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GENERAL INTRODUCTION

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is the most common neurological disorder among young adults affecting about 1 per 1,000, with an age of onset typically between 20 and 40 years. Women are affected more often than men with approximately two women to every one man being diagnosed[1]. The hallmarks of MS pathology include areas of demyelination (lesions), glial scar formation, oligodendrocyte death and axonal degeneration. MS lesions can be found throughout the CNS with a predilection to the optic nerve, periventricular white matter, brain stem, cerebellum and spinal cord[2]. Most lesions are found in the white matter and occasionally in grey matter, but the grey matter lesions are much less obvious and generally do not appear on magnetic resonance imaging (MRI), possibly because of the relatively small amounts of myelin present , and less intense inflammatory reactions[3]. Sensitive measurements with MRI have also shown abnormalities in normal-appearing white matter (NAWM, defined as macroscopically normal tissue without infiltrating immune cells) and normal-appearing grey matter (NAGM) in the majority of MS cases. These abnormalities occur early in the course of MS, can be detected in the early stages of MS lesions, may develop months or even years before lesion formation and are related with the clinical states[4-6]. The abnormalities include reduced myelin density and the most important event is microglial activation, which are the innate immune cells of the brain[7]. Different studies have shown that microglia activation occurs in NAWM at early stages of MS lesions before breakdown of the blood brain barrier (BBB), suggesting that the innate immune responses play an important role in the early stages of the development of MS lesions.

Apart from demyelination, a major problem of MS is axonal damage, since axonal loss significantly contributes to permanent functional deficits, and is irreversible[8]. Axonal damage has already been described in the very early literature of MS pathology (Charcot, 1868), but little is known about the mechanisms involved. Axonal injury has been considered for a long time to be a late phenomenon, but increasing evidence demonstrates that axonal injury occurs in patients already at the earliest stages of MS[9-15]. Axonal injury leads to transection of axons and formation of axon spheroids at their proximal ends[16]. This is not only found in active demyelinating lesions but also in remyelinating and inactive MS lesions. Although the exact cause of axonal damage is still unknown there is evidence to suggest that microglia and macrophages play a crucial role in axonal injury by producing toxic agents [17,18].

Clinical coures and diagnosis

MS can cause many different symptoms depending on which area of the central nervous system (CNS) is damaged. They include changes in sensations, loss of muscular coordination, stiffness, impaired vision or even loss of vision, depression, bladder or bowel dysfunctions, sexual difficulties and fatigue[19,20]. MS can cause impaired mobility and disability in more severe cases. MS is

difficult to diagnose in its early stages and cannot be definitely diagnosed until there is evidence of at least two anatomically separate demyelinating lesions occurring at least thirty days apart. Currently, the McDonald criteria represent international efforts to standardize the diagnosis of MS using clinical, laboratory and imaging data[21]. Magnetic resonance imaging (MRI) of the brain and the spinal cord for instance can be used to reveal active inflammation[22,23], and testing of cerebrospinal fluid (CSF) can be used to reveal the presence of oligoclonal bands, which are immunoglobulins found in 85% to 95 % of patient with definitive MS[24-26]. At least initially, most patients (85%-95%) have the relapsing-remitting (RR) MS form, which is characterized by unpredictable relapses followed by periods of months to years of relative remission with no new signs of disease activity. Later, neurological impairment progresses continuously, with or without superimposed relapses, in what is called secondary progressive (SP) MS (80 %). A small proportion of patients, approximately 15 %, experience primary progressive (PP) MS, characterized by a progressive course from onset, with absence of clinically evident relapses, and less inflammation on MRI[27]. Five percent of patients experience progressive relapsing (PR) MS with progression from onset and suffer acute relapses in the latter form[28]. Although MS relapses are often unpredictable, some attacks are preceded by triggers such as infections with influenza, or emotional or physical stress.

Etiology

Although the exact cause of MS is still unknown, both environmental and genetic components contribute to disease susceptibility. Several epidemiological findings have revealed that the prevalence of MS generally increases with distance from the equator, and that decreased sunlight exposure and possibly decreased vitamin D production may influence disease susceptibility and course [29-31]. High prevalence of MS is found in European countries, Canada, North America and south-eastern Australia, whereas in Asia and South America, the frequency is much lower. In sub-Sahara areas MS is extremely rare. Environmental factors such as infections, climate and diet during childhood may also play an important role in the development of MS. Several studies of migrants have shown that if migration occurs before the age of fifteen, the migrant acquires the new region's susceptibility to MS. If migration occurs after the age of fifteen, the migrant keeps the susceptibility of his home country[32]. Through the years, several infections have been associated with MS, including Epstein-Bar virus (EBV), human herpes virus (HHV 6) and *Chlamydophila pneumoniae*. Different epidemiological studies have shown that almost 100 % of MS patients are EBV-seropositive compared to 95 % of controls, that the risk for MS is significantly increased after infectious mononucleosis, and that MS patients have higher serum antibody titers against EBV[33-35]. Recently, it has been shown that high levels of CD8+ T-cell activation against EBV occurs early in the course of MS, suggesting that EBV may be associated with the onset of MS. Genetic factors also play a role in susceptibility to MS[36]. Twin studies have demonstrated that

the risk to develop MS is higher in monozygotic twins (approximately 30 %) than in dizygotic twins (approximately 3-5 %)[37]. Genome-wide studies have revealed that a number of genes are involved in MS susceptibility, including specific HLA types, particularly HLA class II alleles DR15/DQ6 (DRB1*1501, DQA1*0102, DQB1*0602) on chromosome 6p21[38,39]. Different genetic studies reported association with genes such as the IL7R and IL-2R genes and more recently also with the KIF1B gene, which are involved in T-cell functions and in axonal functions, respectively[40-42].

There is a general agreement that MS is an autoimmune disease in which auto-reactive CD4+ T cells cross the blood-brain barrier to enter the central nervous system wherein they participate in the initiation of a cell-mediated immunological response to CNS myelin. Previously, the myelin-associated small heat shock protein αB-crystallin was identified as an immunodominant myelin antigen in MS[43,44]. In addition, large-scale sequencing of cDNA libraries derived from brains of MS patients indicated that transcripts for αB-crystallin were the most abundant transcripts unique to MS plaques. αB-Crystallin expression was also found to be upregulated within lesional areas in oligodendrocytes and astrocytes. Only in early MS lesions, however, oligodendrocytes were found to express αB-crystallin[45]. In addition, different studies demonstrated that αB-crystallin has anti-apoptotic and anti-neurotoxic functions. Recently, it has been shown that administration of recombinant αB-crystallin ameliorated experimental autoimmune encelphalomyelitis (EAE) and suppressed autoimmunity. These observations suggest that αB-crystallin not only plays an important role in adaptive immune responses but also in protective and repair processes[46].

THE IMMUNE SYSTEM AND THE CNS

The human immune system can be classified into two interactive branches; the adaptive immune system and innate immune system. Both systems cooperate to protect the body from invading pathogens. Adaptive or 'specific' immunity mediates a delayed and specific response to antigens, while the innate immunity is less antigen specific and develops immediately. The adaptive immune responses can be activated by the innate immune system and it is essential for controlling pathogens that escape elimination by the innate immune response, and for the development of immunological memory. Adaptive immunity develops by clonal selection from a vast repertoire of lymphocytes bearing antigen-specific receptors that are generated via gene rearrangements, and thereby becomes able to respond to a wide range of potential antigens.

The innate immunity and Toll-like receptors

The innate immune system is the vital first line of defense against a wide range of pathogens and tissue injury, and to some extent it is able to discriminate between 'self' and 'non self' antigens[47,48]. In addition, innate immune responses are a prerequisite for the induction of acquired immunity[49]. While cells of the adaptive immune system must be activated, undergo cell division and expansion, and travel to the site of infection before pathogen elimination can take place, the cells of the innate immune system do not require cell expansion to eliminate pathogens. The responses are immediate and usually sufficient to prevent establishment of infection. Innate immune cells recognize highly conserved microbial structures, so called pathogen-associated molecular patterns (PAMPs), from bacteria, viruses and fungi, as well as some host molecules, via a limited numbers of germline-encoded pattern-recognition receptors (PRRs). Several different PRRs have been identified, each detecting microbes or microbial components, initiating anti-pathogen responses and promoting adaptive immune responses. PRR receptors include CD14, β2-integrins, C-type lectins, macrophage scavenger receptors, complement receptors, and the more recently identified members of the mammalian Toll-like receptor family (TLR)[50,51,52].

Toll-like receptors

The toll protein was first identified as a development protein in Drosophila and later demonstrated to be important in immune responses against fungal and gram positive bacterial infection[53]. Mammalian homologues of toll were identified through database searches and so far, 13 members of the TLR family have been identified in mammals. TLRs are type 1 transmembrane proteins which have a conserved cytoplasmic domain known as Toll/IL-1R (TIR) similar to the cytoplasmic domain of the interleukin 1 receptor (IL-1R)[54]. The TIR domains have a conserved region of ~ 200 amino acids and are characterized by the presence of three highly homologous motifs, which are involved in different aspects of signaling and/or receptor interactions[55]. TLRs are key components of the innate immune system that can be activated by both exogenous pathogenic ligands as well as by endogenous ligands (Table 1) [56-58]. They trigger host defense responses and control adaptive immune responses[59,60]. As summarized in Table 1, TLRs recognize a wide variety of PAMPs and endogenous proteins, and each differs in their specificity for PAMPs. For instance TLR1 and TLR2 can be activated by bacterial lipoproteins[61-63], whereas TLR7 and TLR8 are activated by viral RNA[64,65]. There is increasing evidence that TLRs also recognize endogenous proteins that emerge upon tissue damage or inflammation. These include breakdown products of the extracellular matrix such as fragments of hyaluronan[66], fibronectin[67], heparan sulphate [68] and fibrinogen[69]. Also heat-

shock proteins have been identified as TLR agonists, in particular for TLR2 and TLR4[70-72].[12] Tissues involved in immune functions such as spleen and peripheral blood leukocytes and other organs such as lungs, testis and the brain express several different TLR. However, the expression of individual TLRs is different between cell types and often dependent on the activation state of the cell. In addition, the different TLRs show differences in cellular localization, partially depending on the cell type. They may be either expressed on the cell surface, or intracellularly in endosomal vesicles.

TLR signalling

After ligand binding, TLR TIR domains recruit adaptor molecules and initiate the signaling process (Fig. 1). Currently there are 5 TLR adaptor molecules that have been identified. Myeloid differentiation primary-response gene 88 (MyD88) was the first identified and is utilized by all the TLRs except for TLR3. It is the only adaptor molecule identified for TLR5, TLR7, TLR8 and TLR9, while TLR2 and TLR4 use MyD88 along with other adaptor molecules. The association of TLRs and MyD88 leads to the activation of mitogen-activated protein kinases (MAPKs) and transcription factor NF-κB to control the expression of cytokine genes including TNF-α, IL-6, IL-1β and IL-12, chemokines and co-stimulatory molecules. Another adaptor molecule in TLR signalling closely related to MyD88 is the TIR domain-containing adaptor protein/MyD88 adaptor-like protein (TIRAP/MAL), which is utilized in the downstream signalling of TLR2 and TLR4 but not other TLRs. The TIR-domain-containing adaptor protein inducing IFN-β (TRIF) or TIR-domain-containing adaptor molecule (TICAM) 1 was simultaneously identified by two different groups. TRIF was identified as the adaptor utilized in MyD88-independent production of IFN-β and induction of IFN-stimulated genes. TRIF signaling is activated by both TLR3 and TLR4 and results in phosphorylation of transcription factor IRF3 as well as activation of NF-κB. Recently two more potential adaptor molecules were identified, namely TRIF-related adaptor molecule (TRAM) or TICAM and sterile α motif and HEAT-Armadillo motifs (SARM). TRAM associates with TRIF in the TLR4 signaling but not in the TLR3 signaling pathway. Together, the existence of different TLR adaptor molecules, and the ability to utilize different combinations of adaptor molecules, allows several different signalling pathways to be initiated by TLRs[55,73-81]. In addition, many proteins are essential for activation of TLRs by their agonists. For example, TLR4, together with MD-2, CD14 and LPS-binding protein, recognizes LPS from Gram-negative bacteria[82]. TLR4 can also associate with the class B scavenger receptor CD36. TLR2 forms heterodimers with TLR1, TLR6 and the non-TLRs CD36 and dectin-1 to discriminate between a wide range of PAMPs, including peptidoglycan, lipoproteins, lipopeptides and zymosan[62,63,83,84]. In addition, TLR10 is thought to form heterodimers with TLR2 and TLR1[85]. To make the picture more complicated, TLRs can influence each other. For example, TLR5 activation by flagellin inhibits TLR9-induced cell activation and cytokine production[86]. In addition, co-expression of TLR1 inhibits the TLR2-

mediated response to phenol-soluble modulin, while co-expression of TLR6 enhances the TLR2 response[87]. TLR1 also associates with TLR4 and inhibits its activation of endothelial cells[88].

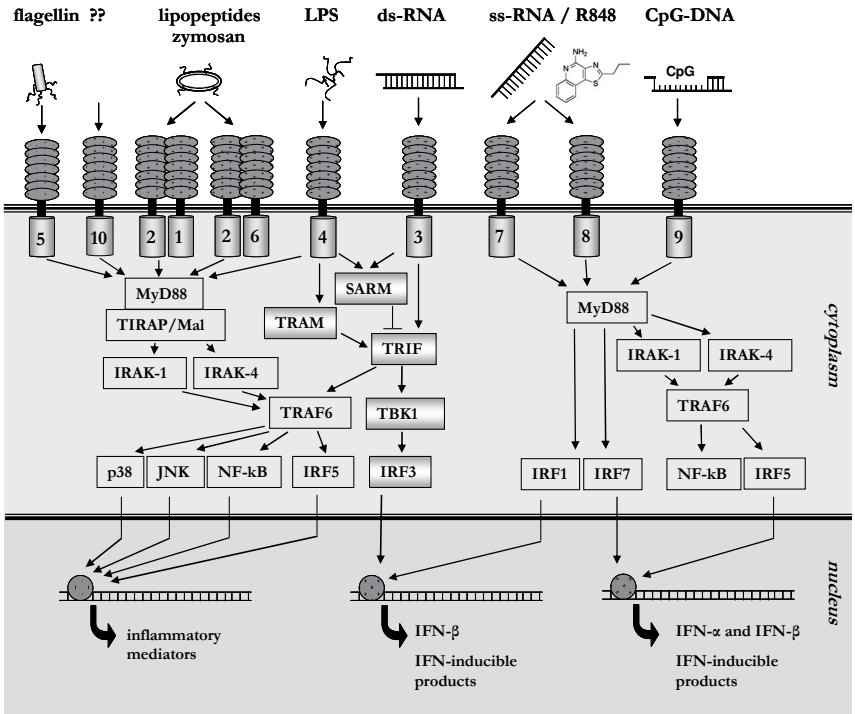

Figure 1: TLR signaling pathway.

The apparent use by the immune system of specific combinations of different PRRs for recognition of certain agonists has at least three major consequences. First, by using unique functional combinations of different types of PRRs for specific recognition of certain agonists, the innate immune system is able to create a considerable combinatorial repertoire which can selectively respond to a much wider range of structures than would be possible by using the limited set of individual PRRs on their own. Secondly, the range of cell types involved in the response to certain agonists can be controlled more precisely, since such responses become dependent on simultaneous expression on multiple co-receptors by a single cell. While TLR2 or TLR4, for example, may be expressed on many different cell types, simultaneous expression of either of these TLRs with all the other co-receptors required by some agonists occurs much less frequently. Agonists requiring a specific combination of PRRs will thus activate a much more restricted population of target cells. Finally, functional interactions between combinations of PRRs will allow fine-tuning of the nature and/or strength of the intracellular signal triggered by an agonist. Traditionally, responses mediated by individual PRR have been seen as largely pro-inflammatory host-defence responses only. Yet, functional combinations of different PRR may allow for more subtle types of cellular responses, notably involving also the activation of cellular pathways which inhibit inflammation, promote repair, and even participate in developmental programs. Thus, by creating functional combinations of different PRRs, the innate immune system can operate at a much more sophisticated level than would be possible by using only individual PRRs. This may well be of particularly relevance for the function of macrophages, which tend to express a much wider range of different PRRs than other cell types, and whose control is of particular importance at the final stages of inflammation, when adequate resolution of the degradative process is required, and a return to homeostasis.

Table 1: Overview of TLRs, their ligands, co-receptors and the activated signaling pathway

TLR	Exogenous ligand	Endogenous ligand	Co-receptors	Signaling pathway
TLR1	Bacterial lipoproteins, tri-acyl lipopeptides	Not known	Not known	NF-κB via MyD88
TLR2	Bacterial lipoproteins, Peptidoglycan, lipoteichoic acid,glycoinositolphospholipids, glycolipids, porins, zymosan	Hsp70, necrotic cells, High mobility group box protein (HMGB1)	Dectin-1, CD36, CD14, TLR1, TLR6	NF-κB via MyD88 and TIRAP/MAL
TLR3	Double-stranded viral RNA	mRNA	Not known	NF-κB and IRF3, 5, 7 via TRIF
TLR4	Lipoproteins, LPS,	Hsp60, hsp70, hyaluronan, fibronectin, fibrinogen, heparin, HMGB1, beta-defensin-2, lung surfactant protein A	CD14, MD2, MSR-A	NF-κB, IRF5 via MyD88 and TIRAP/MAL NF-κB and IRF3 via TRAM and TRIF
TLR5	Flagellin	Not known	Not known	NF-κB via MyD88
TLR6	Bacterial lipoproteins, zymosan in combination with TLR2	Not known	Not known	NF-κB via MyD88
TLR7	Single-stranded viral RNA	Not known	Not known	NF-κB, IRF5, 7 via MyD88
TLR8	Single-stranded viral RNA	Not known	Not known	NF-κB via MyD88
TLR9	CpG DNA (bacterial or viral)	Not known	Not known	NF-κB, IRF1, 5, 7 via MyD88
TLR10	Not known	Not known	Not known	Not known
TLR11	Profilin, uropathogenic bacteria	Not known	Not known	Not known
TLR12	Not known	Not known	Not known	Not known
TLR13	Not known	Not known	Not known	Not known

IMMUNITY IN THE CNS

For a long time the human CNS was considered to be an immunologically privileged organ, inaccessible to circulating lymphocytes. This was based on several anatomical and functional observations: the absence of conventional lymphatic drainage, the presence of a blood-brain barrier (BBB), the presence of a blood-cerebrospinal fluid barrier, the absence of rejection of grafts into the CNS, the absence of major histocompatibility complex (MHC) in the normal brain and the absence of macrophages and dendritic cells[89-91]. Other mechanisms that have been proposed to contribute to CNS immune privilege include local production of anti-inflammatory mediators, e.g., transforming growth factor-β (TGF-β)[92], α-melanocyte-stimulating hormone (α-MSH) and constitutive expression of Fas ligand responsible for Fas (CD95)-mediated killing of CNS-infiltrating immune cells[93]. It is important to point out that the immune privileged state of the CNS also refers to lack of initiation of adaptive immune responses. Innate immune responses however, are readily initiated within the CNS by tissue damage, bacterial components and cytokine overexpression[94,95]. However, this immune-privilege is not absolute. It is now clear that the CNS is organized into different compartments: an immune privileged compartment which is the brain parenchyma, and much less immune-privileged compartments which are the ventricles containing choroid plexus and cerebrospinal fluid (CSF), meninges and subarachnoid space[96]. In addition, macrophages and dendritic cells have been identified in meninges and choroid plexuses and sentinel functions at the BBB are ensured by perivascular macrophages[97].

The immune function of resident CNS cells

The central nervous system consists of neurons and glial cells. Neurons constitute about 50 % of CNS cells and glial cells make up for the rest. They are known as the supporting cells of the nervous system. For a long time, glial cells were thought to have only a maintenance role: surrounding and supporting neurons, supplying nutrients and oxygen to neurons, maintaining a healthy balance of ions in the brain, and eliminate pathogens that evaded the immune system. There is increasing evidence that glial cells play a far more important role than historically presumed. It is now clear that glial cells can communicate with neurons and with each other, have the power to influence the formation of synapses and help to determine which neuronal connections get stronger or weaker over the time. In the following section, I will discuss the most important immune function of the two major CNS resident glial cells, viz. microglia and astrocytes.

Immune functions of microglia

Microglia cells are considered to be the primary immune effector cells in the brain[98]. They are of hematopoietic origin and belong to the myelomonocytic lineage. In the early stages of development microglia cells invade the brain and exist in a resting state, characterized by ramified morphology and a slow turnover rate[99-101]. Resting microglia cells have long multiple processes which interweave the parenchymal nervous tissue and show high constitutive motility. The processes of microglia cells directly contact astrocytes, neuronal cell bodies and blood vessels. The brain parenchyma is continuously screened by resting microglia cells. Under normal conditions, microglia cells can become activated to play a classic role as "scavengers" for the maintenance and restoration of the CNS. Resting microglia display an immunologically quiescent phenotype characterized by the lack of endocytic and phagocytic activity and low or undetectable levels of membrane receptors that are essential for normal macrophage functions[102-105]. After CNS infection or other forms of insults or damage, ramified microglia undergo rapid and dramatic morphological changes including shortening of cellular processes and enlargement of their soma. Under such conditions, they become phenotypically and functionally similar to peripheral macrophages and perform several innate immune functions[106-109]. To do that, activated microglia acquire the expression of numerous receptors including pattern recognition receptors (PRR), major histocompatibility antigens (MCH class I and II), Fc receptors, complement receptors, and co-stimulatory molecules[110].

The pattern recognition receptors that microglia express, either constitutively or induced through activation, include a wide range of TLRs (this thesis). These TLRs play an important role in innate immune responses against both endogenous and exogenous ligands[58,111-114]. In addition, microglia cells also express the integrin CD11b/CD18 (complement receptor type 3 (CR3)), CD14, scavenger receptors, protease-activated receptors, and receptors which mediate or enhance phagocytosis through recognition of serum components deposited on microbes or altered host components. These include, Fcγ receptors I, II and III and the complement receptors CR-1, -3, -4 and C1qRp[110,115-118]. Microglia also express, either constitutively or induced upon activation, many different cytokine and chemokine receptors[119,120]. The IFN-γ receptor for example is constitutively expressed on resting microglia. Binding of IFN-γ to its receptor triggers the activation of the classical IFN-γ signalling cascade involving activation of the transcription factor STAT-1[121]. TNF-α is another important mediator in microglia functions which is produced during CNS inflammation by activated Th1 cells, macrophages, astrocytes and activated microglia themselves[110,122]. The biological activities of TNF-α are mediated by two structurally related but functionally distinct receptors, p55 (TNFRI) and p75 (TNFRII), which are independently expressed on the cell surface. TNF-RI is expressed in most tissues and can be activated by both membrane-bound