming pump (Barber *et al.*, 2004).

Calcium enters cells by ionotropic-operated calcium channels, which are predominately gated by glutamate receptors of the N-methyl-D-aspartate (NMDA) type and by voltage-sensitive calcium channels. When glutamate is released from presynaptic endings, it activates two types of ionotropic glutamate receptors, the NMDA type and the non-NMDA type, with the latter being selectively activated by amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). The AMPA receptor (AMPAR) gates a channel that is permeable to monovalent cations (Na^{+}, K^{+} and H^{+}), while the NMDA-gated channel is also permeable to Ca^{2+}. This channel is blocked by Mg^{2+}, which is released when the cell membrane depolarizes following AMPAR activation. Depolarization also allows Ca^{2+} to enter via voltage-sensitive calcium channels (Siesjo, 1992a).

The decline in CBF and the accompanying loss of oxygen supply result in impaired energy metabolism and measurable decreases in ATP and phosphocreatine. The combination of ATP breakdown and compensatory anaerobic glycolysis leads to cellular acidification. With energy depletion, the membrane potential is lost and neurones and glia depolarize (Katsura *et al.*, 1992). This triggers Ca^{2+} influx through voltage-sensitive Ca^{2+} channels which further depolarizes the membrane, and excitatory amino acids are released into the extracellular space. Energy-dependent processes such as glutamate re-uptake are impaired, which further increases extracellular glutamate, resulting in prolonged activation of membrane glutamate receptors and Ca^{2+} influx (Dirnagl *et al.*, 1999). Sodium and Cl^{-} enter the neuron via channels for monovalent ions (Tyson *et al.*, 1996). Water follows passively, and the ensuing oedema can affect the perfusion of regions surrounding the focally injured brain and have more remote effects that produce increased intracranial pressure and vascular compression. The consequences of the unregulated rise in intracellular cytoplasmic Ca^{2+} during ischaemia are linked with glutamate excitotoxicity to a number of biochemical processes that result in further detrimental injury to ischaemic brain tissue (Dirnagl *et al.*, 1999). Some of these events continue

irrespective of re-oxygenation, while others are provoked or triggered during reperfusion.

The activation of *lipolysis* produces an increase in free fatty acids (FFAs), which are potentially neurotoxic, and the oxidation of arachidonic acid (catalysed by cyclooxygenase and lypooxygenase) yields prostaglandins, leukotrienes and thromboxane A_2. These agents, together with PAF, can damage cellular membranes and act additionally as chemoattractants (Dirnagl *et al.*, 1999). The activation also produces oxygen free radicals. These reactions can produce sustained damage to the cell membranes and receptors, further accelerating the toxic effects of glutamate by impaired uptake.

Many of the enzymatic processes involved in the *phosphorylation* of proteins are Ca^{2+} mediated. An alteration in protein kinase and phosphatase activity during ischaemia provokes changes in ion membrane and receptor activity, and affects gene transcription and translation. The effect of these processes may perhaps explain the long-term effects of ischaemia on membrane function and integrity, facilitating free radical peroxidation as well as unmasking processes leading to programmed cell death (apoptosis).

During ischaemia, other Ca^{2+}-mediated reactions are accelerated such as *proteolysis*, and the assembly and reassembly of microtubules (Baudry and Vicaut, 1993). Cytoskeletal proteins are degraded when Ca^{2+} rises intracellularly. Several Ca^{2+}-dependent enzymes trigger reactions that produce reactive oxygen species (ROS). These events proceed during ischaemia if there is residual blood flow but are greatly accelerated during reperfusion. Some of the reactions involved result in the oxidation of arachidonic acid (by cyclooxygenase and lipooxygenase), the oxidation of xanthine and hypoxanthine to $O_2^{\bullet}$ and H_2O_2 (via xanthine oxidase) and those reactions leading to the formation of $NO^{\bullet}$ (by nitric oxide synthase). However, the vast majority of ROS are produced by the mitochondrial respiratory chain, and because mitochondria contain superoxide dismutase the result is the formation of $O_2^{\bullet}$ and H_2O_2.

Mitochondria have been increasingly implicated in the pathophysiology of ischaemic brain injury. Free radical–mediated disruption of the inner mitochondrial membrane and the oxidation of proteins that mediate electron transport impair their function. The mitochondrial membrane becomes leaky, and eventually mitochondria become overloaded with Ca^{2+}, resulting in impaired ATP production and ultimately energy and membrane failure (Budd and Nicholls, 1996). Cytochrome C is released from the mitochondria and has been suggested as a trigger for apoptosis (Dirnagl *et al.*, 1999).

Free radical formation

Free radicals are produced in small quantities in cells during aerobic metabolism. Mitochondria are the major producer of free radicals in biological systems. Superoxide ($O_2^{\bullet}$) is formed during the operation of complex I and complex II of the mitochondrial electron transport chain. However, they are

extremely toxic, causing damage to DNA, proteins, lipids and components of the extracellular matrix. Polyunsaturated fats and sulphur-containing amino acids that are found in high concentrations within the brain are particularly vulnerable. Free radical scavengers such as tocopherol and vitamin C enzymes, which metabolize free radicals (via superoxide dismutase), help maintain the balance physiologically.

During severe ischaemia, insufficient O_2 is available to accept electrons passed along the mitochondrial electron transport chain. Free radicals are also generated during ischaemia by the release of iron from ferritin stores within ischaemic neurones (Davalos *et al.*, 1994). As the cerebrospinal fluid has a low concentration of ferritin-binding proteins, much of the iron released from damaged brain cells remains unbound and is therefore available to catalyse the generation of radical hydroxyl ($OH^{\bullet}$), leading to iron-induced lipid peroxidation. This process is further compounded by the fact that the central nervous system is relatively depleted of superoxide dismutase, which scavenges $OH^{\bullet}$ and controls the release of iron from intracellular stores.

The commonest free radicals produced during cerebral ischaemia are $O_2^{\bullet}$ and $OH^{\bullet}$, and these, like other free radicals, react with and exacerbate the damage of proteins, DNA and lipids, particularly the fatty acid component of the cell membrane, causing changes in the permeability and integrity of the cell membranes (lipid peroxidation). It has been shown using salicylate trapping of $OH^{\bullet}$ that brain undergoing ischaemia reperfusion produces oxygen free radicals during the reperfusion phase after ischaemia (Cao *et al.*, 1988). Activated leucocytes produce oxygen free radicals which, during ischaemia, become localized on the brain microvessels through the action of P-selectin, exposing the microvascular endothelium to high levels of oxygen free radicals and causing oxidative damage to specific local sites. This contributes to the breakdown of the BBB and to brain oedema.

The ischaemic inflammatory response

Focal ischaemia is associated with differentiated cellular responses involving microglia, neurons, astrocytes and endothelial cells (Rothwell, 1997). The immediate inflammatory response is primarily coordinated by the resident microglia. These cells appear to be activated within seconds and minutes of the onset of ischaemia. Their activation promotes the release of the important inflammatory cytokines and chemokines, including tumour necrosis factor alpha (TNFα) and various interleukins. Central to the cellular propagation of the inflammatory response are Toll-like receptors (TLRs); these recognize endogenous interleukins termed damage-associated molecular patterns (DAMPs) (Arslan *et al.*, 2010; Iwata *et al.*, 2010). TLR2 and TLR4 have been specifically shown to be up-regulated in stroke and are essential components of the adaptive immune response following ischaemic injury. Neurons, within seconds and minutes of the onset of ischaemia, depolarize and become

overloaded with calcium in a process termed 'excitotoxicity'. They also produce free radicals, which are involved in DNA damage resulting in cellular necrosis. When injury is less severe, neurons may die by a process called programmed cell death (apoptosis), an energy-dependent process requiring ATP. Astrocytes swell and also depolarize, and their injury disturbs the normal homeostasis and buffer of excitatory amino acids; the end result is a propagation of the excitotoxic injury. The endothelium becomes activated by cytokines which up-regulate adhesion molecules which result in the attraction, adhesion, rolling and then diapedesis of peripheral inflammatory cells such as polymorphonuclear leukocytes. The endothelium is also involved in the matrix metalloproteinase of up-regulation that results in BBB disruption and eventual breakdown which can result in a spectrum of injury: in ascending order, these injuries are paracellular movement of water, protein extravasation and, in the extreme situation, red blood cells that are observed clinically as haemorrhagic transformation.

In addition, there is evidence for the production of cytokines by peripheral blood mononuclear cells, including monocytes, T lymphocytes, natural killer cells and PMN leukocytes, which can exacerbate CNS inflammation and gliosis. In support of this, peripheral irradiation or treatment with colchicine attenuates inflammation, wound healing and gliosis (Giulian *et al.*, 1989). The early accumulation of neutrophils in the ischaemic brain has been confirmed histologically (Hallenbeck *et al.*, 1986; Clark *et al.*, 1993; Dereski *et al.*, 1993; Liu *et al.*, 1993) by biochemical studies (Barone *et al.*, 1991, 1995), and in radiolabelled leukocyte studies (Pozzilli *et al.*, 1985; Dutka *et al.*, 1989; Hsu *et al.*, 1993). A rapid transient peripheral neutrophilia precedes brain infiltration (Chapman *et al.*, 2009), and the degree to which this peripheral response has been shown in humans correlates with infarct size (Huang *et al.*, 2006; Buck *et al.*, 2008).

Focal ischaemia is a very powerful stimulus to elicit genomic responses in the brain in the form of multiple early gene expression. In addition to the genes expressed for cytokines, TNFα, IL1β and many other inflammatory genes are expressed during focal ischaemia. Depending on the severity of the ischaemia and the intrinsic nature of the neuronal populations, it is thought that a stress response and changes in gene expression are elicited, which may be vital to cell survival and repair. Following focal ischaemia, immediate early genes are the first to be regulated, as shown by the identification of *c-fos*, *c-jun* and zinc finger genes, but this expression is only transient (Hsu *et al.*, 1993; Wang *et al.*, 1995). A further phase consists of heat shock protein expression, and increased cytokine gene expression, for TNFα and IL1β but also IL6 (Wang *et al.*, 1995) and IL1 receptor antagonist (IL1ra) (Wang *et al.*, 1997). Chemokines such as IL8 (Liu *et al.*, 1993), CXCL10 (IP10) and CCL2 (MCP1) (Wang *et al.*, 1998) are also elevated, and they play important roles in neutrophil and mononuclear cell infiltration, respectively. Subsequent gene expression involves the transcription of proteolytic enzymes

(metalloproteinases (MMPs)), which are implicated in the remodelling of the extracellular matrix (Rosenberg *et al.*, 1996; Romanic *et al.*, 1998; Wang *et al.*, 1998) and their endogenous proteinase inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (Cao *et al.*, 1988). This regenerative phase also involves the transcription of mediators such as transforming growth factor beta (Rosenberg *et al.*, 1996) and osteopontin (Ellison *et al.*, 1998), which appear to be important in tissue remodelling and in creating a barrier to protect the residual intact brain tissue.

The post-ischaemic inflammatory response can contribute to secondary brain injury in several ways. Under the influence of inflammatory mediators, the cerebral endothelial microvasculature becomes actively pro-thrombotic (Hallenbeck *et al.*, 1986). The rheologic effects of 'sticky' leukocytes in the blood interfere with microvascular perfusion. Platelets, fibrin deposition and PMN leukocytes can cause aggregates within the microvasculature (Dutka *et al.*, 1989. Leukotriene and prostaglandin synthesis may contribute to ischaemic injury by causing vasoconstriction and activating platelets and neutrophils, a process that can cause additional leukocyte activation by a 'vicious circle' phenomenon (Nishida and Markey, 1996). Infiltrating neutrophils produce inducible nitric oxide synthase (iNOS), an enzyme that produces toxic amounts of nitric oxide (NO), the pathogenic importance of which is reflected by the observation that pharmacological inhibitors of iNOS attenuate ischaemic damage (del Zoppo and Mabuchi, 2003; Iadecola, 2004).

The inflammatory process is also linked to apoptosis. It has been observed that antibodies against adhesion molecules attenuate ischaemic injury and reduce apoptotic cell death (Iadecola and Alexander, 2001). Central to this apoptotic cell death is nuclear factor kappa B **(**NF-κB), a well-characterized, ubiquitous and inducible transcription factor that plays an important role in inflammation, and also in apoptosis and the cell cycle (Baeuerle and Baltimore, 1996; Carroll *et al.*, 2000). In non-stimulated neuronal cells, the inhibitor (IκB) masks the nuclear transport signal by binding to NF-κB, forming a dimer. Upon activation, IκB is degraded by the proteasome complex, revealing the nuclear localization signal, and NF-κB translocates to the nucleus where it activates the transcription of target genes, including cell adhesion molecules, iNOS, cyclooxygenase-2 (COX2), p53, MnSOD and Bcl2.

In vivo, NF-κB is strongly induced in animal models of focal cerebral ischaemia (Coulter *et al.*, 1984; Salminen *et al.*, 1995; Carroll *et al.*, 2000). Different experiments suggest that NF-κB can promote either cell death or survival, depending on the paradigm. Mice lacking the NF-κB subunit p50 have significantly smaller lesions after focal cerebral ischaemia (Schneider *et al.*, 1999). Inhibition of NF-κB by a proteasome inhibitor (CVT-634) also reduces ischaemic damage (Buchan *et al.*, 2000). Both of these experiments support the idea that, overall, NF-κB is detrimental for neuronal survival. On the contrary, the capacity of NF-κB to promote expression of anti-apoptotic

genes such as Bcl2 and MnSOD suggests that NF-κB can also promote cell survival (Mattson *et al.*, 1997). Along these lines, overexpression of NF-κB *in vitro* renders neurons more resistant to glucose deprivation and glutamate excitotoxicity (Yu *et al.*, 1999). NF-κB also promotes expression of NAIP (nuclear apoptosis inhibitor protein), which renders neurons more resistant to ischaemia *in vivo* (Xu *et al.*, 1997; Stehlik *et al.*, 1998). NF-κB activates iNOS and COX2, and it plays a major role in later stages of inflammatory ischaemic brain injury (Iadecola and Alexander, 2001). The interplay between NF-κB-induced cell survival and death has important implications, and hence limitations, for disease-modifying approaches targeting NF-κB.

The neurovascular unit

While our understanding of the molecular and biochemical responses that ensue following cerebral ischaemia in individual cell types, such as neurons and glia, has grown appreciably, along with our understanding of the acute responses at the microvascular level, there is a demand to understand these processes together. More recently, there has been a conceptual and practical approach that links the microvessel with neuron interactions, referred to as the neurovascular unit (NVU) (del Zoppo and Mabuchi, 2003; Zonta *et al.*, 2003). The components of the NVU are considered as those involving vascular cells (endothelium, pericytes, vascular smooth muscle cells, and glia, astrocytes, microglia and oligodendroglia), which can contribute to brain damage from acute ischaemic stroke. The control and modulation of regional and local blood flow in the absence of cerebral injury depend upon neurovascular coupling (Iadecola, 2004; Koehler *et al.*, 2009), and local changes in regional CBF relate to microvessel responses and reflect the presence of neuronal activation, which in itself requires functional intact neurons (Zonta *et al.*, 2003). Astrocytes and endothelial cells interact to form an intervening basal lamina barrier and enhance inter-endothelial tight junctions as part of the permeability barrier of the capillaries.

One of the implications of this conceptual framework is that signalling processes and communication between components of the NVU is extremely well integrated (Milner *et al.*, 2008). The most important consequence clinically is that there will be limited recovery of the ischaemic tissue unless blood flow is re-established because of the selective vulnerability of individual components of the unit. Strategies that attempt to preserve components of the unit such as neurons or glia are likely to be unhelpful if flow is not preserved. During stroke, alterations in endothelial cell integrity and activation of the coagulation system are interrelated, and they contribute to disturbance of blood flow and complete vascular occlusion. All components of the NVU appear to support and respond to the inflammatory processes occurring during cerebral ischaemia, including the endothelium, astrocytes, the vascular extracellular matrix, pericytes, neurons and their axons.

Alterations in endothelial cell integrity and activation of the coagulation system are interrelated, and they contribute to disturbance of blood flow and complete vascular occlusion. At the cellular level, endothelial cells rapidly convert into a pro-inflammatory and pro-thrombotic state by the up-regulation of various humoral intermediaries, such as proteinase activated receptor-1 (PAR1), tissue factor and MMPs (Tagaya *et al.*, 2001; Rosenberg, 2002); gene expression in the ischaemic core and boundary, which facilitates inflammation; and BBB dysfunction (del Zoppo and Milner, 2006; Rosell *et al.*, 2008; Rosell and Lo, 2008). This process facilitates the accumulation of fibrin, platelets and neutrophils and results in microvascular obstruction. On the abluminal side, MMPs degrade the neurovascular matrix, leading to acute BBB disruption. Release of endogenous ligands, termed DAMPs, from damaged cells leads to the activation of TLRs (principally 2 and 4) which signal through several mediators, involving kinase-signalling cascades to promote the production of pro-inflammatory cytokines via the activation of transcription factors such as NF-κβ and AP1, perpetuating a cycle of neurovascular damage (Figure 13.1) (Chakravarty and Herkenham, 2005; Marsh *et al.*, 2009; Abe *et al.*, 2010).

Significant alterations in both microvessel and neuronal integrity occur within the first minutes of flow cessation. An early inflammatory cascade occurs within the microvasculature which is typified by a series of pathophysiological events and consists of two components: a biochemical inflammatory component characterized by the production of inflammatory cytokines, chemokines and eicosanoids by the individual cellular components of the NVU, and cellular inflammation characterized by the recruitment of inflammatory cells (Stanimirovic and Satoh, 2000; Del Zoppo, 2010; Stanimirovic and Friedman, 2012). Endothelial cells facilitate selective leukocyte recruitment and are selectively recruited by a sequence of interactions with brain endothelial cell adhesion molecules (Greenwood *et al.*, 2011) controlling leukocyte rolling, tethering, adhesion along endothelial cells and ultimately transmigration from the luminal to the abluminal side of the endothelial layer (Figure 13.1).

One consequence of these events is that microvessels become obstructed within the territory at risk, with focal loss of permeability barriers and changes in endothelium–astrocyte–neuron relationships. The obstruction is most prominent in end arteries, for instance the microvasculature of the striatum (del Zoppo *et al.*, 1991; Mori *et al.*, 1992). This focal 'no reflow' results when the endothelium is activated, leading to expression of the leukocyte adhesion receptors P-selectin and intercellular adhesion molecule-1 (ICAM1), the activation of PMN leukocytes and their lodgement in the microvessel bed (del Zoppo and Mabuchi, 2003). Activated platelets and fibrin, caused by the generation of thrombin, are also inherently involved in the microvessel obstruction. The microvessel wall undergoes rapid and dynamic changes affecting matrix integrity of the basal lamina and matrix receptors,

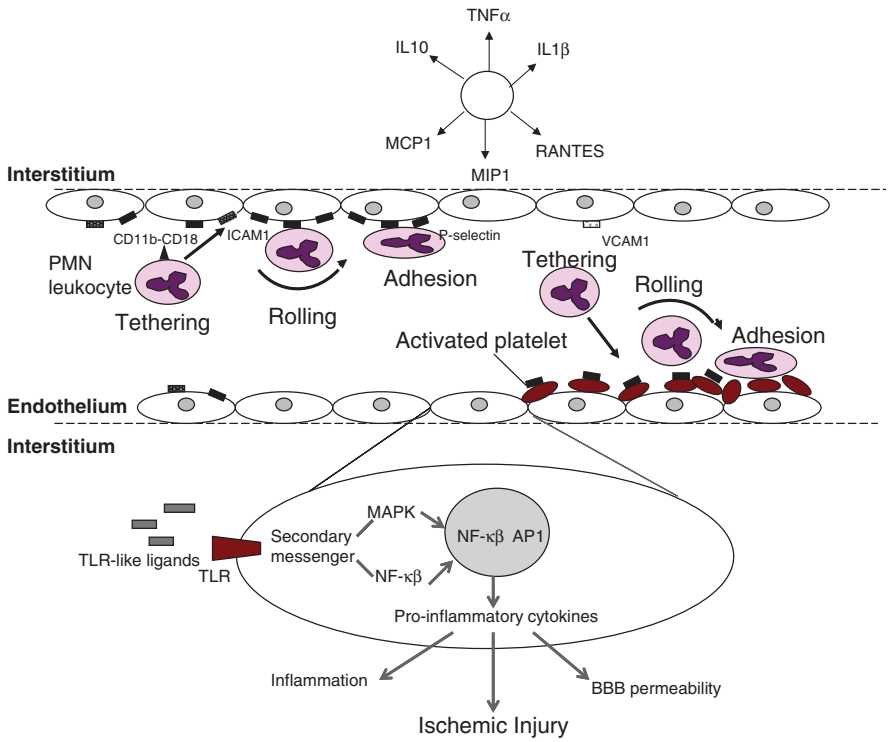

Figure 13.1 During ischaemia, endothelial cells express PAR1, tissue factor (TF) and matrix metalloproteinases (MMPs). Together, these facilitate the endothelial inflammatory response causing the aggregation of platelets, fibrin degradation and leukocyte recruitment resulting in the microvascular 'no reflow' phenomenon. Also leukocytes adherent to endothelium can cause endothelial dysfunction, transvascular protein leakage and oedema leading to brain injury. MMPs contribute to the degradation of extracellular matrix causing an increase in BBB permeability. Endogenous ligands from damaged cells cause the expression of Toll-like receptors (TLRs). Through complex signalling pathways, pro-inflammatory cytokines are produced via transcription factors such as NF-κβ and AP1.

processes that appear to occur at a similar time as the neuronal injury. The expression of the matrix constituents (basal lamina, laminin, collagen IV, cellular fibronectin and perlecan) decreases substantially (Okada *et al.*, 1994). It has also been established that endothelial and astrocyte cytoskeletal structures are compromised by a decrease in endothelial cell β_1-integrin receptor and integrin $\alpha_6\beta_4$ on astrocyte end feet in the first hour following middle cerebral artery (MCA) occlusion (Mori *et al.*, 1992; del Zoppo and Mabuchi, 2003).

Blood–brain barrier permeability

The BBB is the critical, physical, metabolic and neurological barrier that separates the CNS from the peripheral circulation (Reese and Karnovsky, 1967). As mentioned in this chapter, microvascular endothelial cells act in concert

with adjacent astrocytes, pericytes, neurons and the extracellular matrix to create a 'neurovascular unit' to restrict the entry of potentially damaging blood-borne substances into the brain's fragile microenvironment (Abbott *et al.*, 2006; Neuwelt *et al.*, 2011). Cerebral microvascular endothelial cells that form the BBB are distinguished from most peripheral vascular endothelial cells by the absence of fenestrations, increased mitochondria, reduced pinocytosis and the presence of tight junctions that connect apposing microvascular endothelial cell membranes (Hawkins and Davis, 2005; De Boer and Gaillard, 2007). Initial steps of leukocyte brain endothelial cell interactions have been well documented in the context of ischaemia and inflammatory disease (Greenwood *et al.*, 2011), but this belies the mechanisms of their abluminal extravasation. It was once considered that leukocyte migration occurred principally by the paracellular route, contributing to the degradation of tight junction proteins (Kebir *et al.*, 2007). This early concept has somewhat been revised recently with recent histological electron microscopy studies demonstrating that leukocytes have been observed to migrate through the transcellular pathway, leaving the tight junctions morphologically intact (Engelhardt and Wolburg, 2004). Once leukocytes have migrated into the abluminal space, several deleterious mediators released by activated leukocytes include free radicals, MMPs and eicosanoids which have been implicated in further endothelial injury, disruption of the basement membrane and transient opening of the BBB (Stanimirovic and Satoh, 2000; Del Zoppo, 2010).

The barrier to paracellular diffusion created by tight junction proteins is capable of rapid disassembly in response to extracellular stressors such as pain, inflammation and hypoxia (Huber *et al.*, 2001; Balda and Matter, 2008). Both claudin and occludin are critical for tight junction BBB function and paracellular diffusion (Hawkins and Davis, 2005). Aging in experimental animals and humans is associated with significant structural functional alterations in the BBB (Greenwood *et al.*, 2011). One study that addressed this found that the amount of occludin expression was reduced in elderly rats of age 24 months by approximately 30%, but the number of tight junctions did not change with age (Greenwood *et al.*, 2011). There are many factors contributing to BBB disruption in ischaemia, including the generation of oxygen radicals (Mooradian *et al.*, 2003; Haorah *et al.*, 2007) and nitric oxide (Mark *et al.*, 2004; Han *et al.*, 2006), the production of vascular endothelial growth factor (Zhang *et al.*, 2000; Yeh *et al.*, 2007) and changes in intracellular calcium (Ikeda *et al.*, 1997; Brown and Davis, 2005; Kuhlmann *et al.*, 2009). Focal cerebral ischaemia, as is observed in acute stroke, is responsible for the loss of endothelial cell integrity resulting in an increase of vascular permeability (Haorah *et al.*, 2005). The disruption of the BBB results in the formation of a vasogenic oedema, which causes further damage in the surrounding tissue. Several mediators may contribute to the stroke-induced alterations of the BBB: reactive oxygen species (ROS) (Mattson *et al.*, 1997; Han *et al.*, 2006), platelet-activating factor, TNFα (Marchal *et al.*, 1993), vascular endothelial growth factor (Marchal *et al.*, 1995) and MMPs (Ding *et al.*, 2004). Under

physiological conditions, the endothelial cells of the BBB form a tight barrier that is sealed by tight junctions, junctional adhesion molecules and adherens junctions (Engelhardt and Wolburg, 2004). Besides a down-regulation of these molecules, another possible explanation for the stroke-induced opening of the BBB appears to be the activation of the endothelial cell contractile machinery (Mattson *et al.*, 1997). Endothelial cells contain the contractile elements actin and myosin and other regulatory proteins (Goeckeler and Wysolmerski, 1995). Phosphorylation of the regulatory myosin light chain (MLC) leads to activation of the endothelial contractile elements (Garcia *et al.*, 1995), and the MLC phosphorylation state is controlled by the MLC kinase (MLCK). A major contributory factor leading to disruption of the BBB after hypoxia is the contraction of the actin–myosin cytoskeleton. A recent study demonstrated that inhibition of actin–myosin contraction protected the BBB after hypoxic stress specifically by inhibition of MLCK (Kuhlmann *et al.*, 2007), confirming an involvement of the endothelial contractile machinery to stroke-related BBB disruption *in vitro* and *in vivo*. Hypoxia-induced BBB disruption was prevented by inhibition of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase, or by chelation of intracellular calcium (Mark and Davis, 2002; Koehler *et al.*, 2009). These protective effects were correlated with a decrease in the amount of phosphorylated myosin light chain (pMLC) detected by immunofluorescence. Although there is substantial evidence that inhibition of MLCK will be an effective way to inhibit BBB damage, not known is its importance in ischaemic stroke or whether such an approach can be used to expand the treatment window, especially when conflicting factors such as tissue plasminogen activator, ischaemia in the elderly brain and ischaemic severity may be involved (Kaur *et al.*, 2011).

In conclusion, this review has outlined a complex cascade of biochemical and associated electrophysiological mechanisms involved in ischaemic brain injury. Several experimental approaches to the study of neuronal ischaemic injury have produced evidence suggesting that neuronal death may be mediated by the effects of excitatory neurotransmitters, such as glutamate, which promote Ca^{2+} entry into the cells, the release of oxygen free radicals and the accumulation of lactic acid during a switch to anaerobic metabolism. In addition to these processes, there are a number of microcirculatory processes which precede them or occur simultaneously and which may exacerbate injury. Central to this microvascular response is the activation of the vascular endothelium by tissue factor, thrombin and pro-inflammatory cytokines that initiate adhesion of polymorphonuclear leukocytes, platelets and fibrin compromising blood flow in the microcirculation, BBB dysfunction and secondary tissue injury (Del Zoppo *et al.*, 1998; Frijns and Kappelle, 2002). However, the inflammatory response is complex, with each tissue, brain region and individual potentially having its own timing and magnitude of response. Also fundamental to the microvascular response is the reorganization of the NVU following tissue hypoxia and inflammatory activation that includes the disruption

of end-to-end endothelial tight junctions, the retraction of pericytes from the abluminal surface of the capillary, the breakdown of the basal lamina and the transmigration and infiltration of the inflammatory cells. Polymorphonuclear leukocytes play several roles in the maturation of focal ischaemia, especially during reperfusion (Jin *et al.*, 2010). They contribute to the local haemostatic changes in the microcirculation, oedema formation, increased post-capillary venule endothelial permeability, free radical generation and ultimately infarct maturation. These cellular events are accompanied by increased expression of endothelial cell leukocyte adhesion receptors, the loss of endothelial cell and astrocyte integrin receptors, the loss of their matrix ligands, the expression of members of several matrix-degrading protease families and the appearance of receptors associated with angiogenesis and neovascularization (Del Zoppo, 2010). This remodelling contributes to functional disorganization at the NVU, which includes BBB dysfunction, impaired neurovascular coupling and leukocyte adhesion and infiltration as well as prothrombotic activation.

Adaptive inflammatory response in the context of ischaemia reperfusion can be either interrupted or pathologically perpetuated, resulting in attenuation or an amplification of the initial pathological stimulus. Certain strategies may be required to intervene early in brain inflammation to reduce injury and neurodegeneration, and other interventions may be needed to facilitate the repair and recovery of regeneration processes after CNS injury (Lazarov-Spiegler *et al.*, 1998). Amongst the injury mechanisms specifically targeted for therapy are calcium regulation and transit, neurotransmitter release, cell death pathways and inflammation (Barber *et al.*, 2003; Chauhan *et al.*, 2003). Table 13.1 summarizes some of the individual biochemical, cellular and

Table 13.1 Pathways and therapies in clinical trials

Target	Therapies in clinical trials
Cerebral blood flow	• Thrombolytic agents • Platelet anti-aggregating agents • Anticoagulants • Carotid and vertebral artery angioplasty • Angioplasty for vasospasm
Metabolic • Tissue acidosis • Free radicals • Impaired protein synthesis	• Prevention of hypoglycaemia • Free radical scavengers • Lipid peroxidase inhibitors • Growth factors
Inflammation	• Inhibition of leukocyte migration • Inhibition of prostaglandin synthesis
Excitotoxicity and intracellular calcium	• Inhibition of leukocyte migration • Inhibition of prostaglandin synthesis
Thermoregulation	• Physical cooling • Pharmacological cooling

molecular pathways involved in experimental studies, and preclinical strategies to mitigate them.

In contrast to experimental studies, neuroprotective therapies assessed in clinical trials have been unequivocally negative, despite evidence in experimental studies of positive results related to the effects of therapies targeting specific mechanisms on traditional outcomes such as histological injury, and in some cases animal behaviour (Hallenbeck, 1995; Gladstone *et al.*, 2002). The commonly cited reasons for this incongruity include lack of rigorous pre-clinical investigation, specifically the control of physiological confounders such as changes in systemic blood pressure and temperature, and clinical trial design inconsistent with pre-clinical studies may have led to the failure of the clinical trials (Gladstone *et al.*, 2002; Barber *et al.*, 2003, 2004; O'Collins *et al.*, 2006). Evidence from these trials suggests that neuroprotection alone without restoration of tissue perfusion and vascular integrity may have compromised the therapeutic benefit of promising neuroprotective agents for the treatment of acute stroke (Lees *et al.*, 2006).

The timing of specific interventions may be critical to the development of significant neuroprotective, anti-inflammatory therapy. Establishing the importance of the early steps in the inflammatory process involved within the cerebrovasculature will be critical to understanding secondary tissue injury following stroke.

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14
Spinal Cord Injury

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Overview of neuroinflammation and spinal cord injury

Most traumatic spinal cord injuries (SCIs) are caused by contusion or bruising of the spinal cord as a result of fracture and/or dislocation of the spine. Individuals with an SCI experience paralysis, abnormal sensation, autonomic dysfunction and compromised bowel, bladder, sexual and/or respiratory function at or below the level of injury. These effects are usually permanent as the mammalian central nervous system (CNS) has a limited capability for endogenous repair and axon regeneration. Although the incidence is small relative to other CNS disorders, SCI tends to affect people early in life with the average age at the time of injury around 30–37 years old (DeVivo and Chen, 2011). Healthcare costs associated with managing life after SCI are substantial: lifetime costs range from three to five million US dollars per individual and represent a significant healthcare burden (Cao *et al.*, 2011).

The dysfunction associated with SCI depends in large part on the initial damage inflicted at the time of SCI; however, delayed neurodegeneration and endogenous repair processes occur after SCI that can further exacerbate or ameliorate neurological impairments. The initial injury causes tissue destruction and cell loss due to mechanical shearing and breaking of cells and anatomical structures. This 'primary injury' is commonly incomplete, leaving some tissue spared from the damage (DeVivo, 2012). Primary injury occurs at the time of SCI and is not amenable to therapeutic interventions. Subsequent to the primary trauma, however, a series of therapeutically amenable,

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pathophysiological and cellular events are triggered. These events contribute to both a progressive loss of initially spared tissue (i.e. 'secondary injury') and endogenous repair of injured tissue (Beattie *et al.*, 1997, 2000). Mechanisms contributing to secondary injury include excitotoxic cell death and oxidative stress, among others. Endogenous repair consists of, for example, remyelination of denuded axons, axon sprouting, revascularization and axon regeneration.

Secondary injury, as evidenced by cellular apoptosis, occurs within hours of injury and continues for many weeks (Crowe *et al.*, 1997; Liu *et al.*, 1997). Axonal degeneration and demyelination occur within weeks of injury with persistent conduction impairments indicative of chronic demyelination for months after SCI (James *et al.*, 2011; Powers *et al.*, 2012). During this time, reparative events also take place. Endogenous axonal sprouting, cellular proliferation, remyelination and wound repair begin within days of SCI and persist at chronic stages (Beattie *et al.*, 1997; Hill *et al.*, 2001; Mctigue and Tripathi, 2008; James *et al.*, 2011; Powers *et al.*, 2012). SCI activates adaptive and innate immune responses that overlap spatially and temporally with secondary injury and endogenous repair processes (Donnelly and Popovich, 2008). It is not surprising, therefore, that the immune response to SCI is central to post-SCI degeneration and repair processes.

The innate immune response is triggered at the time of injury; tissue resident microglia and astrocytes become activated, proliferate and migrate to the site of injury. Within a day of SCI, neutrophils invade from the blood and fill the lesion (Popovich *et al.*, 1997; Taoka *et al.*, 1997; Fleming *et al.*, 2006; Kigerl *et al.*, 2006; Beck *et al.*, 2010). The neutrophil response subsides within a few days of SCI; however, macrophages (consisting of blood monocytes and endogenous, activated microglia) soon proliferate and home to the injury site (Popovich *et al.*, 1997; Fleming *et al.*, 2006; Kigerl *et al.*, 2006; Beck *et al.*, 2010). There they persist indefinitely and there is no evidence that this aspect of the innate immune response ever subsides. The adaptive response is more delayed, with B and T cell infiltration delayed until 1–2 weeks post SCI (Popovich *et al.*, 1997; Kigerl *et al.*, 2006; Ankeny and Popovich, 2009; Beck *et al.*, 2010). B and T cell numbers increase in the injured spinal cord through to at least 9 weeks post injury and, similar to macrophages, persist indefinitely (Ankeny and Popovich, 2009).

The magnitude and timing of the immune response differ based upon the spinal level of injury (thoracic vs. cervical), injury severity, strain and species, age at the time of injury, and mode of injury (i.e. contusion, compression or ischemic). Regardless of these factors, however, these immune responses persist after SCI and contribute to pathological and reparative processes. Throughout this chapter, we will discuss the role that individual aspects of the innate and adaptive immune responses may be playing in repair or pathology after SCI. Where appropriate, current clinical therapies targeting these immune responses will be highlighted.

Role of the innate immune response: neutrophils

Neutrophils are the first leukocytes to enter the injured spinal cord. Subtle differences in the kinetics and magnitude of neutrophil recruitment have been observed between different species and strains of animals; however, intra-spinal accumulation peaks ~1 day post injury (dpi), then resolves within 2–3 days (Donnelly and Popovich, 2008; Beck *et al.*, 2010). Neutrophils remove damaged tissue via phagocytosis and the release of proteases, oxidative metabolites and other microbicidal chemicals. Due to this non-specific mode of action, bystander damage of intact and spared tissue is often associated with neutrophil activation after SCI (Taoka *et al.*, 1997; Taoka and Okajima, 2000). The consensus among researchers, therefore, is that neutrophils contribute to secondary injury after SCI. Experimental manipulations that target neutrophils after SCI by and large support their detrimental role, and there is no evidence that boosting neutrophil activation can improve recovery; however, neutrophils also likely play an important role in initiating the wound repair process.

Most experimental manipulations examining neutrophil function after SCI do so by limiting accumulation in the spinal cord. In response to SCI, neutrophils and endothelial cells increase expression of surface adhesion and migration proteins, for instance P-selectin and intercellular adhesion molecule-1 (ICAM1) (Isaksson *et al.*, 1999). These receptor interactions between neutrophils and endothelial cells facilitate neutrophil entry into the injured spinal cord. Interfering with these interactions through the delivery of monoclonal antibodies (mabs) can limit neutrophil accumulation after SCI (Hamada *et al.*, 1996; Taoka *et al.*, 1997; Bao *et al.*, 2004; Gris *et al.*, 2004; Stirling *et al.*, 2009; Lee *et al.*, 2011). Similar neutrophil depletion in the injured spinal cord has also been achieved using anti-neutrophil serum, anti-inflammatory drugs or drugs that prevent blood–brain barrier disruption (Holtz *et al.*, 1989; Bartholdi and Schwab, 1995; Lee *et al.*, 2012).

The results of these depletion studies largely support a negative role for neutrophils in repair processes after SCI. Acute post-injury injections of mabs against the neutrophil adhesion proteins CD11d and CD18 reduce neutrophil accumulation in the injured spinal cord and are associated with improved tissue sparing and functional recovery (Bao *et al.*, 2004; Gris *et al.*, 2004; Bao *et al.*, 2005, 2011; Ditor *et al.*, 2006; Geremia *et al.*, 2012). Similar administration protocols after SCI of antibodies against ICAM1 and P-selectin reduce neurological impairments and tissue loss (Hamada *et al.*, 1996; Taoka *et al.*, 1997; Naidu *et al.*, 2003).

Mab treatments, however, do not always yield consistent results. In rabbits, mab treatment targeting CD11 and CD18 did not improve recovery from ischemia-induced SCI (Forbes *et al.*, 1994). Stirling *et al.* (2009) used Ly6G–Gr1 antibodies to deplete neutrophils in mice after SCI and found that the decrease in neutrophil recruitment resulted in less spared tissue, reduced

wound healing and worse motor recovery compared to controls. This paper provided the first evidence that neutrophils may be playing a beneficial role when responding to SCI.

Collectively, anatomical observations and experimental manipulations provide evidence for and against neutrophils playing a detrimental role in recovery after SCI.

Neutrophils are difficult to identify *ex vivo* on tissue samples; myeloperoxidase (MPO) activity is generally used to approximate neutrophil accumulation. There are limitations, however, to this indirect approach. Activated macrophages can include high levels of MPO activity (Rodrigues *et al.*, 2002). In addition, the molecules used to deplete neutrophils can also target microglia, macrophages and endothelial cells. Collectively, these confounding factors make it difficult to determine the true pathological or reparative role of activated neutrophils in recovery from SCI.

The inflammatory response to SCI is complex. Even focusing on a single cell population after injury can yield conflicting results. When deciphering the role of inflammation after injury, one must consider how heterogeneity may make interpretations difficult. With respect to neutrophils, most depletion and antibody protocols do not reduce the presence of a homogeneous population of cells at the site of injury; it is likely that microglia and monocytes are also affected. After SCI, ICAM1 expression peaks at 2 days and is significantly increased at 1 week (Isaksson *et al.*, 1999). These later times are after the peak in neutrophil migration and activation. Since an ICAM1 counter-receptor, CD11b, is increased on monocytes and microglia after SCI, the increased expression at later time points likely reflects changes in activated macrophages and not neutrophils (Dusart and Schwab, 1994; Isaksson *et al.*, 1999). The timing of the mab depletion protocols mentioned here overlaps with onset of macrophage activation. It is therefore difficult to ascertain, through alterations in these heterogeneous populations of cells (e.g. neutrophils, microglia and monocytes), how individual cell types contribute to the repair or pathology associated with antibody treatment. As with most components of the inflammatory response, it is difficult to modulate one cell type in isolation.

Role of the innate immune response: astrocytes

Astrocytes, in combination with tissue resident microglia, coordinate the earliest phases of post-SCI inflammation. Within hours of SCI, astrocytes proliferate and migrate to the injury site and the lesion penumbra (Gwak *et al.*, 2012). Astrocytes release cytokines, growth factors, chemokines and other signalling molecules that influence the progression of the inflammatory response (Gwak *et al.*, 2012). Within weeks of severe injury, astrocytes wall off the area around the lesion forming a glial scar, similar to the one formed by fibroblasts in cutaneous wounds. This glial scar limits the migration of leukocytes and tissue macrophages into areas of spared, intact tissue.

Historically, astrocytes have been implicated as playing a negative role in repair processes after SCI (Clemente and Windle, 1954). The glial scar has been seen as a physically barrier to axon growth and other endogenous repair processes (Liuzzi and Lasek, 1987). Indeed, astrocytes can inhibit axon growth through physical and chemical means (McKeon *et al.*, 1991). The potential for astrocytes to play a negative role after injury has been reviewed extensively and will not be discussed in detail here (for a comprehensive review, see White and Jakeman, 2008). Recently, however, genetic manipulations of astrocytes have revealed a more complex role for astrocytes in initiating and maintaining reparative processes after SCI.

Up-regulation of the intermediate filament, glial fibrillary acid protein (GFAP), is a hallmark of reactive, scar-forming astrocytes after SCI (Pekny and Pekna, 2004; White and Jakeman, 2008; Gwak *et al.*, 2012). Using a transgenic mouse in which proliferating, GFAP-expressing astrocytes are ablated following administration of the antiviral agent ganciclovir, Faulkner and colleagues revealed that the astrocyte response to SCI might be essential for coordinating endogenous repair efforts (Faulkner *et al.*, 2004). Following a moderate spinal cord crush injury, transgenic mice treated with ganciclovir had more cellular degeneration and larger lesions, with associated persistent motor deficits, compared to untreated genetic controls or ganciclovir-treated wild-type (WT) controls. Increased leukocyte infiltration was also noted in astrocyte-ablated animals. Collectively, the authors conclude that astrocytes are essential for wound healing. They postulate that astrocytes facilitate repair of a compromised blood–brain barrier after SCI, thereby restricting the potentially destructive inflammatory response to the already damaged lesion foci (Faulkner *et al.*, 2004).

One limitation of this model is that ganciclovir treatment causes proliferating astrocytes to undergo apoptosis; this cellular debris is a pro-inflammatory stimulus that can drive microglia and macrophage activation independent of the loss of astrocyte function (Bianchi, 2007). Indeed, following astrocyte ablation, the macrophage response to SCI was increased sevenfold (Faulkner *et al.*, 2004). The authors conducted intra-spinal NMDA injection studies to kill neurons *in vivo* and determine if the cellular debris is sufficient to cause this increased inflammation. Based on a small increase in macrophages after these injections, the authors conclude that the presence of dead cells alone cannot account for the increased inflammation and that loss of astrocyte function, not increased cell death, leads to a prolonged infiltration of pathological macrophages (Faulkner *et al.*, 2004).

Results of a recent, more selective, genetic astrocyte depletion study support this potentially positive role. Using knockout animals, Lepore *et al.* genetically reduced the expression of the astrocytic glutamate transporter, GLT1, *in vivo* (Lepore *et al.*, 2011). GLT1 facilitates removal of extracellular glutamate, thereby maintaining homeostasis and preventing excitotoxicity. Mice heterozygous for GLT1 had worse recovery, increased apoptosis

and neuronal cell loss, increased lesion size and decreased tissue sparing compared to wild-type controls following thoracic crush SCI. While the microglia and macrophage response was not reported, it is less likely that non-specific microgliosis may be confounding the results. Indeed, a similar detrimental effect of selective genetic depletion of astrocytes has been reported after mouse thoracic crush SCI (Herrmann *et al.*, 2008). In that study, astrocyte activity was attenuated through selective deletion of the cytokine and signal transducer and transcription factor, STAT3, under the GFAP promoter. The authors detected reduced astrocytic activation with an increased spread of inflammation and increased lesion size in transgenics compared to controls. Collectively, based on the results of these selective depletion studies, researchers concluded that astrocytes play a protective role after SCI, potentially through limiting both immune infiltration and glutamate excitotoxicity (Herrmann *et al.*, 2008; Lepore *et al.*, 2011).

Other genetic studies, however, supported a detrimental role for astrocytes after SCI. Similar to the global suppression approach used by Faulkner *et al.* (2004), Toyooka and colleagues administered adenovirus vectors expressing GFAP small interfering RNA (siRNA) to suppress astrogliosis after rat thoracic spinal cord contusion injury (Toyooka *et al.*, 2011). This astrocytic suppression was associated with increased recovery from urinary dysfunction. Although there were no functional or anatomical differences among the siRNA-treated versus control groups, these data are in contrast to the detrimental results reported by Faulkner *et al.* (2004). There is less potential that siRNA administration caused indirect effects on macrophage activation than ganciclovir treatment; however, it should be noted that Toyooka *et al.* did not examine microglia and macrophage activation in response to treatment. Due to this potential confound, more work is needed to determine the direct role that astrocytes are playing in repair processes.

More targeted genetic approaches also provide evidence for a detrimental role of astrocytes after SCI. The connexin43 (Cx43) hemichannel allows astrocytes to release adenosine triphosphate (ATP), a stimulus for astrogliosis, cell death and microglial activation. Using a cre–lox system, Huang and colleagues removed the Cx43 gene under the GFAP promoter (Huang *et al.*, 2012). After thoracic contusion SCI, Cx43ko mice had significantly less astrocyte activation, smaller lesion volumes, increased axonal conductance and increased functional recovery compared to Cx43wt controls (Huang *et al.*, 2012). Interestingly, these effects were not associated with increased endogenous microglia activation. There was a significant decrease in blood-derived macrophage activation, however, suggesting that under normal circumstances, ATP release from astrocytes may drive pathological macrophage activation after SCI (Huang *et al.*, 2012). These results are in accordance with studies finding that selective deletion of suppressor of cytokine signalling-3 (SOCS3) or knockdown of nuclear factor kappa B (NF-κB), specifically in astrocytes, reduces intra-spinal inflammation, protects tissue from secondary cell death

and improves functional recovery in mouse thoracic contusion models of SCI (Brambilla *et al.*, 2005, 2009; Okada *et al.*, 2006).

Both global and targeted astrocyte ablation and suppression techniques provide evidence for a dual role of astrocytes after SCI. It is likely that astrocytes limit damage and promote endogenous repair mechanisms while also contributing to secondary injury. As illustrated with the ganciclovir studies and other studies discussed here, it is often difficult to disassociate the role of astrocytes from other cellular responses to SCI. The response of astrocytes to trauma is heterogeneous, with complex signals from a variety of different sources contributing to the response in a time- and location-specific manner. Signals from endothelial cells, microglia, neurons, oligodendrocytes and infiltrating leukocytes stimulate astrocytes that in turn release cytokines, growth factors and other molecules that can further exacerbate or reduce secondary death cascades. In addition, astrocytes adopt species-specific functional phenotypes that vary depending upon the mode of injury (Gwak *et al.*, 2012). For example, bone morphogenic proteins are growth factors that exert different effects on human and rodent astrocytes (Davies *et al.*, 2011; Wang *et al.*, 2011). The majority of the genetic studies discussed in this chapter demonstrating a potentially positive role for astrocytes utilized crush SCI models; evidence for detrimental roles was collected using contusion SCI models. Future research should try to account for these astrocyte–glia interactions, species difference and injury mode–specific effects if the role of astrocytes in repair and pathological processes after SCI is to be fully understood.

Role of the innate immune response: macrophages

Microglia, along with astrocytes, are the first resident cells to respond to SCI. Microglia alter their morphology, phenotype and secretions in response to the environmental disruption caused by SCI. A key change is the release of pro-inflammatory chemokines and cytokines that recruit leukocytes to the injury site. Following these cues, within a few days of SCI, monocytes from the blood migrate to the damaged tissue and differentiate into tissue macrophages. By one week post SCI, monocyte- and microglia-derived macrophages populate the lesion site and penumbra; both adopt a round, phagocytic phenotype, making them morphologically indistinguishable (Jones *et al.*, 2005). Collectively, in the context of SCI, microglia- and monocyte-derived macrophages are often referred to as macrophages or CNS macrophages. Regardless of the cause of SCI, in both animal models and clinical observations, macrophages persist at the site of SCI indefinitely (Donnelly and Popovich, 2008).

Macrophages contribute to both pathological and reparative functions after SCI. Over the past two decades, SCI researchers have tested these dichotomous effects. Increasing macrophage activation after SCI, through transplantation or administration of pro-inflammatory mediators, improves regeneration, myelin clearance, tissue preservation and recovery (Prewitt

et al., 1997; Rabchevsky and Streit, 1998; Rapalino *et al.*, 1998; Perrin *et al.*, 2005; Gensel *et al.*, 2009). Decreasing macrophage activation, through depletion or neutralization, is neuroprotective, improves recovery and increases axon growth (Blight, 1994; Popovich *et al.*, 1999; Gris *et al.*, 2004; Bao *et al.*, 2005; Gray *et al.*, 2007; Fleming *et al.*, 2008; Stirling *et al.*, 2009; Mukaino *et al.*, 2010; Bao *et al.*, 2011, 2012; Iannotti *et al.*, 2011). These various experiments, and the controversies surrounding the role of macrophages in SCI, have been reviewed previously (Jones *et al.*, 2005; Alexander and Popovich, 2009; Donnelly and Popovich, 2008; Popovich and Longbrake, 2008) and will not be discussed further. To gain better insight into the role that macrophages play in SCI, factors that influence their functional potential are discussed in this chapter.

Macrophage functions, and their associated molecular phenotypes, are dynamic and change in a time- and context-dependent manner in response to signalling moieties present at sites of inflammation (Stout and Suttles, 2004). Thus, it is challenging to determine their role in recovery from SCI. Complex activation by cytokines, cellular debris, blood products and extracellular matrix fragments influences macrophage functions and phenotypes (Kigerl and Popovich, 2009). Once appropriately activated, CNS macrophages can produce neurotrophic and neurotropic molecules that are necessary for promoting cell survival and axon regeneration (i.e. GDNF, BDNF and oncomodulin) (Batchelor *et al.*, 1999; Yin *et al.*, 2006). Macrophages also cause oxidative stress and proteolytic degradation via the release of superoxide, nitric oxide (NO) and proteases (Bao *et al.*, 2004, 2005). In addition, it is possible for a single population of macrophages, activated in the spinal cord, to concurrently promote regeneration and cause cell death (Gensel *et al.*, 2009). Unfortunately, for researchers trying to understand the role of macrophages after SCI, macrophages with the potential to both increase regeneration and also promote cell death are present in the injured spinal cord (Kigerl *et al.*, 2009).

The pathological or reparative properties of activated macrophages are influenced by the time after injury and the lesion environment. Acutely after SCI, mixed populations of pro-regenerative and neurotoxic macrophages are present in and around the injury site (Kigerl *et al.*, 2009). Seven to 14 days after SCI, the majority of macrophages adopt a neurotoxic phenotype (Kigerl *et al.*, 2009). Environmental cues present at these later times contribute to this delayed neurotoxic polarization. Macrophages pre-differentiated to have a pro-regenerative phenotype adopt a pathological phenotype when they are transplanted into the injured spinal cord and exposed to the lesion environment (Kigerl *et al.*, 2009).

Age at the time of injury may also be a key determinant for the role that macrophages play after SCI. Levels of interferon gamma (IFNγ), the primary cytokine associated with pathological macrophage activation, increase with age in the spinal cord (Chung *et al.*, 2010). Accompanying these increases in pro-inflammatory cytokines is a decrease in anti-inflammatory cytokines (IL4 and IL10) that drive pro-regenerative M2-type macrophage activation

(Jurgens and Johnson, 2012). It is not surprising, therefore, that the magnitude and pro-inflammatory polarization of macrophages increases with age in CNS trauma. Compared to young animals, aged animals have a robust CNS macrophage response after intracerebral haemorrhage, cortical stab injury and peripheral nerve injury (Gilmore and Kane, 1998; Kyrkanides *et al.*, 2001; Zhu *et al.*, 2003; Lee *et al.*, 2006, 2009a; Wasserman *et al.*, 2008). To see whether the same pathological polarization occurs after SCI, we examined macrophage responses 7 days post contusion SCI in young (3–4 months old) versus aged (16–18 months old) animals. Overall, we detected more macrophages in the injured spinal cord of aged animals (Figure 14.1). In addition, fewer macrophages adopted a pro-regenerative phenotype in these aged animals (Figure 14.1). Therefore, it is possible that being of an older age at the time of SCI may polarize macrophages towards a pathological phenotype.

Genetics are also important determinants for macrophage function. Although the time course of the macrophage response to SCI is similar across species (Sroga *et al.*, 2003; Fleming *et al.*, 2006), subtle genetic differences have the potential to affect recovery. The macrophage response to SCI in Lewis rats

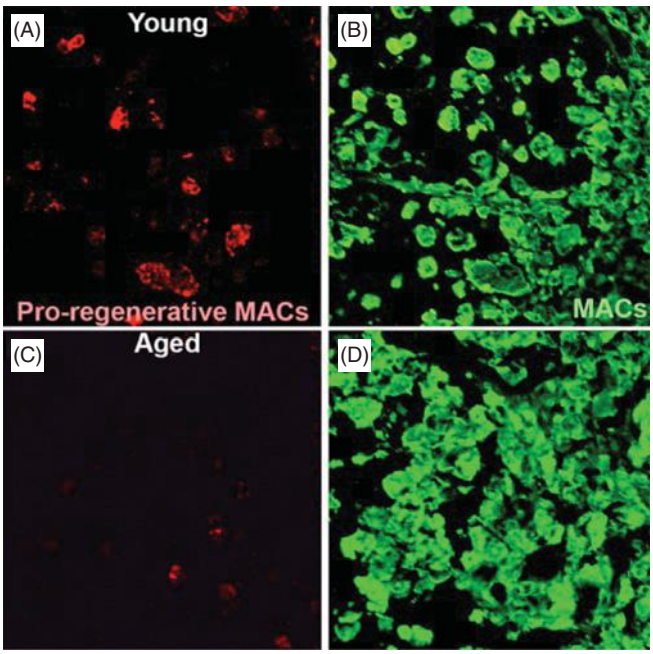

Figure 14.1 Fewer pro-regenerative macrophages respond to spinal cord contusion injury in aged (14–16 months old) versus young (4 months old) mice. Representative images of macrophages (green = MAC1; red = arginase) in the lesion epicentre 7 days after thoracic SCI in 4-month-old (A–B) and 14–16-month-old (C–D) mice. Pro-regenerative macrophages identified as arginase-positive cells (A,C). This demonstrates that SCI at an older age may result in a more destructive macrophage response.

is prolonged and greater in magnitude compared to in Sprague–Dawley rats (Popovich *et al.*, 1997). The magnitude of the macrophage response to SCI is smaller in BALB/c compared to other mouse strains (Kigerl *et al.*, 2006). For both rats and mice, strains exhibiting less macrophage activation often have better recovery (Basso *et al.*, 2006; Schmitt *et al.*, 2006). There are limited data regarding the macrophage response to SCI in humans (Fleming *et al.*, 2006), and it is difficult to predict which animal models most closely represent the human condition (Mestas and Hughes, 2004).

Given that time, age and genetics may influence macrophage function after SCI, it is important that a variety of models, strains and species continue to be utilized in order to understand the role of macrophages after SCI and develop immunomodulatory therapies that will have the broadest potential for treating humans with SCI.

Role of the adaptive immune response: T and B cells

T and B cell numbers are negligible or suppressed acutely after SCI (Jones *et al.*, 2005; Donnelly and Popovich, 2008). During the sub-acute phase (3–7 dpi), these lymphocyte numbers increase and remain chronically elevated (Popovich *et al.*, 1997; Sroga *et al.*, 2003; Ankeny and Popovich, 2010). Although controversy surrounds how lymphocytes should be manipulated after SCI, there is agreement that the endogenous lymphocyte response is not sufficient for facilitating repair and recovery (Schwartz *et al.*, 1999; Popovich and Longbrake, 2008). Limiting lymphocyte activation after SCI, through genetic or pharmacological manipulations, results in increased tissue sparing and functional recovery. There are no published reports documenting the negative consequences associated with depletion or reduction of T and B cells after SCI.

The controversy surrounding the adaptive immune response and SCI relates to whether T cells can be used as a tool for improving recovery after SCI. As discussed in Chapter 9, T cells that are autoreactive towards CNS proteins (specifically myelin proteins) amplify and target pathological inflammatory functions towards neurons and oligodendrocytes and therefore negatively contribute to CNS disorders. There is evidence that CNS injury can trigger pathological autoimmunity through activation of T cells (Jones *et al.*, 2002; Ankeny *et al.*, 2006). The negative role for T cells after injury is further supported by reports of attenuated neuropathology when the T cell response to SCI is reduced (Ankeny and Popovich, 2009). In contrast, Schwartz and colleagues propose that the T cell response can be advantageous after injury, but the magnitude is inefficient to promote repair (Martiñón *et al.*, 2012). Work done primarily by this group demonstrates that boosting this 'protective autoimmunity' through passive or active myelin basic protein (MBP)

immunizations after SCI limits secondary neurodegeneration (Schwartz, 2001). While these conflicting findings illustrate that our understanding of T cell functions after SCI is likely incomplete, there is a growing body of evidence in support of both these dichotomous roles for T cells after SCI.

Numerous, independent laboratories have reported that pharmacological interventions aimed at reducing T cell activation and spinal cord infiltration after SCI are beneficial. Sphingosine-1-phosphate (S1P) is a bioactive lysophospholipid mediator that regulates peripheral lymphocyte circulation, differentiation, activation and tissue infiltration (Bode and Gräler, 2012). Oral administration of the S1P antagonist, FTY720 (fingolimod), after SCI improves white matter sparing and locomotor recovery in mouse and rat models of contusion SCI (Lee *et al.*, 2009b; Norimatsu *et al.*, 2012). Interestingly, although FTY720 is reported to have broad immunomodulatory properties, only T cell infiltration, and not that of other inflammatory cell populations, is reduced after FTY720 treatment in SCI (Lee *et al.*, 2009b; Norimatsu *et al.*, 2012). Similar improvements in tissue sparing and functional recovery after SCI have been reported after antibody neutralization of CXCL10, a T cell chemoattractant (Gonzalez *et al.*, 2003). Although T cell infiltration was reduced, it should be noted, however, that CXCL10 is expressed by macrophages and microglia in the CNS and this cell population was also significantly reduced after antibody treatment (Gonzalez *et al.*, 2003).

Genetic models also point towards pathological T cells being activated after SCI. Athymic (nude) rats are homozygous recessive for the rnu gene required for normal thymus development (Festing *et al.*, 1978). Homozygous rats therefore have fewer mature T cells compared to heterozygous controls. Following T10 spinal cord transection, nude rats show better functional recovery, reduced secondary damage and a lessened macrophage response compared to controls (Potas *et al.*, 2006).

It is possible that autoreactive T cells contribute to the deficits after SCI. T cells isolated from the lymph node and spleen have increased proliferation to MBP when isolated from SCI rats versus sham-injured rats (Popovich *et al.*, 2001). In humans, autoreactive T cells that recognize MBP occur in higher frequency in patients with SCI compared to controls and persist for >10 years after SCI (Kil *et al.*, 1999; Zajarías-Fainsod *et al.*, 2012). To better understand if these autoreactive T cells could be contributing to secondary injury events after SCI, Popovich and colleagues examined the effects of SCI in transgenic (Tg) mice where >95% of the T cells were autoreactive for MBP (Jones *et al.*, 2002). When compared to mice with normal T cell responses, Tg mice had worse recovery, exacerbated tissue loss and hyper-inflammatory responses after SCI (Jones *et al.*, 2002). These data, and work done by others looking at autoreactive T cells after SCI (Marcondes *et al.*, 2005), demonstrate that SCI can trigger T cell autoimmunity with neurotoxic and pathological potential.

In contrast, starting in the late 1990s Schwartz and colleagues began championing the idea of protective autoimmunity. This concept, that T cells

responding to SCI are beneficial but too few in number to be effective, is based upon a number of studies in which animals receiving systemic administration of T cells specific to myelin-associated antigens after SCI showed better recovery than those left untreated (Moalem *et al.*, 1999; Schwartz *et al.*, 1999; Schwartz, 2001; Schwartz and Moalem, 2001). Greatest improvements were observed with active immunization of MBP or passive administration of MBP-specific T cells. This led to calls for immunization-based therapies to treat SCI in humans (Schwartz and Moalem, 2001). Initial independent replication, however, failed to detect the same beneficial effects; MBP-immunized rats had worse recovery and lesion pathology, greater neuron loss and macrophage activation, and more intra-spinal T cell accumulation compared to non-treated SCI controls (Jones *et al.*, 2004).

Recently, other labs have replicated certain aspects of protective autoimmunity. For instance, Wang and colleagues report that passive immunization with MBP-activated T cells can reduce axon retraction after transection SCI (Wang *et al.*, 2012). Despite these neuroprotective improvements, however, no significant differences in axon regeneration or functional recovery were detected (Wang *et al.*, 2012). Immunization with neural-derived peptides, for example A91, also confers neuroprotection and improves recovery after SCI by limiting the toxic potential of activated inflammatory cells (Ibarra *et al.*, 2010; García *et al.*, 2012; Martiñón *et al.*, 2012). Collectively, these data support the concept of protective autoimmunity, but the effectiveness of immunization as a treatment for SCI may depend upon the spinal level of injury, injury severity, genetic disposition, and timing and type of immunization (Martiñón *et al.*, 2007; Lü *et al.*, 2008; Donnelly and Popovich, 2008).

Inherent in most T cell responses is the activation of B cells and subsequent antibody secretion. Antibodies to CNS proteins are present in the serum of SCI individuals chronically (Zajarías-Fainsod *et al.*, 2012); this parallels the activation and maintenance of B cells observed in rodent models of SCI (Ankeny *et al.*, 2006, 2009; Donnelly and Popovich, 2008). B cell accumulation is detectable in the injured spinal cord in areas of dense intra-spinal antibody labelling (Ankeny *et al.*, 2009). The presence of antibodies in the cerebrospinal fluid (CSF) of injured mice increases along with B cell activation, and antibodies are absent in B cell knockout mice (Ankeny *et al.*, 2006, 2009).

Interestingly, when purified antibodies isolated from animals with SCI are injected into the intact spinal cord, they bind to cells with glial and neuronal morphologies and cause extensive tissue damage and functional impairments (Ankeny *et al.*, 2009). This, and the improved anatomical and functional recovery detected in B cell knockout mice after SCI, provides evidence that B cells, through the production of autoantibodies, may be playing a detrimental role after SCI (Ankeny *et al.*, 2006; Ankeny *et al.*, 2009; Ankeny and Popovich, 2009; Ankeny and Popovich, 2010).

The magnitude of the B cell response, and the associated B cell–mediated impairments, varies as a function of time and the level of SCI (Ankeny and

Popovich, 2010). Acutely after neurotrauma, B cell activity is blunted and the cells are non-responsive to immune challenges (Meisel *et al.*, 2005; Riegger *et al.*, 2009). As noted above, however, in the weeks to months following SCI, B cells accumulate in the spinal cord with a corresponding increase in CSF and serum antibodies (Ankeny *et al.*, 2006, 2009). This initial suppression is specific to the spinal level of injury. Sympathetic innervation of the spleen and other peripheral lymphoid tissues is regulated in part through innervation from the thoracic spinal cord. Therefore, injuries above or below the thoracic level of innervation differentially regulate B cell suppression and antibody synthesis. After high-thoracic SCI (T3), antibody synthesis is suppressed relative to after mid-thoracic injury (T9) (Lucin *et al.*, 2007).

In conclusion, the adaptive immune response to SCI is complex. There is compelling evidence that SCI activates T and B cells that have pathological and beneficial properties. The challenge remains to develop therapies that maximize the beneficial properties while not limiting their reparative effects or host defence abilities. Future efforts should focus on how differences between species, level of injury, timing, genetic disposition and type of immunization may reveal conserved beneficial targets for therapeutic exploitation.

Current clinical approaches

Given the potential for immune and inflammatory cells to enhance or limit endogenous repair processes, clinical approaches are being developed to better understand the role of neuroinflammation in SCI. For instance, the levels of inflammatory cytokines in the CSF of SCI individuals are indicative of injury severity (Kwon *et al.*, 2010b). An ongoing clinical trial is profiling this cytokine response in acutely injured SCI individuals to determine if specific inflammatory biomarkers are predictive of functional outcomes. In a similar biomarker-based clinical trial, blood levels of macrophage migration inhibitory factor (MIF) are being examined in individuals with SCI (Gensel *et al.*, 2011). Ideally, results of these clinical experiments can help elucidate the role of neuroinflammation in the pathogenesis of SCI. Through this understanding, immune therapies can then be developed to treat individuals suffering from SCI.

To date, only one acute drug therapy has been approved to treat individuals with SCI. Methlyprednisolone (MP) is a synthetic glucocorticoid with potent anti-inflammatory properties. In rodent models of SCI, MP administration decreases neutrophil and macrophage accumulation at the site of injury and improves tissue sparing and functional recovery (Behrmann *et al.*, 1994; Constantini and Young, 1994; Bartholdi and Schwab, 1995). Clinical administration of MP improves recovery for individuals with SCI (Bracken *et al.*, 1990). Although the anti-inflammatory effects of MP likely underlie its neuroprotective properties, depending upon the route of administration and dose, MP may also have adverse effects if the immune system remains suppressed

(Qian *et al.*, 2000). Nonetheless, the fact that the only clinically viable therapy for treating SCI has anti-inflammatory properties demonstrates that effectively manipulating the inflammatory response to SCI holds promise for improving recovery.

Accordingly, there are clinical trials underway examining the safety and efficacy of multi-mechanistic therapies that can reduce the inflammatory response to SCI. Minocycline is a second-generation tetracycline derivative with anti-inflammatory and neuroprotective effects when administered after experimental SCI (Kwon *et al.*, 2010a). The safety and tolerance of minocycline administration after acute SCI in humans are currently being evaluated (Gensel *et al.*, 2011). Hypothermia reduces cellular energy consumption and can slow inflammatory processes. Mild hypothermia after SCI improves recovery in rodent models (Dietrich, 2009). Optimized approaches for inducing hypothermia in humans after SCI have been developed, and the efficacy of this approach is being tested in humans (Lotocki *et al.*, 2009; Gensel *et al.*, 2011). Animals and humans treated with omega-3 polyunsaturated fatty acids after SCI show improved functional recovery compared to untreated controls (Javierre *et al.*, 2006; Michael-Titus, 2007; Lim *et al.*, 2012b). The effects are associated with decreased excitotoxic cell death, lipid peroxidation and macrophage accumulation in the injured spinal cord (Huang *et al.*, 2007; Hall *et al.*, 2012; Lim *et al.*, 2012a). Dietary supplementation with omega-3 fatty acid is being clinically evaluated as a neuroprotective therapy for SCI.

A number of therapies in clinical trials for SCI have anti-inflammatory effects that, although not the primary mode of action, may prove to be effective for treating SCI in part due to their ability to dampen the inflammatory response. The potential for current therapies to affect inflammation has been comprehensively reviewed (Gensel *et al.*, 2011), and only one example is presented here.

Clodronate is a first-generation (non-nitrogenous) bisphosphonate that, if taken up by monocytes, causes the cells to undergo apoptosis and is therefore an effective means of depleting monocyte-derived macrophages (Van Rooijen and Hendrikx, 2010). When administered after experimental SCI, clodronate reduces intra-spinal macrophage accumulation and results in improved recovery and tissue sparing (Popovich *et al.*, 1999; Kotter *et al.*, 2005; Horn *et al.*, 2008; Iannotti *et al.*, 2011; Lee *et al.*, 2011). Currently, two bisphophonates with similar monocyte depletion potentials, Zoledronic Acid and Fosamax™ (Russell *et al.*, 2008; Roelofs *et al.*, 2010), are being tested as therapies for osteoporosis after SCI. Bisphophonates cause osteoclasts to undergo apoptosis, slowing bone breakdown and loss. In addition to the therapeutic effects through apoptosis of osteoclasts, it is possible that these drugs could also improve function after SCI through a reduced macrophage accumulation in the injured spinal cord.

Clinical therapies have also been proposed and tested that boost the inflammatory response to promote recovery and tissue repair. ProCord is a

proprietary procedure where autologous macrophages from SCI individuals are isolated and then stimulated *ex vivo* (through co-activation with skin biopsies) to adopt a reparative phenotype. These presumptively pro-regenerative macrophages are then surgically transplanted into the injured spinal cord (Kigerl and Popovich, 2006; Jones *et al.*, 2010). Based on the success of a phase 1 clinical trial and in rodent models, this technique was tested in a phase 2 clinical trial from 2003 to 2006 (Schwartz and Yoles, 2006; Lammertse *et al.*, 2012). Although the trial was prematurely terminated for unrelated financial reasons, analysis failed to show a significant improvement between untreated and treated controls (Lammertse *et al.*, 2012). While the results of the study are potentially disappointing, the clinical techniques and infrastructure were created to support future studies examining macrophage manipulations in a clinical setting (Jones *et al.*, 2010).

Protective autoimmunity has been proposed as means of promoting recovery through a boost in the immune response to SCI (Schwartz, 2001). No clinical trials are yet underway testing this hypothesis in SCI individuals. Copaxone, however, activates a variety of self-reacting T cells to induce protective autoimmunity and, when administered to individuals with multiple sclerosis, significantly improves outcomes as tested in a phase 3 clinical trial (Fox *et al.*, 2012). A patent was issued to use Copaxone to treat neurological disorders, and it is conceivable that this drug will be tested in SCI individuals in the future.

Conclusion

The neuroinflammatory response to SCI is complex. Multiple cell types interact in a time- and context-dependent manner to coordinate different aspects of CNS repair. Non-specific functions of activated immune cells can also create bystander damage and secondary injury. Information is being gained through basic and clinical science research that will inform the field about this complex response. As we learn more about the role of neuroinflammation in the repair and pathology in SCI, hopefully therapeutic interventions can be developed with maximal benefits for individuals suffering from spinal cord injuries.

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15
Immune Responses to Tumours in the CNS

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Tumours of the CNS

Compared to tumours elsewhere in the human body, the diversity and complexity of CNS tumours have been described as being unrivalled. Based on systematic histopathological analysis for more than a century, a World Health Organization (WHO) scheme of CNS tumours has emerged that is now accepted worldwide as the gold standard for classification of these neoplasms (Louis *et al.*, 2007). An important distinction in the spectrum of CNS tumours is that of primary (i.e. arising from the CNS or its coverings) versus secondary neoplasms (i.e. metastases in or around the CNS from malignant tumours elsewhere in the body). Although primary tumours are much less common than CNS metastases of, for example, lung and breast cancer, they are on the top of the list of 'average number of years of life lost' by cancer (Burnet *et al.*, 2005). This can at least partly be explained by two important facts: (i) the most frequent primary brain tumour is glioma, and most gliomas show very extensive, diffuse infiltrative growth in the surrounding CNS parenchyma, precluding curative therapy (Claes *et al.*, 2007); and (ii) CNS tumours are relatively frequent in children, and many of those are highly aggressive.

Although CNS tumours have been, up till now, classified based on their histopathological characteristics, during the last decade insight into the molecular aberrations underlying their oncogenesis has increased in a

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revolutionary way. It is increasingly clear that a combination of morphological and molecular characteristics will soon allow for a much more robust and clinically meaningful 'taxonomy' (typing and grading) of CNS tumours, especially of gliomas and embryonal tumours. Also, the molecular underpinnings provide information on the pathways that might be targeted with different therapeutic compounds.

Increased knowledge about the genetic and molecular aberrations underlying oncogenesis has accumulated in a landmark article summarizing the tasks that a cancer cell generally needs to accomplish to successfully survive and grow as a tumour: sustain proliferative signalling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis and activate invasion and metastasis (Hanahan and Weinberg, 2000). More recently, conceptual progress resulted in the addition of two hallmarks: reprogramming of energy metabolism and evading of immune destruction. Furthermore, it was acknowledged that genome instability in tumour cells and inflammation are characteristics facilitating or even enabling the hallmarks just listed (Hanahan and Weinberg, 2011).

The most frequent CNS tumours will now be briefly introduced.

Primary tumours of the CNS

Figure 15.1 compares the frequency of primary tumours of the CNS.

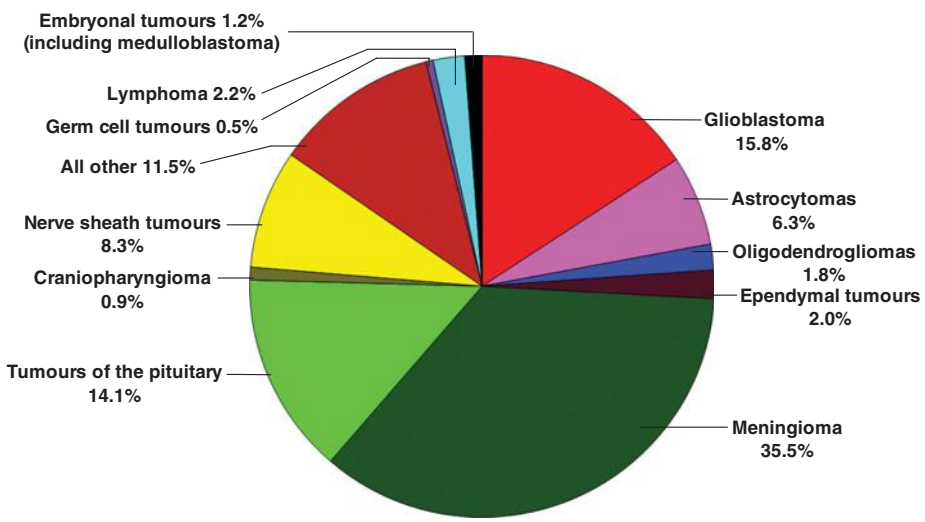

Figure 15.1 Relative frequency of primary tumours of the CNS in 311,202 patients from 2005 to 2009 as registered by the Central Brain Tumor Registry of the United States (CBTRUS) (see Dolecek *et al.*, 2012).

Gliomas Gliomas are considered to originate from glial cells or their precursors and may show astrocytic, oligodendroglial or ependymal differentiation or a combination thereof. In adult patients, most gliomas belong to the category of 'diffuse gliomas' (i.e. glial tumours showing extensive, diffuse, infiltrative growth in the surrounding brain parenchyma). The diffuse gliomas are astrocytic, oligodendroglial or mixed oligoastrocytic in nature. Based on histopathological parameters such as marked mitotic activity, a peculiar form of angiogenesis ('florid' or even 'glomeruloid' microvascular proliferation) and/or necrosis, a malignancy grade can be assigned to these tumours. The least malignant diffuse glioma is designated as WHO grade II, and the most malignant (and unfortunately also the most frequent) astrocytic tumour is traditionally coined as glioblastoma (WHO grade IV). In between these is a category of anaplastic (WHO grade III) diffuse gliomas. Both low-grade (WHO grade II) and high-grade (WHO grade III and IV) diffuse gliomas occur in all age groups, but glioblastomas are relatively frequent in patients older than 50 years of age, whereas young adults are more often diagnosed with a diffuse low-grade glioma. Unfortunately, these latter tumours have a strong tendency to progress to more malignant neoplasms in the course of years.

Important other groups of gliomas include the ependymomas and 'variant astrocytic gliomas'. These 'other' gliomas tend to show more circumscribed growth and to occur much more frequently in the paediatric and young adult age group. Although ependymomas can be low- or high-grade malignant, most 'variant astrocytic gliomas' are low grade (WHO grade I or II) and, in contrast to diffuse gliomas, do not show progression to a more aggressive lesion in the course of time. By far the most frequent 'variant glioma' in the paediatric age group is pilocytic astrocytoma, a WHO grade I lesion that most frequently occurs in the cerebellum, the hypothalamic region and/or the optic nerve.

About 5 years ago it was demonstrated that WHO grade II and III diffuse gliomas in adult patients often carry an isocitrate dehydrogenase 1 (*IDH1*) or (less frequently) *IDH2* mutation. Earlier, complete co-deletion of the short arm of chromosome 1 and of the long arm of chromosome 19 (complete 1p/19q co-deletion) was reported as a characteristic molecular aberration for oligodendroglial tumours, whereas high copy amplification of the epidermal growth factor receptor (*EGFR*) and the presence of its variant III (*EGFRvIII*) are typically found in glioblastomas (Riemenschneider *et al.*, 2010). Diffuse gliomas in the paediatric age group have a different molecular background, with relatively frequent mutations in *Histon-H3* or the *ATRX* (alpha thalassemia–mental retardation syndrome X-linked) gene (Sturm *et al.*, 2012). Pilocytic astrocytomas often harbour aberrations related to *BRAF* (the *BRAF V600E* mutation or *KIAA1549–BRAF* fusion gene) (Pfister *et al.*, 2010).

Using gene expression data from the Cancer Genome Atlas Project, gliomas can be divided into four subgroups: classical, neural, proneural and mesenchymal. These subtypes are associated with aberrations of *EGFR*, neurofibromin-1 (*NF1*), platelet-derived growth factor receptor alpha

(*PDGFRA*) and *IHD1*. Furthermore, a link was described with response to therapy, with aggressive treatment being the most effective for the classical subtype and having a more limited effect on tumours of the proneural subtype (Verhaak *et al.*, 2010).

Meningiomas Tumours originating from meningothelial cells and arachnoid cap cells in the (lepto)meninges are traditionally designated as meningiomas. The vast majority of these tumours are benign (WHO grade I) and show a compact, expansive growth pattern, allowing for relatively easy surgical removal (or highly focussed irradiation in cases where the tumour cannot be adequately removed by surgery). Based on increased mitotic activity and/or a number of other microscopic features (including necrosis, high cellularity and patternless growth) a small subset of meningiomas is graded as WHO grade II ('atypical'; up to 8% of meningiomas) or even as WHO grade III ('anaplastic' or malignant; 1–2% of meningiomas). Also, meningiomas showing infiltrative growth in the brain parenchyma are known to be associated with a substantially higher recurrence rate, comparable to atypical meningiomas, and are therefore also graded as WHO grade II.

Embryonal tumours CNS tumours characterized by high cellularity with small, (apparently) poorly differentiated, highly proliferative tumour cells are grouped together as the high-grade malignant (WHO grade IV) category of 'embryonal tumours'. Many of these tumours show signs of neuronal differentiation at the immunohistochemical level and can be designated as primitive neuro-ectodermal tumours (PNETs). PNETs are rare in adult patients but relatively frequent in the paediatric age group (about 15% of CNS tumours in children aged 0–14 years) (Dolecek *et al.*, 2012). By far, the most common representative in this category is medulloblastoma, a neoplasm that by definition originates in the posterior fossa/cerebellum. Morphologically, medulloblastomas are subtyped as classic, desmoplastic/nodular, extremely nodular, and large cell/anaplastic. The desmoplastic and extremely nodular variants are associated with a significantly better prognosis, whereas the large-cell or anaplastic subtype shows an even more aggressive behaviour compared to classic medulloblastomas. Recently, molecular characteristics were elucidated that not only provide more robust information on the clinical behaviour that is to be expected, but also may help to design and implement more effective, molecularly targeted therapies (Northcott *et al.*, 2012).

Other relatively frequent embryonal tumours of the CNS are supratentorial PNETs, pineoblastomas and atypical teratoid–rhabdoid tumours (AT/RTs). Supratentorial PNETs and pineoblastomas are often morphologically and immunohistochemically indistinguishable from classic medulloblastomas, and it is then its site of origin that determines the precise name that is given to the lesion. It is increasingly clear, however, that these tumours indeed differ not only in their location but also in their molecular underpinnings.

AT/RTs are relatively recently defined as a separate group of embryonal tumours, with typically 'rhabdoid features' (globular eosinophilic condensations) in the cytoplasm of (part of) the tumour cells. Of note, microscopic delineation of PNETs from AT/RTs can be difficult, especially in cases in which the rhabdoid features are not that prominent. Here, too, molecular markers are very helpful as most AT/RTs show loss of function of the *SMARCB1* tumour suppressor gene (SWI–SNF (switch/sucrose nonfermentable) related, matrix-associated, actin-dependent regulator of chromatin subfamily B member 1; also called *INI1*) or, less frequently, of the *SMARCA4* gene (Hasselblatt *et al.*, 2011).

Other primary tumours Relatively frequent other primary tumours of the CNS are pituitary adenomas (generally benign, but diverse with regard to hormonal production), schwannomas (benign, originating around the CNS from the roots of the cranial nerves (relatively frequently in the cerebellopontine angle) or from the nerve roots in the spinal canal) and primary CNS lymphomas (over 95% of these being high-grade malignant, diffuse large B cell lymphomas and relatively frequently occurring in immune-compromised patients). Less frequent neoplasms include primary germ cell tumours of the CNS, a very heterogeneous group of variable malignancy (with germinomas, embryonal carcinomas, choriocarcinomas and yolk sac tumours being high-grade malignant, and mature teratomas being benign), and haemangioblastoma (benign, extremely well-vascularized and often partly cystic neoplasms that occur in the context of Von Hippel–Lindau disease in about one-quarter of patients with a haemangioblastoma).

Secondary tumours

Secondary or metastatic tumours in and around the CNS are the most frequent CNS neoplasms. Some cancers show a significantly higher propensity to form CNS metastases (particularly carcinoma of the lung, breast, kidney and melanoma) than others (e.g. colorectal and prostate cancer). Also, the metastatic lesions may be single (and thereby more easily amenable to local surgical therapy and/or intense irradiation) or multiple, and metastases may occur in different compartments of the CNS (intraparenchymal, leptomeningeal and intraventricular).

Blood–brain barrier in CNS tumours

A very important aspect in clinical neuro-oncology is the fact that the CNS microvasculature normally forms a blood–brain barrier (BBB). This barrier is elicited by the interaction between the microvascular endothelial cells and the perivascular astrocytic endfeet. The barrier function is established by a combination of tight junctions between the endothelial cells, only limited

trans-endothelial transport via pinocytotic vesicles and the presence of transporter molecules on the endothelial cells that are specialized in keeping out compounds that are harmful for the brain parenchyma (Daneman, 2012). Understandably, this barrier function contributes to reduced efficacy of chemotherapeutic compounds for CNS tumours. Furthermore, because of the presence of the BBB, the CNS has for many years been considered as an immune-privileged site (De Micco, 1989; Mitchell *et al.*, 2008) (see Chapter 1).

When performing T1-weighted magnetic resonance imaging (MRI) after intravenous injection of the paramagnetic contrast agent gadolinium–diethylenetriaminepentacetate (gadolinium–DTPA, an agent that passes the blood vessel wall only when the BBB is defective), contrast enhancement of a CNS tumour unequivocally means loss of the barrier function in the enhancing area. In diffuse low-grade gliomas, contrast enhancement is generally absent, indicating that the infiltrating glioma cells in the brain parenchyma leave the BBB relatively unaffected. In many other CNS tumours, however, the BBB function is incomplete or even lost (Claes *et al.*, 2007). This is true not only for high-grade malignant tumours located in the brain parenchyma ('intra-axially') such as high-grade gliomas and metastases, but also for benign and WHO grade I neoplasms such as pilocytic astrocytomas and haemangioblastomas. Meningiomas generally show intense contrast enhancement but these neoplasms are located outside the brain parenchyma ('extra-axially'). In a subset of meningioma patients, the surrounding brain tissue shows oedema but without contrast enhancement.

The inflammatory infiltrate in CNS tumours

A traditional neuropathologist's perspective

Based on examination of haematoxylin-and-eosin (H&E)-stained sections, neuropathologists are well aware of the fact that striking inflammatory infiltrates may occur in CNS tumours with a highly variable contribution of lymphocytes, plasma cells, macrophages and neutrophilic and/or eosinophilic granulocytes. Generally, the presence or absence of such an infiltrate does not have an impact on the diagnosis. Rather, the histopathological diagnosis is based on the characteristics of the tumour cells themselves and on the results of some other tumour–microenvironment interactions such as infiltrative growth, necrosis and microvascular changes, whereas the inflammatory infiltrate is often considered as something that 'just happens'. This is especially true in cases where inflammatory cells can be explained as a logical consequence of, for example, hypoxia and necrosis (macrophages). Microglial cells, the resident, antigen-presenting cells of the CNS, are inconspicuous in most routinely performed histochemical stainings.

Some CNS tumours are well known for the occurrence of a particularly prominent lymphocytic infiltration. Examples are diffuse astrocytic tumours

with gemistocytic change (i.e. 'ballooning' of the cytoplasm of the astrocytic tumour cells because of accumulation of glial fibrillary acidic protein (GFAP) filaments) and 'variant' gliomas such as pleomorphopic xanthoastrocytoma and ganglioglioma that often show dispersed, prominent perivascular cuffs of small lymphocytes. Furthermore, germinoma (the most frequent primary germ cell tumour of the CNS, occurring especially in the pineal gland of adolescent boys) classically shows a combination of clusters of large tumour cells and dense infiltrates of small lymphocytes. Thereby, germinomas look exactly the same as their counterparts originating in the testis (seminoma) and ovary (dysgerminoma). Like lymphomas elsewhere in the body, primary CNS lymphomas are composed of a mixture of lymphoid tumour cells (in over 95% of the cases, large and immature B cells) and non-neoplastic inflammatory cells, especially lymphocytes. In Langerhans cell histiocytosis (LCH) (a rare 'haematopoetic tumour' that most often occurs in children and young adults), remarkable accumulation of eosinophilic granulocytes is often seen. The presence of prominent infiltrates of lymphocytes (or eosinophilic granulocytes) may thus guide the neuropathologist towards the correct diagnosis and occasionally even allows for the diagnosis of a specific tumour subtype: in the lymphoplasmacyte-rich subtype of meningioma, the accumulation of lymphocytes and plasma cells can be so intense and widespread that the meningothelial tumour cells are difficult to identify (Louis *et al.*, 2007).

Revelations by immunochemistry

Soon after immunohistochemical staining became available, it was clear that the number of inflammatory cells in CNS tumours was often much higher than had been recognized in H&E-stained sections (Figure 15.2). For instance, in an immunohistochemical study using antibodies for microglial cells and macrophages, large numbers of these cells could be demonstrated in gliomas (Roggendorf *et al.*, 1996). Furthermore, the inflammatory cells in CNS tumours could be further characterized, and differences between different (sub)types of tumours could be investigated. For example, in a study of glioblastomas and pilocytic astrocytomas, glioblastomas exhibited

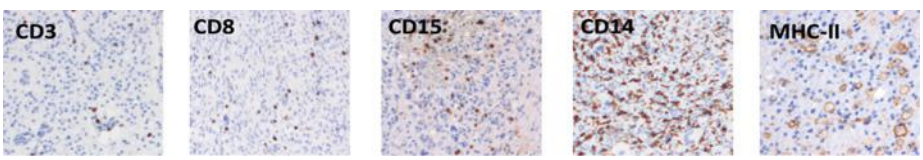

Figure 15.2 Examples of immunohistochemical detection of immune cells in glioblastoma; histological sections of formalin-fixed, paraffin-embedded tumour tissue showing the presence of T cells (CD3), cytotoxic T cells (CD8), granulocytic cells (CD15) and microglia and macrophages (CD14, MHC-II).

significantly higher perivascular cytotoxic $CD8^+$ T cell infiltration, as well as more perivascular and intratumoural $CD56^+$ natural killer (NK) cells and $CD68^+$ macrophages. The CD3, CD20 and leukocyte common antigen (LCA)-positive cells did not differ between glioblastoma and pilocytic astrocytoma (Yang *et al.*, 2011). The same group reported that a relatively high $CD8^+$ T cell infiltrate in newly diagnosed glioblastoma was associated with prolonged survival (Yang *et al.*, 2010b). In another study of glioblastoma patients, a moderate but not significant inverse association between regulatory T cells (Tregs) and survival was noted (Jacobs *et al.*, 2010).

Using fluorescence-activated cell sorting of freshly obtained human glioblastoma tissue, microglial cells and macrophages were reported to be the predominant immune cell type in gliomas (about 1% of total number of cells) (Hussain *et al.*, 2006). Using gene expression data from the Cancer Genome Atlas Project and the Gene Expression Omnibus database, an enrichment of immune response–related gene expression was found in the 'mesenchymal' subtype of adult patients with glioblastoma that is associated with poor prognosis. Immunostaining of these tumours indeed revealed significantly higher cell numbers of microglial cells and macrophages in this subtype (Engler *et al.*, 2012). Using antibodies to CD68 and human leukocyte antigen (HLA)-DR in immunohistochemistry, a significant number of microglial and macrophage cells was observed in most epilepsy-associated glioneuronal brain tumours, the density of activated microglial cells being correlated with the duration of epilepsy and with the frequency of seizures prior to surgical resection (Aronica *et al.*, 2005).

Relatively little is known about the exact composition of the immune cell infiltration in non-glial CNS tumours. In one study investigating a series of 67 intracranial neoplasms including glioblastomas, meningiomas, medulloblastomas, metastases of carcinoma and melanoma, the level of $CD68^+$ microglial cells and macrophages was higher in glioblastomas and adenocarcinomas than in medulloblastomas and meningiomas. Lymphocytes (as detected by staining for LCA) were described to be rare in all tumour types, generally not exceeding 2% of tumour cells and not bound to morphologically distinct tumour regions (Strik *et al.*, 2004). Jacobs *et al.* (2009) reported that the percentage of Tregs (characterized as being $CD4^+$ $FoxP3^+$ CD25 high and CD127 low) in a spectrum of brain tumours showed a strong correlation with WHO grade.

Biology of immune responses to CNS tumours

Present and potent?

The CNS has been considered as an immune-privileged site because immune cells were thought to less easily pass the BBB and because of the lack of an obvious lymphatic drainage system in the brain. However, animal studies showed that antigens from the brain can be drained to the cervical lymph

nodes (Cserr and Knopf, 1992) (see Chapter 1). Nowadays, it is generally accepted that continuous immunosurveillance is also present in normal brain tissue, and it is an open question if tumour tolerance in the brain is really fundamentally different from that of non-CNS sites of tumourigenesis (Johnson *et al.*, 2012).

Based on recent advances in understanding CNS immunity, it is more accurate to regard the CNS as an immunologically specialized site which can be divided into three different compartments: the brain parenchyma, the ventricles containing choroid plexus and cerebrospinal fluid, and the meninges. Whereas the immune reactivity within the ventricles and meninges is more similar to that elsewhere in the body, within the brain parenchyma immune responses are often delayed or even abrogated (Grauer *et al.*, 2009). This might be related to the absence of MHC II^+ dendritic cells (DCs) in the healthy brain parenchyma (Hart and Fabre, 1981), whereas these cells are present in the choroid plexus and meninges (Mcmahon *et al.*, 2006). Different DC subsets do accumulate within the brain parenchyma under various inflammatory conditions, suggesting a role in antigen presentation. The exact role of microglial cells, the brain resident antigen-presenting cells (APCs), in modulating intracranial immune responses is still unclear, but it may be much more diverse and dynamic than previously thought and result in containment as well as aggravation of the disease process (Graeber *et al.*, 2002; Hanisch and Kettenmann, 2007; Yang *et al.*, 2010a).

Humoral responses against CNS tumours may be elicited against tumour-associated antigens. Examples of such antigens are cancer–germ line genes (CGGs) and cancer–testis genes, self-antigens, differentiation antigens and stromal antigens (Zhang *et al.*, 2007). CGGs are physiologically expressed in germ line cells, mostly intracellularly. Over 40 CGG antigen family members have been identified, most of them in melanomas. Interestingly, several of these melanoma antigen-encoding (MAGE) genes (e.g. *MAGE-1*, *MAGE-3* and *MAGE-E1*) can also be detected in malignant gliomas. The fact that glial cells and melanocytes are both from neuroectodermal origin may help to explain this phenomenon. Other CGGs that have been described in gliomas include *Homo sapiens* testis-14 (*HOM-TES-14*) (also known as stromal cell–derived protein-1 (*SCP-1*)), synovial sarcoma X breakpoint-1 (*SSX1*), *SSX2*, *SSX4*, *HOM-TES-85* and *Sry* (sex-determining region Y)–related high-mobility group (HMG) box-containing gene 6 (*SOX-6*).

Self-antigens in gliomas with restricted protein expression encompass EGFRvIII, the interleukin-13 receptor α chain-2 (IL-13Rα2) and transferrin receptors. These latter receptors are especially overexpressed on rapidly dividing cells including glioblastoma cells. Also, self-antigens can be expressed on the cell surface facilitating targeting by specific antibodies. Relatively recently, cytomegalovirus (CMV) antigens were reported to be expressed in high-grade gliomas but not in surrounding non-neoplastic brain tissue (Scheurer *et al.*, 2008).

Examples of differentiation antigens (i.e. antigens that are normally expressed only in particular phases of cell differentiation) are tyrosinase-related protein-1 (TRP1), TRP2, gp100, antigen isolated from immunoselected melanoma-2 (AIM2), human epidermal growth factor receptor-2 (HER2, also known as ErbB2), ADP ribosylation factor-4-like protein (ARF4L) and UDP–Gal:betaGlcNAc beta1, 3-galactosyltransferase, polypeptide-3 (GALT3).

Stromal antigens are expressed on stromal components of the tumour such as microvascular cells (endothelial cells and pericytes) and the extracellular matrix (ECM) (Grauer *et al.*, 2009). An interesting stromal component in this respect is tenascin, a polymorphic ECM glycoprotein that is expressed in the vast majority of high-grade gliomas (Behrem *et al.*, 2005).

In some patients, T cells directed against some of the antigens mentioned here were found (Liu *et al.*, 2004). Necrosis in the CNS may well facilitate antigen drainage and activate T cells that can enter the brain parenchyma. Destruction of the BBB makes the neoplasm even more accessible to the immune system. The attraction of immune cells is directed by a number of molecules, which may well be produced by oxygen-deprived tumour cells surrounding necrosis. Hypoxia inducible factor-1α (HIF1α) has been identified as a key factor in recruiting immune cells to gliomas via chemokines including C–X–C motif ligand (CXCL)5, CXCL8–IL8, CXCL12–stromal cell–derived factor-1 and different members of the C–C motif ligand (CCL) family (Grauer *et al.*, 2009). A recent study demonstrated increased infiltration of all T cell subsets in high-grade gliomas, and within glioblastomas a significant correlation between elevated numbers of effector T cells (Teffs) and better survival was seen (Lohr *et al.*, 2011).

Or rather: present but permissive?

In the 'Present and potent?' section, several antigens were highlighted against which an immune reaction might be beneficial to attack the tumour. However, not all targets might be potent enough to do so. Although tumours expressing CGGs tend to co-express different CGG antigens, these antigens may be present in only a small percentage of the total population of tumour cells. Also, efficient immune recognition of CGG antigens on glioma cells may be compromised by the limited presence or even absence of classical MHC molecules and/or inefficient processing and presentation of such antigens (Grauer *et al.*, 2009). Importantly, these genes are generally expressed intracellularly and therefore more difficult to target with antibodies.

Tumours can also diminish expression of self-antigens expressed on the cell surface to avoid detection by the immune system, either by down-regulation or by decreased trafficking to the cell surface. In studies using EGFRvIII as a target molecule, it was found that the majority of the recurrent tumours had lost EGFRvIII expression (Sampson *et al.*, 2010), indicating the need for

multi-target approaches. The value of antigens that are the product of differentiation genes as targets for anti-glioma therapy might be limited, as these antigens often are expressed in a broader range of tissues.

Apart from evading the immune system, gliomas can also directly and indirectly contribute to an immunosuppressive microenvironment via production of soluble factors, enzymes and/or surface molecules (Grauer *et al.*, 2009). Glioma cells were reported to produce immunosuppressive soluble factors like vascular endothelial growth factor (VEGF), IL10, transforming growth factor beta (TGFβ) and enzymes such as indoleamin-2,3-dioxygenase (IDO), cyclooxygenase-2 (COX2) and inducible nitric oxide synthase (iNOS), whereas production of immune-stimulatory molecules like IL12, IL18 or interferon gamma by these cells is relatively lacking. Immuno-inhibitory molecules that can be abundantly expressed on the surface of glioma cells include the non-classical MHC molecules HLA-G and HLA-E, the co-inhibitory molecule programmed cell death-1 ligand-1 (PDL1) (also known as B7 homolog-1 (B7H1)), minor brain gangliosides like GM2 and GM3, and galectin-1 and -3. Gliomas can also express other members of the B7 family, including B7H3 and B7H4. There are also reports of low expression of costimulatory B7 molecules (CD80/86) on glioma cells which may antagonize immunity by inducing T cell anergy (Anderson *et al.*, 2007). Although functional analysis demonstrated that glioma-infiltrating microglial cells and macrophages may have a few intact innate immune functions, their capacity to be stimulated via Toll-like receptors (TLRs), secrete cytokines, up-regulate costimulatory molecules and in turn activate anti-tumour T cells is apparently not sufficient to initiate effective immune responses (Hussain *et al.*, 2006).

Recent evidence suggests that glioma cells may exploit rather than be inhibited by microglial cells. *In vitro* studies revealed that the glioma-derived chemokine CCL2 acts upon C–C chemokine receptor (CCR)-2-bearing microglia, which then produce IL6 to stimulate the invasiveness of gliomas in a three-dimensional gel matrix (Zhang *et al.*, 2012). Also, the presence of lymphocytes in glial tumours was originally considered as an attempt of the body to attack the tumour cells and thus a beneficial response. The occurrence of perivascular lymphocytic infiltrates in 'variant gliomas' with a relatively indolent clinical behaviour might be considered as evidence for this hypothesis. However, gemistocytic astrocytomas also are known for prominent perivascular lymphocytic infiltrates but tend to carry a poorer prognosis compared to their non-gemistocytic counterpart of the same malignancy grade. Indeed, the correlation between the presence of different immune cell infiltrates in the tumour tissue and the clinical course of glioma patients is not clear. Although it was found that elevated numbers of Teffs correlate with better survival in glioblastoma patients, it is also known that when these cells reach the CNS parenchyma through the intact or damaged BBB, resident CNS cells such as astrocytes and microglial cells are able to suppress T cell proliferation and to induce T cell anergy or apoptosis, by the secretion of immunosuppressive

molecules and the expression of death receptor ligands such as Fas ligand (FasL). T cell effector functions in the brain (including crossing of the BBB) may be further impeded by constitutive immunosuppressive factors such as TGFβ (Lohr *et al.*, 2011).

Furthermore, Teffs in and around gliomas are confronted with other populations of immune cells with suppressor activity that are attracted to the tumour site (Grauer *et al.*, 2009; Lindau *et al.*, 2013) (Figure 15.3). Detailed

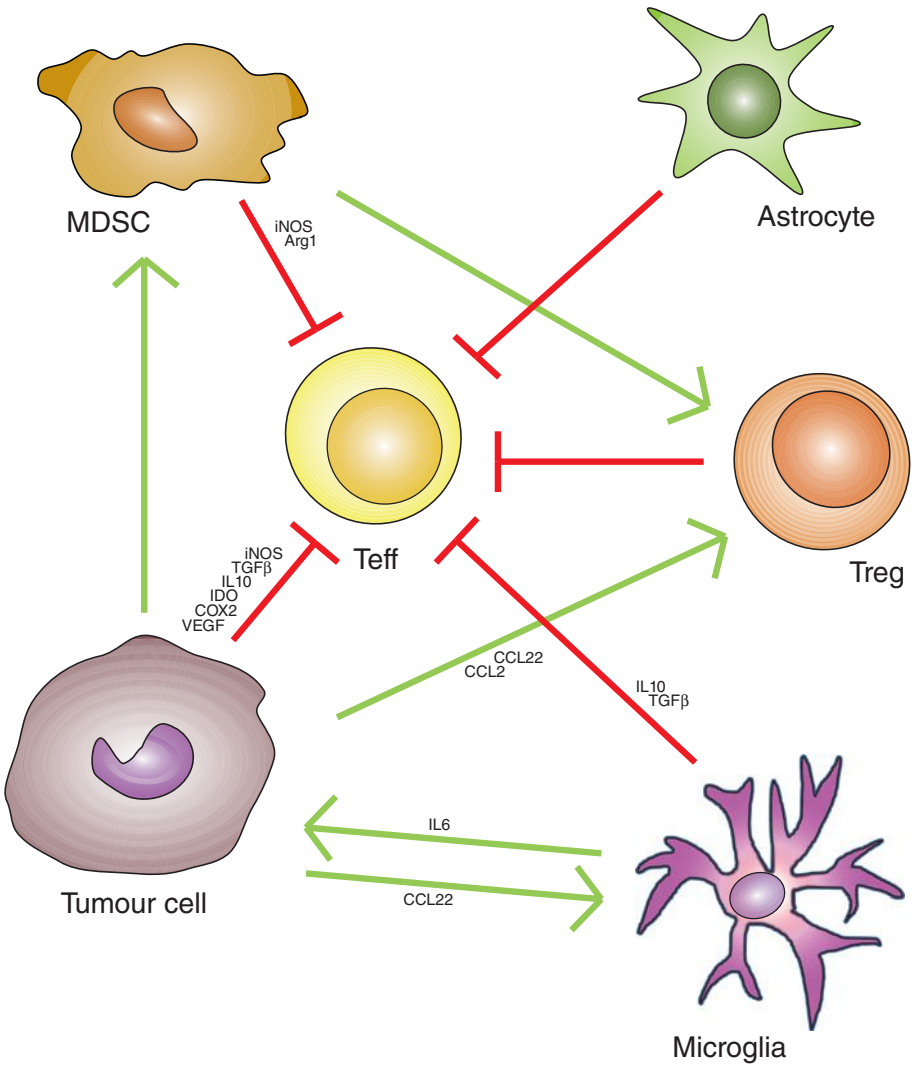

Figure 15.3 Simplified scheme of crosstalk between cells in a glioblastoma microenvironment suppressing an effective T cell response. Suppressive cells can activate one another, resulting in multiple ways of suppressing Teff cells.

immunohistological and functional analyses of diffuse gliomas demonstrated that $CD4^+$ Tregs infiltrate and accumulate within gliomas. Tregs are well known to suppress an effective cytotoxic response of other T cells. Tregs have been reported to differ in homing receptor expression and chemokine responsiveness. The Treg accumulation in glioblastomas might be explained by the fact that (in contrast to other tumour-infiltrating lymphocytes) these cells have a high level of expression of the CCR4 chemokine receptor, whereas its ligand CCL22 is secreted by glioblastoma cells (Jacobs *et al.*, 2009, 2010). More recently, an important role was attributed to myeloid-derived suppressor cells (MDSCs) in creating an immunosuppressive microenvironment. MDSCs represent a heterogeneous population in which generally monocytic and granulocytic subpopulations are recognized. These cells have been reported within the tumour of both the *de novo* and transplantable syngeneic GL261 cell line mouse models where depletion of these cells inhibited the development of gliomas (Kohanbash and Okada, 2012). Granulocytic MDSCs were reported to be increased within the peripheral blood of glioma patients (Raychaudhuri *et al.*, 2011). Furthermore, these cells were described in tumour tissue of patients with cancers for example, breast and gastrointestinal tract and to correlate with tumour stage and/or Treg levels. Recently, a study was published reporting MDSC infiltration in glioma as well (Kohanbash *et al.*, 2013). Recent studies indicate that MDSCs can promote induction of Tregs. The partially overlapping target cell populations of both suppressor cell types indicate flexibility in immune suppression under pathological conditions (Lindau *et al.*, 2013).

Systemic aspects

Apart from the accumulation of 'immune cells' in the CNS tumour tissue, these tumours may also exert systemic effects on the immune status of the patients. Again, this phenomenon has been studied most extensively in patients with gliomas and glioblastomas. Patients with glioblastomas are reported to suffer from a decreased systemic activity of their immune system (Gousias *et al.*, 2010), a phenomenon that is ascribed to the immunosuppressive effect of cytokines released by the tumour cells. More precisely, reduced cellular immune responses in brain tumour patients were reported with profound depression of $CD4^+$ helper lymphocytes. Although the absolute count of both $CD4^+$ and $CD4^+$ Tregs is diminished in glioma patients, the Tregs frequently represent an increased proportion within the $CD4^+$ compartment (Fecci *et al.*, 2006). This predominance of Tregs may partly explain the immunological defects that have been described in glioma patients, such as abnormal delayed hypersensitivity responses, depressed mitogen responsiveness of T and B cells, decreased antibody responses and impaired T cell cytotoxicity. A recent study provided further evidence of systemic immune suppression by showing accumulation of MDSCs in peripheral blood and

increased arginase activity (arginase being a known immunosuppressive enzyme) within the serum of glioma patients (Raychaudhuri *et al.*, 2011).

Immunotherapy of CNS tumours

Different strategies

There is ample evidence that in glioma patients, the differentiation, maturation and function of tumour-infiltrating DCs and other APCs as well as the generation and activation of immune effector cells, and the cytolytic activity of macrophages, NK cells and cytotoxic T cells are suppressed. The ultimate goal of anti-tumour immunotherapy is to induce an immune response leading to the complete eradication of tumour cells without serious negative side effects. For gliomas, passive, adoptive and active immunotherapeutic approaches have been evaluated (for detailed information, see Mitchell *et al.*, 2008, Grauer *et al.*, 2009, Xu *et al.*, 2012, and references therein) (Figure 15.4).

Passive immunotherapy To target glioma-specific structures by passive immunotherapy, different monoclonal antibodies (Mabs) have been designed that are coupled to radionucleotides (radio-immunoconjugates) or exotoxins (immunotoxins) which are administered locally. An ideal target for immunotherapy is an antigen that is specifically and stably expressed by the tumour, absent from normal tissues and crucial for the survival of the cancer cell. CGG antigens have the potential to match such requirements, but a drawback is that these genes are generally expressed intracellularly. In some studies, a survival benefit in glioma patients has been documented by performing passive immunotherapy. However, the effectiveness of the therapy seems to be restricted to patients with a low tumour burden. Furthermore, a disadvantage of this approach is that the penetration of these molecules into the tumour tissue appears to be quite limited, due to high interstitial pressure in the tumour and its surrounding tissue. Intra-tumoural inoculation and convection-enhanced delivery (CED) might be needed to efficiently deliver these molecules to the more peripheral parts of diffuse infiltrative gliomas.

Adoptive immunotherapy Using adoptive immunotherapy, transfer of *ex vivo* expanded and activated effector cells into the patients with malignant gliomas has been investigated. Treatment approaches have differed in the types of cells administered, the route of administration and the activation status of the cells. An advantage of adoptive immunotherapy is that it circumvents deficits of anti-tumour effector cell populations in the host. However, despite good tolerability, clinical efficacy has rarely been seen with the local administration of lymphokine-activated killer cells (LAK cells), mitogen-activated killer cells (MAKs), NK cells, tumour-infiltrating lymphocytes (TILs) and allogeneic or autologous tumour-specific cytotoxic T cells. Pro-inflammatory cytokines such

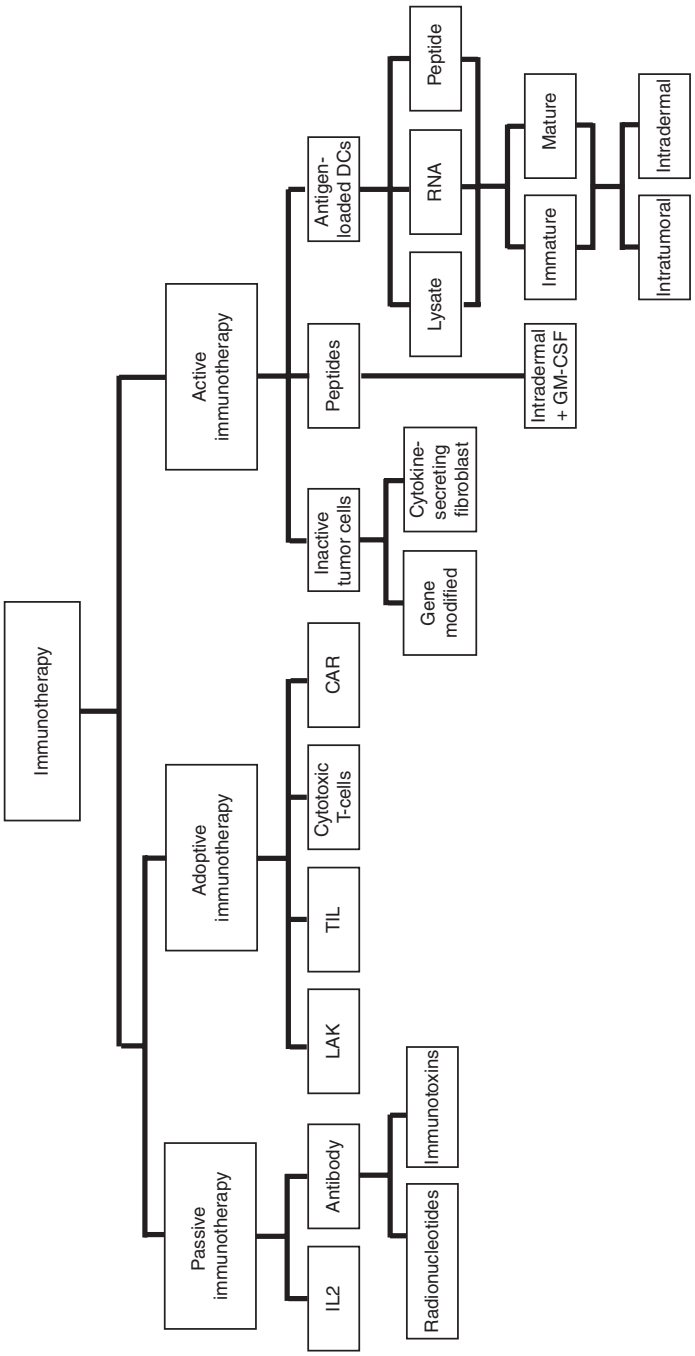

Figure 15.4 Scheme of different immunotherapeutic approaches for CNS tumours.

as IL2 have been co-administered locally to enhance anti-tumour responses, but have been reported to induce significant toxicity, particularly in patients with high tumour burden.

More recently, the use of genetically engineered autologous T cells expressing a novel T cell receptor or chimeric antigen receptor (CAR), which can specifically recognize a tumour-associated antigen, has been explored. CARs, sometimes referred to as 'T-bodies', can recognize intact cell surface antigens and selectively kill the tumour cells in a way that is independent of HLA recognition. More recently, also second- and third-generation CARs have been developed to enhance the signalling of the CAR by engineering additional CD28 and OX40 domains (Essand and Loskog, 2013). CARs have been developed to recognize EGFRvIII (Morgan *et al.*, 2012), and a clinical trial is currently underway using CARs targeting HER2 in glioblastoma patients (NCT01109095).

Active immunotherapy Active immunization of patients with malignant gliomas is regarded as a powerful strategy to induce a potent anti-tumour response. As a vaccine, autologous inactivated tumour cells have been used, either gene-modified or applied together with cytokine-secreting fibroblasts. Alternatively, tumour-specific peptide vaccines targeting EGFRvIII have been administrated intradermally along with granulocyte–monocyte colony-stimulating factor as adjuvants. One promising approach to amplify tumour-specific T cell responses is to use *ex vivo*–generated, autologous tumour antigen-loaded DCs as a vaccine. The induction of effective immune responses is dependent upon proper DC maturation. Tumour antigen loading with tumour lysate, tumour RNA or synthetic tumour-specific peptides occurs at either the immature or mature DC stage. Mature, tumour antigen–loaded DCs are then injected back into patients. Several phase I and II studies have been performed with this approach, and although these clinical trials differed in terms of DC generation, loading of tumour antigens and the route of application, robust intra-tumoural cytotoxic and memory T cell infiltration could be detected in patients who underwent re-operation after vaccination. DC vaccinations appeared to be mainly beneficial for younger patients with minimal residual tumour burden and with low levels of TGFβ2 expression. A recent study revealed increased patient survival following DC vaccination, especially for patients who had a glioblastoma with the mesenchymal gene expression signature, and that these tumours harboured higher numbers of CD3 and CD8 positive TILs compared to glioblastomas with other gene expression signatures (Prins *et al.*, 2011).

To improve the efficacy of glioma immunotherapy, preferably higher numbers and more potent glioma-specific effector cells should be induced that home into the glioma, including into the peripheral, diffusely infiltrative areas. Some clinical studies indicate that additional intra-tumoural DC application is more beneficial in the treatment of glioma patients than intradermal DC

injections alone. The immunogenicity of DCs may also be enhanced by including immune response modifiers, such as TLR agonists, into the maturation cocktail and/or by local administration of (combinations of) TLR agonists (Prins *et al.*, 2011). However, as such agonists induce not only beneficial activating but also potentially suppressive effector responses (like up-regulation of PDL1 on DCs), selective inhibition of immunosuppressive molecules induced during TLR immunotherapy may be needed (e.g. by anti-PDL1 antibodies).

Different strategies have been used to eliminate Tregs in preclinical murine models, including CD25-specific mAbs, immunotoxins or different chemotherapeutic agents. In a GL261 murine glioma model, treatment with anti-CD25 mAb resulted in elimination of Tregs within the brain and complete protection against orthotopic GL261 tumour challenge. Treated mice showed infiltration of $CD11b^+$ myeloid cells in the brain which were identified as $F4/80^+$ macrophages and granulocytes and not as MDSCs (Maes *et al.*, 2013).

Another interesting approach to reduce the number of tumour-infiltrating Tregs is to selectively block their trafficking to the tumour site. Furthermore, IL12 was shown to directly inhibit Treg differentiation from naïve T cells. The efficacy of immunotherapy for gliomas might thus be improved by using mature DCs with retained capacity to produce IL12 or by co-administering IL12. Chemotherapeutic agents such as temozolomide (i.e. the standard-of-care chemotherapeutic agent used in glioblastoma patients) and carmustine were shown to reduce the production of CCL2 by glioma cells, CCL2 being the ligand for the CCR4 chemokine receptor on Tregs. Implementation of CXCR4 inhibitors may also improve the efficacy of immunotherapeutic strategies, as CXCR4 expression was reported to be up-regulated on glioma-infiltrating Tregs during tumour growth.

Another way to overcome glioma-induced tolerance mechanisms involves direct targeting of immunosuppressive molecules such as TGFβ2 within the tumour microenvironment (Lohr *et al.*, 2011). Several phase I and II studies have been performed applying TGFβ2-antisense-oligonucleotides intratumourally by CED in patients with high-grade gliomas and confirmed the safety of this approach as well as long-term clinical benefit in several individual patients. An alternative approach is to prevent downstream signalling by interfering with TGFβ receptor kinase activities. Furthermore, targeting of other immunosuppressive molecules and enzymes such as signal transducers and activators of transcription 3 (Stat3), IDO and COX2 may improve antitumour immunity against gliomas. Selective iNOS inhibitors such as SD3651 or arginase inhibitors might be attractive to restore Teff cell functions.

Stromal cell subpopulations, which can capture and present tumour-derived antigens, appear to display less immune escape mechanisms than the tumour cells themselves and are therefore an interesting target for immune effector cells. Additional studies are needed to define the protective mechanisms of

action of distinct innate immune receptor agonists and their potential toxicity, because immune response modifiers that activate a wide range of innate and adaptive immune cells can have severe adverse effects.

Recent data show that tumour cell death triggered by chemotherapy or radiotherapy initiates an immune-adjuvant pathway that contributes to the success of cytotoxic treatment. Numerous endogenous danger signals transferred by dying tumour cells to innate immune effector cells may account for the immunogenicity of tumour cell death. Alkylating chemotherapeutics such as temozolomide have been shown to induce immunogenic cell death by triggering innate receptors such as TLRs, thereby enhancing the ability of DCs to present tumour antigens from dying tumour cells to T and B cells. Moreover, activation of innate immune receptors leads to the up-regulation of MHC molecules on tumour cells, thus increasing their sensitivity to T cell–mediated killing. Also, radiotherapy and chemotherapy may contribute to more effective T cell stimulation by removing local suppressor cells such as tumour-specific Tregs. Chemotherapy has been shown to induce lymphopenia, thereby allowing thymic-independent antigen-driven T cell regeneration within the context of T cell homeostasis. The concept of tumour-specific immunization at the time of immune reconstitution after chemotherapy has been successfully tested in different animal models and phase I and II clinical studies, demonstrating that the availability of tumour antigens during homeostatic T cell proliferation leads to effective anti-tumour immunity and enhanced memory T cell responses. Indeed, recent studies indicate that the clinical responsiveness of patients with malignant gliomas to chemotherapy is increased after DC vaccinations, maybe because these vaccinations help eliminate the chemoresistant tumour cells. In this context, a low-dose metronomic temozolomide regime in association with a cancer vaccine could be an attractive strategy for the treatment of malignant glioma and might be superior in efficacy to conventional temozolomide chemotherapy alone.

Conclusions

Although our understanding of the molecular biology of glial tumours of the CNS is rapidly growing, our knowledge of the complex pathological interactions within the local tumour microenvironment is still far from complete. Further elucidation of the functional and spatio-temporal organization of the different players in tumour-induced immunosuppression is of utmost importance for improving intervention strategies that boost potent anti-tumour responses. Current vaccination protocols are still of limited efficacy for suppressing glioma growth and need to be improved (e.g. by a combination of therapeutic strategies aimed at breaking the local immunosuppressive environment of gliomas without inducing autoimmunity). With further unravelling of glioma immunobiology, immunotherapeutic strategies have the opportunity to become a standard component in the multimodal treatment of

malignant gliomas. Elucidation of the underlying mechanisms of immune evasion in individual patients is critical as it will ultimately enable the development of tailored therapies to overcome these mechanisms.

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