Immune modulation in MS models by peptidoglycan, CD97 and CD44

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General introduction

Multiple sclerosis

General features

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). The oligodendrocyte is the myelin-producing cell of the CNS and encircles its lipid bilayer membrane around multiple axons. Myelin serves as an isolation sheath for axons. This insulation is critical for protection against harmful components and establishes proper neuronal signaling and transmission. In MS, immune-mediated attacks against components of the myelin sheath and axons are thought to eventually lead to impaired nerve conduction and motor functions[1-3]. Demyelinated, multifocal sharp-edged lesions (plaques) are the pathological hallmark of MS. Processes of astrocytes fill up demyelinated areas and form scars, a process referred to as astrogliosis. The presence of multiple scars in the CNS has led to the name multiple sclerosis.

Clinical assessment of myelin and axonal damage

A specific feature of MS is the appearance and sometimes disappearance of CNS lesions in time and space. The distribution of these lesions can be monitored by various magnetic resonance imaging (MRI) techniques[4], which visualize the expanded extracellular space within the plaque. In the CNS of MS patients, so-called T1- or T2-weighted MR images show black holes or white spots, respectively. Furthermore, blood-brain barrier damage can be detected by gadolinium enhancement of T1-weighted lesions, with the use of the tracer gadolinium diethylene-triamine-pentaacetic acid (DTPA). This tracer can pass the compromised blood-brain barrier (BBB) in active MS lesions. T2-weighted lesions represent areas with demyelination and/or astrogliosis. MRI-detected lesions are not directly correlated with disease activity[5]. Especially cerebral lesions can be clinically silent, whereas cerebellar, brain stem and spinal cord lesions are in general accompanied by clinical signs of deficit. In the post-inflammatory phase, burnt-out lesions can be partially repaired. This repair process involves the regeneration of myelin around axons by oligodendrocytes, leading to shadow plaque formation. Permanent scars are formed when astrogliosis precedes remyelination.

In contrast to the possible regeneration of myelin and immigration of precursor oligodendrocytes into demyelinated lesions, axonal and/or neuronal damage is an irreversible event. Axonal damage is present already in the early stages of MS[6], but can be clinically silent, since the CNS is capable to fully compensate for a certain amount of functional loss[7,8]. In MS, it is known that axonal loss accumulates in time. After reaching a certain threshold of axonal/neuronal degeneration, functional damage becomes permanent. It is assumed that axonal/neuronal degeneration plays a central role in disease progression towards the irreversible secondary-progressive phase[9]. The extent of neuronal and axonal damage can be reflected by magnetic resonance spectroscopy, using the decreased concentration of *N*-acetyl

aspartate as a measure for brain atrophy. In addition, T1-weighted black holes on MRI are also thought to represent areas with axonal damage.

Disease course

MS mostly affects young adults. The clinical disease course is highly heterogeneous. In general, the disease course can be categorized into four distinct forms; relapsing-remitting, primary-progressive, secondary-progressive and progressive-relapsing MS (Figure 1)[10].

In relapsing-remitting MS (85-90% of patients), periods of decline (relapses) are alternated by periods of complete or incomplete remission, with a stable course between attacks. Seventeen percent of relapsing-remitting MS patients have a benign form of MS. This form is defined by a fully functional neurological system, for at least fifteen years after disease onset. After a certain period of time, about 40% of relapsing-remitting MS patients develop a progressive neurodegenerative secondary disorder, known as the progressive phase and categorized as secondary-progressive MS. Primary-progressive MS patients (10-15%) develop a gradual and nearly continuous disease progression from onset, with minor fluctuations. Plateaus, remissions and acute relapses are all absent. In the course of time, primary-progressive MS can also convert into a secondary-progressive MS phase. It is not known what triggers the transition into a secondary-progressive phase. The transition might be explained by the fact that irreversible axonal and neuronal damage accumulate over time, into the extent of permanent disability and extensive brain atrophy. In this phase, MS patients do not respond to immunotherapy. A small group of MS patients (<5%) develop a progressive-relapsing disease course. This form is defined by a progressive course from onset, with acute relapses. Between relapses, patients show a continuous progression of disability.

Besides the four major forms of MS (Figure 1), also other sub forms of MS exist. In a small proportion of MS patients, an acute, malignant disease course is manifested, characterized by a severe disability or even death within months after disease onset. Acute disseminated encephalomyelitis (ADEM) can be erroneously diagnosed as MS, because ADEM patients also develop acute disability in a short time period[11,12]. However, the CNS pathology of ADEM patients does not show the typical sharp-edged plaques as seen in MS patients. The classification of variants of MS, including the sub forms Marburg's type of acute MS, Devic's type of neuromyelitis optica, Baló's concentric sclerosis, Schilder's myelinoclastic diffuse sclerosis and disseminated encephalomyelitis patients has recently been reviewed and revised by Poser and Brinar[13].

Etiology

As described above, the disease course of individual MS patients is highly variable, and is possibly caused by different immunopathological mechanisms. MS is a disease with unknown etiology and affects about 1 in 1.000 individuals. Prevalence

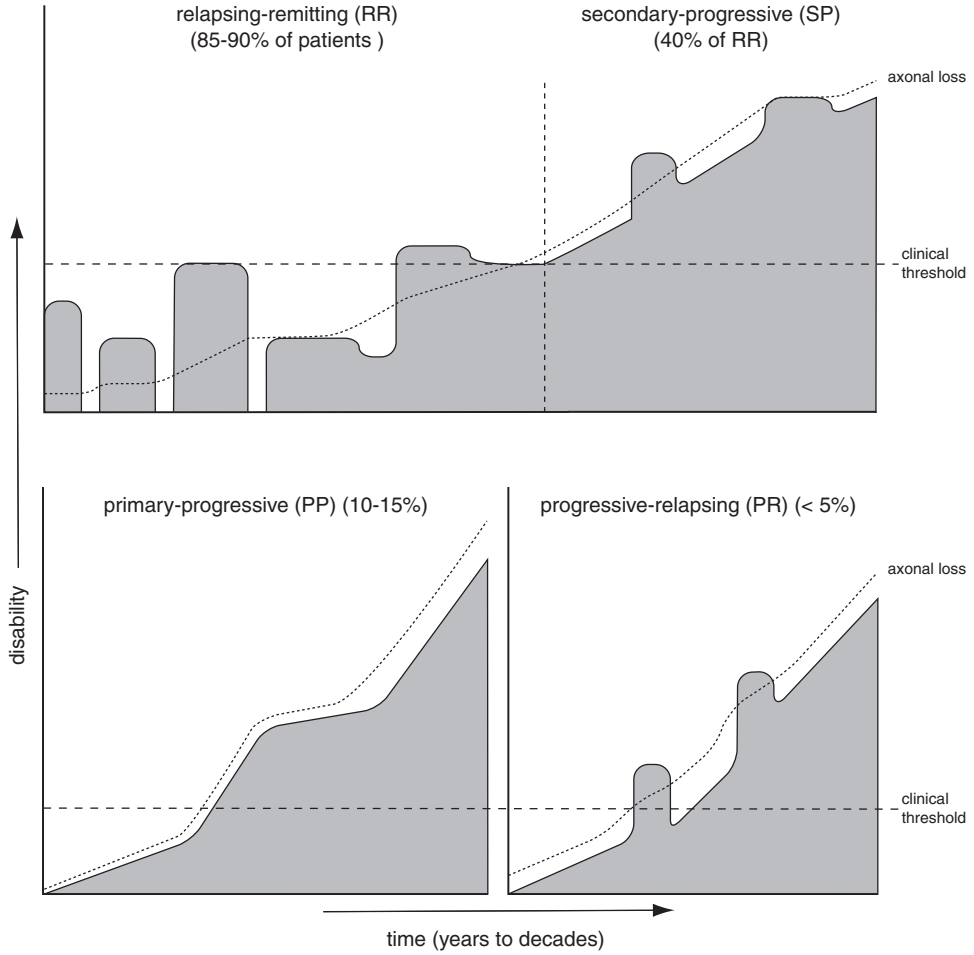

Figure 1. Classification of clinical MS courses.
The disease course in MS patients is heterogeneous and can be subdivided into four main categories. Relapsing-remitting (RR) MS is characterized by acute attacks, followed by full or partial recovery, with periods of clinical silence of the disease between the relapses. RR MS can progress into a secondary-progressive (SP) disease course, which is defined by disease progression with possible relapses and minor remissions. Primary-progressive (PP) and progressive-relapsing (PR) MS both show progression from onset and includes acute relapses in the latter form. Irreversible axonal damage is already present early after MS onset and accumulates over time. Progressive clinical deficit may develop after reaching a certain treshold of axonal loss and brain atrophy. Based on Lublin *et al.*, 1996[10], Compston and Coles, 2002[337].

and incidence studies have shown that the individual susceptibility risk for MS development is variable[14] and is dependent on a combination of host factors (e.g. genes and hormones) and exogenous factors (e.g. infections)[15-18]. A genetic basis

is underscored by a concordance rate of approximately 31% among monozygotic twins and 5% among dizygotic twins[19]. Furthermore, although low (± 2%), the recurrence risk factor in first-degree relatives is increased 20- to 40-fold compared with the risk factor in the general population[20]. In children from conjugal MS pairs, the risk factor is increased by 10-fold, compared to children with one affected MS parent. Many genetic association or linkage studies have consistently shown that in Caucasian Europeans and North Americans the HLA class II alleles DR15/DQ6 (namely DRB1*1501, DQB1*0601) on chromosome 6p21 are associated with an 3.6-fold elevated risk for MS development[21,22]. Likewise, genetic variants for the TNF-α gene, located in the same linkage group, have been identified as a susceptibility factor for MS development. In addition, a considerable number of low-risk, non-HLA genes are also thought to contribute to MS susceptibility. Hormonal factors can influence MS incidence and disease course as well. MS is more common in females than in males, with an approximate ratio of 2:1. Especially during and after pregnancy a reduced number of relapses are observed, which may partially be related to hormonal changes. In sharp contrast, a deterioration of disease symptoms is observed *post-partum*.

The fact that about 60% of monozygotic twins are not concordant for MS, strongly argues in favour of an additional role of exogenous factors in MS susceptibility[23]. From migration studies, it became apparent that besides genetic predisposition, exogenous factors contribute as well to the risk of MS development[14]. Loss of genetic isolation in Sardinia, by the immigration of soldiers during World War II, may have increased MS incidence by transmittable agents. People, who migrate before the age of 15 years, acquire the relative risk factor of the new location. Emigrants who move later in life maintain the susceptibility risk of their original location[24,25]. Moreover, the prevalence of MS can vary within homogeneous genetic populations and is higher in areas with a moderate climate[26]. In summary, there is substantial evidence that genetic predisposition and environmental factors both contribute to MS susceptibility.

Microbial factors

The immune system plays a considerable role in the destruction of myelin and axonal components in the CNS of MS patients. It is not known where and how these inflammatory attacks are initiated. The initial event may take place within the CNS, i.e. by tissue damage initiated by trauma or microbial infection. Alternatively, the initial trigger might take place in peripheral lymphoid organs, i.e. by activation of a CNS-specific immune response. In general, there are two points of view on the formation of MS lesions. The outside-in model suggests that inflammation is followed by demyelination and subsequent axonal damage. The inside-out model, on the other hand implies that axonal injury triggers inflammation and demyelination. Hence, the immune response in MS could be a secondary event in which lesions develop from the axons to the myelin. Several studies imply that

axonal damage may actually occur first, as reviewed recently[27,28]. In both models, loss of CNS tissue integrity, in genetically susceptible hosts, can induce release of CNS antigens to the periphery, where priming or reactivation of autoreactive T and B cells can take place in secondary lymphoid organs[29-32].

Other CNS inflammatory demyelinating diseases are clearly associated with infections, such as ADEM (associated with a post-viral or post-vaccination inflammation), human T-lymphotropic virus type 1 (HLTV-1)-associated myelopathy/tropical spastic paraparesis and subacute sclerosing panencephalitis, caused by the reactivation of latent measles virus. Theiler's murine encephalomyelitis virus and mouse hepatitis virus both induce an immune-based demyelinating encephalomyelitis in mice with clinical and pathological features of human MS[33]. However, so far no specific pathogen has been identified as relevant in the development of MS. This does not exclude a microbial involvement in the etiology of MS, since it is known that diverse antecedent viral and bacterial infections are frequently associated with MS relapses[34-36].

An encounter with microbes may activate autoreactive T and B cells and subsequently initiate chronic CNS inflammation. Both in healthy individuals and MS patients, myelin-specific T and B cells are present in the periphery[31,37]. This suggests that suppressive mechanisms are operational which maintain the immune system in a state of tolerance to self-antigens of the CNS. $CD4^+$ and $CD8^+$ regulatory T cells play an important role in the down regulation of inflammation in experimental autoimmune encephalomyelitis (EAE), an animal model for MS[38,39]. Interestingly, it was recently demonstrated that the functional CNS-specific $CD4^+$ and $CD8^+$ T cell responses were distinct in MS patients and healthy individuals[40]. Compared to healthy subjects, CNS-specific $CD4^+$ T cell responses of MS patients were associated with elevated expression levels of IFN-γ, whereas IL-4 and IL-5 levels were decreased. These data suggest that in MS patients $CD4^+$ CNS-specific T cell responses are polarized towards the T helper (Th) 1 phenotype. In MS patients, $CD8^+$ CNS-specific T cells expressed elevated levels of both IFN-γ and IL-10. It is unclear at present whether this cytokine-profile represents pathogenic and/or regulatory $CD8^+$ T cells. Myelin-specific $CD4^+$ and $CD8^+$ T cells can potentially be activated or reactivated by infectious agents through diverse mechanisms[41]:

a. molecular mimicry
b. bacterial and viral superantigens
c. modulation of antigen presenting cell (APC) function

Molecular mimicry and superantigens

Autoantigen-specific T cells and B cells can cross-react with foreign antigens, a concept known as molecular mimicry[42-44]. Some examples of viruses that share conserved sequences with myelin proteins in humans are Epstein-Barr virus, human herpes virus type 6, measles and papilloma virus. By using autoantigen-specific T cell clones from MS patients, it was functionally demonstrated that cross-reactivity

is possible with peptides from several viral and bacterial strains[45]. Activation of autoreactive T cells by bacterial superantigens has also been functionally demonstrated in EAE. EAE can be induced in susceptible animals upon active immunization with myelin components in adjuvant. Additional administration of the superantigen *staphylococcal* enterotoxin B induced relapses and exacerbations of mouse EAE by stimulation of Vβ8 receptors on autoantigen-specific T cells[46].

Modulation of APC function

EAE animal models have furthermore shown that interplay between the innate and adaptive immune system is a prerequisite for development of autoimmunity. A strong adjuvant containing heat-killed *M. tuberculosis* (complete Freund's adjuvant, CFA) is required for the induction of EAE by active immunization with autoantigens. In EAE, a proinflammatory immune response is raised against microbial products present in the adjuvant mix. This immune response can also activate neighbouring T and B cells with other antigen-specific receptors, e.g. for an autoantigen in adjuvant. Bacterial compounds, such as lipopolysaccharides (LPS) can engage innate receptors, such as Toll-like receptors (TLR), expressed on and within APC. Receptor triggering provides a proinflammatory signal and furthermore facilitates (auto)antigen uptake and presentation by MHC-II molecules. In addition to bacterial components, viral components, such as cytosin-guanosin dinucleotides (CpG) oligodinucleotides, can also induce TLR9 activation and exert similar proinflammatory effects on APC. Interestingly, both LPS and CpG have been implicated as proinflammatory modulators in EAE development[47-50].

In the initial inflammatory attack, T and B cells are reactive against the myelin antigen used for immunization. However, at later stages in the disease T and B cell responses are also raised against other myelin peptides, a process referred to as epitope spreading. In EAE and MS, initiation and progression of disease are associated with T and B cell reactivity against new myelin epitopes[51-54]. Epitope spreading is first observed within the same protein (intermolecular spreading) and can proceed to different proteins (intramolecular spreading). Microbial products through bystander activation can influence epitope spreading. Taken together, in initial and in subsequent inflammatory attacks, both bacterial and viral components can modulate APC function and induce bystander activation by inducing proinflammatory cytokines, and by relieving T regulatory immunosupression.

Model of cellular events in the pathogenesis of MS and EAE

Autoantigen-specific T cells are present in the periphery, but are nonpathogenic under normal circumstances. In Figure 2, a hypothetical model is shown in which the sequence of events is schematically represented. In susceptible hosts, insults with microbes or microbial particles (as *M. tuberculosis* in CFA) can create a proinflammatory environment by stimulating innate receptors of APC. APC may

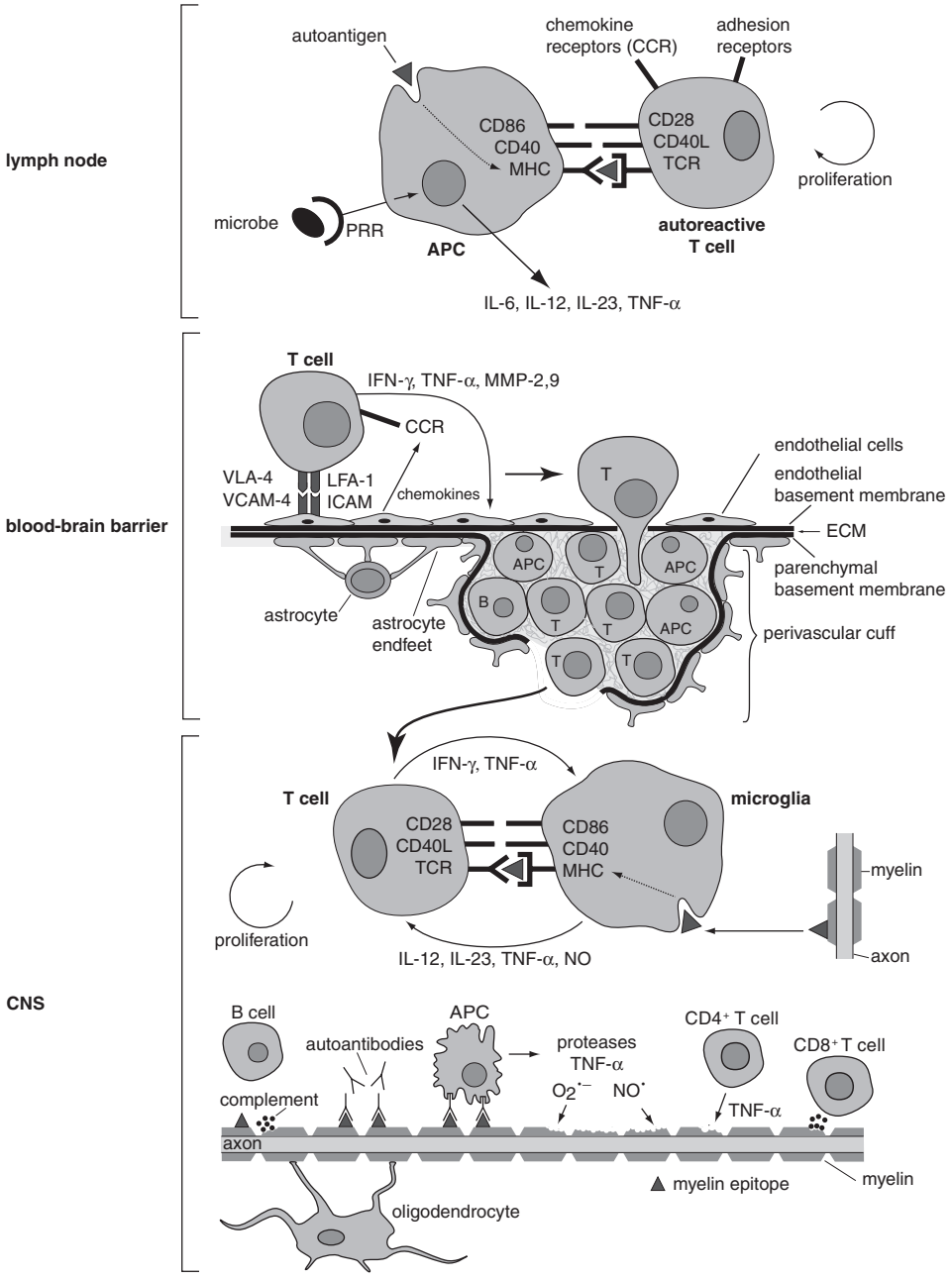
lymph node
autoantigen
chemokine receptors (CCR)
adhesion receptors
CD86
CD40
MHC
CD28
CD40L
TCR
proliferation
microbe
PRR
APC
autoreactive T cell
IL-6, IL-12, IL-23, TNF-α
blood-brain barrier
T cell
IFN-γ, TNF-α, MMP-2,9
CCR
VLA-4
VCAM-4
LFA-1
ICAM
chemokines
endothelial cells
endothelial basement membrane
ECM
parenchymal basement membrane
perivascular cuff
astrocyte
astrocyte endfeet
APC
T
B
CNS
IFN-γ, TNF-α
T cell
microglia
CD28
CD40L
TCR
CD86
CD40
MHC
myelin
axon
proliferation
IL-12, IL-23, TNF-α, NO
B cell
APC
autoantibodies
proteases
TNF-α
CD4+ T cell
CD8+ T cell
complement
O2•−
NO•
TNF-α
axon
myelin epitope
myelin
oligodendrocyte

Figure 2. Model of cellular events in the pathogenesis of MS and EAE.
The sequence of events in the pathogenesis of MS remains largely unknown and current concepts rely predominantly on EAE studies. The initial event may occur in the CNS (i.e. damage mediated by trauma or infection) or in the periphery (i.e. activation of autoreactive lymphocytes). It is generally believed that after an unknown insult naïve autoreactive T cells ($CD4^+$ and $CD8^+$) and B cells are primed by APC in a proinflammatory environment in secondary lymphoid organs (e.g. lymph node). Activated autoreactive T cells express chemokine receptors, which enable the cells to recognize the site of inflammation, where chemokines are secreted. T cells adhere to the endothelial cells of the CNS, by contacting adhesion molecules. T cells can transmigrate through the endothelial cells into the perivascular space, and subsequently form perivascular cuffs. At the basal side of the endothelial cell, the basement membrane is reformed around the T cell. T cell migration through the extracellular matrix (ECM) is facilitated by the production of gelatinases (MMP-2/9). Proinflammatory cytokines (e.g. TNF-α) likely mediate transmigration across the parenchymal transmembrane and astrocyte endfeet. In the CNS parenchyma, T cells recognize their specific peptide targets, presented by MHC molecules on microglia, the APC of the CNS. This interaction initiates a cascade of events, including the attraction of other cell types and initiation of diverse effector functions (antibody-, cytokine-, complement- and cell-mediated damage). This inflammatory cascade leads to the degeneration of myelin and axonal loss. APC represents macrophages or dendritic cells. TCR represents T cell receptor. PRR represents pattern-recognition reception. Modified from Waubant *et al.*, 1997[338].

present microbial antigens that are cross-reactive with myelin antigen(s), which can be present in peripheral lymph nodes[55], in the context of MHC-I or MHC-II. Autoantigens are presented in a proinflammatory setting (IL-1, IL-6, IL-12) to naïve Th cells. IL-12 and the more recently discovered IL-23 and IL-27 are structurally related, heterodimeric cytokines that regulate cell-mediated immune responses and Th1 inflammatory reactions in EAE[56,57]. In this proinflammatory setting, $CD4^+$ Th1 and $CD8^+$ T cytotoxic cells develop. Ligation of costimulatory molecules on APC and T cells, such as CD40-CD154 and CD80-CD28, results in T cell activation and expansion. Autoantigen-specific $CD4^+$ Th1 cells produce proinflammatory mediators such as IFN-γ, TNF-α, osteopontin (OPN) and matrix metalloproteinases (e.g. MMP-2 and MMP-9). $CD4^+$ Th1 cells promote APC activation, provide help for the activation and expansion of autoreactive B cells and $CD8^+$ T cells, and cause delayed-type hypersensitivity (DTH)[58,59]. At this stage of immune activation, immunosuppression by regulatory T cells is blocked via TLR engagement by inflammatory mediators, such as IL-6[60].

Upon activation, antigen-specific T cells can enter the circulation and adhere to the endothelium of the BBB (Figure 2). T cell derived cytokines (e.g. IFN-γ and TNF-α) induce activation of endothelial cells. Activated T cells can normally enter into the perivascular space, but exit when they fail to encounter their respective antigens. T cell migration into the CNS is mediated by adhesion molecule interactions on activated T cells and endothelial cells (tethering: E- and P-selectins, α4 integrin/VLA-1 and transmigration: α4 integrin/VLA-1 and LFA-1/ICAM-1). Subsequent transmigration requires the passage through several barriers, including an endothelial cell layer, endothelial basement membrane, epithelial leptomeningeal cell layer (not depicted in Figure 2), parenchymal basement membrane and astrocyte endfeet[61]. Thus, the first step of transmigration

requires crossing of the endothelial cell layer and endothelial basement membrane. Interestingly, recent studies have demonstrated that T cells migrate through CNS endothelial cells by a transcellular pathway[62]. After endothelial transmigration, the endothelial basement membrane is reformed. Other cell types, such as neutrophils, transmigrate between tight junctions of CNS endothelial cells. Cells have to migrate through the extracellular matrix (ECM) of the perivascular space to reach the CNS parenchyma. This process is facilitated by the gelatinases MMP-2 and MMP-9, which can degrade type IV collagen of the ECM. In order to infiltrate the CNS parenchyma, additional proinflammatory mediators are likely required to pass the parenchymal basement membrane and astrocyte endfeet.

Autoantigen-specific T cells do not recognize intact myelin proteins. In the CNS parenchyma, autoantigen-specific T cells activate resident microglial cells, the APC of the CNS. After phagocytosis and proteolytic processing of myelin by microglia, $CD4^+$ T cells can encounter their respective antigens in the context of MHC-II. This initiates an inflammatory cascade with edema, cytokines (e.g. TNF-α, IL-12 and OPN), chemokines (e.g. CCL-19 and CCL-21), MMP and complement-mediated reactions (e.g. deposition of the membrane attack complex). Antibodies against myelin antigens can cause direct demyelination, possibly in combined action with complement components. In the context of autoantigen presented by MHC-I molecules, $CD8^+$ T cells can directly attack neurons and all glial cell types, including astrocytes, microglia and oligodendrocytes. $CD8^+$ T cells can directly induce target cell death, by the release of cytotoxic granules containing perforines and granzymes.

Counter mechanisms may dampen the inflammatory immune responses in the CNS or the periphery, including induction of apoptosis of inflammatory cells or induction of Th2 cells (IL-4, IL-5, IL-13), Th3 cells (TGF-β) or Tr1 cells (IL-10)[63]. Also resident microglia and astrocytes may restore homeostasis by producing anti-inflammatory cytokines, such as IL-10.

Experimental autoimmune encephalomyelitis

There is an urgent need to further elucidate the mechanisms involved in the immunopathogenesis of MS, since so far no long-lasting beneficial therapy has been developed. Therefore it is important to develop new therapeutic strategies and diagnostic tools to monitor treatment efficacy. In this thesis we describe the contribution of bacterial stimulation by peptidoglycan (PG) and immune modulation of CD97 and CD44variant isoforms in MS and in different animal models for MS. To determine whether these factors play a role in MS, we first assessed whether these bacterial components and molecules are present or expressed in *post-mortem* MS brain tissue, respectively. For obvious ethical and practical reasons, it is not possible to determine the functional aspects of PG, CD97 and CD44 in MS patients. As an alternative, EAE animal models are highly useful to investigate the

molecular events involved in the immunopathogenesis of CNS autoimmunity.

By using EAE models, several aspects of the clinical and pathological features of MS patients can be validated. The disease course in EAE is dependent on the encephalitogen, the protocol of immunization, and the species or strains used. We have analyzed EAE in two different monkey species and two different mouse strains. Thus far, spontaneous EAE development in mice is only observed in proteolipid protein $(PLP)_{139-151}$-specific T cell receptor transgenic mice[64] or myelin basic protein (MBP)-specific T cell receptor transgenic mice with a deficiency for RAG1[65]. In other mice, EAE needs to be induced by immunization or adoptive transfer. Well-known autoantigens used for immunization are total protein or peptides of myelin; MBP, myelin oligodendrocyte glycoprotein (MOG) and PLP. In the non-human primate species used in this thesis, EAE was induced by active immunization with human myelin or recombinant human MOG peptides. EAE in mice was induced by active immunization with MOG or PLP peptides or by the adoptive transfer of autoantigen-specific effector T cells, as described below. By using active (by immunization) versus passive (by adoptive transfer) EAE induction, a distinction can be made between the priming and effector phase of the immune response, and adjuvant effects can be excluded. The fundamentally distinct disease course and pathological presentation of the different EAE models might be representative for different forms and aspects of human encephalomyelitis. As an example, ADEM and acute MS forms may be modelled by acute EAE in rhesus monkeys, immunized with recombinant human MOG_{1-125} in CFA. Characteristics of the EAE models that were used in this thesis are summarized in Table 1.

Mouse EAE models

There are many advantages in using mouse EAE models. Mice are small, easily bred, relatively low in costs and moreover, many mouse-specific reagents are available. Mouse EAE models are well documented and over the years, much knowledge about immunopathogenic mechanisms and immunomodulating therapies in MS has been extrapolated from EAE mouse models. A major advantage of using mouse models is the possibility to genetically modulate genes of interest in EAE susceptible mouse strains. EAE in mice is characterized by mononuclear cell infiltrates in the CNS accompanied by clinical signs of paralysis. The disease can be induced in different susceptible mouse strains by immunization with myelin autoantigen in CFA.

In this thesis we used C57BL/6 and SJL/J mice and induced EAE by active immunization or adoptive transfer (Table 1). To induce EAE in C57BL/6 mice, animals were immunized with MOG_{35-55} in CFA. In this mouse strain, additional injections with *Bordetella pertussis* toxin (PTX) are required for disease development. PTX has been shown to stimulate the innate and adaptive immune response in the development and expansion of autoantigen-specific Th cells[66,67]. Furthermore, via stimulating TLR4, PTX can mediate leukocyte recruitment and adhesion to

Table 1. Characteristics of the EAE models used.

Strain/species	Haplotype/ MHC association	Immunogen	*B. pertussis*	Disease course	Axonal damage/ Demyelination	Adoptive transfer	Reference
C57BL/6 mouse	I-A^{b}	MOG_{35-55}	toxin	monophasic/chronic	yes	yes	66-68
SJL/J mouse	I-A^{s}	$PLP_{139-151}$	none	monophasic/ relapsing-remitting	yes	yes	69-71
			toxin	monophasic	nd	nd	
			dead bacteria	monophasic/chronic	nd	nd	
common marmoset *(Callithrix jacchus)*	*Caja*-DRB*W1201	human myelin	none	relapsing-remitting/ secondary-progressive	yes	yes, between chimeric twins	72-77
		$rhMOG_{1-125}$	none	primary-progressive	yes	nd	74,75,78
rhesus macaque *(Macaca mulatta)*	*Mamu*-DPBI*01	$rhMOG_{1-125}$	none	hyperacute	yes	nd	78,79
		$phMOG_{34-56}$	none	variable	yes	nd	78, 't Hart, *in prep.*

nd not determined
$rhMOG_{1-125}$ recombinant protein, representing the complete extracellular N-terminal domain of human MOG
$phMOG_{34-56}$ synthetic peptide, representing an extracellular N-terminal domain of human MOG

the BBB[68]. For EAE induction in SJL/J mice three different active immunization protocols were used. Mice were immunized with $PLP_{139-151}$ in CFA. Subsequently they were injected with either PTX, heat inactivated *B. pertussis* bacteria or did not receive additional injections. These groups develop a monophasic, chronic, or relapsing-remitting disease course respectively, and mimic different pathological aspects of MS, such as inflammation, demyelination and axonal suffering (Table 1).

Upon active immunization, autoantigen-specific naïve $CD4^+$ T cells are activated and polarized into $CD4^+$ Th1 cells in secondary lymphoid organs draining the injection sites. Already after several days, autoantigen-specific $CD4^+$ Th1 cells can be isolated. Upon *in vitro* re-stimulation with the antigen, highly encephalitogenic effector T cells are re-activated, which expand clonally. In the last decades of MS and EAE research the focus has been on the role of encephalitogenic $CD4^+$ Th1 cells[69,70]. However, it was shown that $CD8^+$ T cells outnumber the $CD4^+$ T cells[71,72] and are clonally expanded in MS brain lesions[73]. Here, $CD8^+$ T cells are in close contact with MHC-I-expressing oligodendrocytes and axons, with their cytotoxic granules in the direction of the contact zone between the T cell and the target cell[74]. Additionally, microglia, astrocytes and neurons can serve as targets for $CD8^+$ cytotoxic T cells. Furthermore, EAE studies have shown that like $CD4^+$ Th1 cells, encephalitogenic $CD8^+$ cells can also transfer disease into naïve recipients[75-77].

In EAE models, immunomodulating treatments can be assessed in different stages of the disease course, e.g. before disease onset, at the peak of the disease burden and during remissions. In our studies, for instance, we assessed the functional contributions of PG, CD97 and CD44variant isoforms 7 and 10 in the development of mouse EAE. In the different phases of the disease course, CNS tissues were isolated for immunopathological or flow cytometric analysis, and secondary lymphoid organs were isolated to determine antigen-specific T cell proliferation and cytokine production (Table 2).

Non-human primate EAE models

EAE susceptible mouse strains are of inbred nature and therefore restricted in immune response variability. This can be seen as an advantage, because isolated effects in cellular responses can be determined. But restriction in variability is a disadvantage with respect to the validation of such effects in comparison to MS patients. In essence, in genetic terms a homogeneous mouse strain represents only one individual. Non-human primates are of outbred nature and genetically and immunologically closely related to humans[78]. In non-human primate EAE models, the efficacy of new treatments for MS can be assessed pre-clinically by using human-specific reagents.

Two frequently used non-human primate EAE models are the rhesus macaque (*Macaca mulatta*) and the common marmoset (*Callithrix jacchus*)(Table 1)[79,80]. Rhesus monkeys are old-world monkeys and have an evolutionary distance to humans of 35 million years. Rhesus monkeys are infected by largely similar

Table 2. Overview of the mouse EAE studies.

Subject studied	Model	Mouse strain	Adjuvant	Encephalitogen/ encephalitogenic T cells	Mab treatment / genetic deletion	Read-outs	Chapter
PG	active immunization	C57BL/6	IFA + PG	MOG_{35-55}	none	EAE course, CNS pathology, T cell proliferation	3
CD97	active immunization	SJL/J	CFA	$PLP_{139-151}$	Mab anti-panCD97 (against EGF1)	EAE course	6
CD44v7	active immunization	SJL/J	CFA	$PLP_{139-151}$	CD44v7 deletion	EAE course, CNS pathology, T cell proliferation	5
		C57BL/6	CFA	MOG_{35-55}	CD44v7 deletion	EAE course, CNS pathology, T cell proliferation	5
	adoptive transfer	SJL/J	no	$PLP_{139-151}$ T cells	CD44v7 deletion	EAE course	5
		C57BL/6	no	MOG_{35-55} T cells	CD44v7 deletion	EAE course	5
CD44v10	active immunization	C57BL/6	CFA	MOG_{35-55}	CD44v10 deletion	EAE course, CNS pathology, brain analyses by flow cytometry, T cell proliferation	5

CFA complete Freund's adjuvant
EGF1 epidermal growth factor domain 1
IFA incomplete Freund's adjuvant

pathogens and follow a similar disease course compared with humans. Both humans and rhesus monkeys share MHC-II DP, DR and DQ loci and moreover, antigens can be presented and recognized across the species barrier. In MOG- and MBP-specific responses, epitope specificity is comparable with humans. In rhesus monkeys, EAE susceptibility is associated with the *Mamu*-DP locus. Upon immunization with recombinant human $MOG_{1–125}$, representing the complete extracellular N-terminal domain, rhesus EAE monkeys develop a hyperacute disease course, similar to acute forms of MS and ADEM[81]. A variable disease course was observed after immunization of rhesus monkeys with a synthetic peptide ($phMOG_{34-56}$), ranging from mild clinical signs to a hyperacute disease[79](B.A. 't Hart, *in prep*).

Marmoset monkeys are new world-monkeys and have an evolutionary distance to humans of 55 million years. Humans and marmoset monkeys share many general and disease-related features, e.g. neuroanatomy, T and B cell receptors, CNS immunopathology and MRI characteristics of lesions, as recently reviewed by 't Hart *et al.*[82]. Disease susceptibility is highly associated with *Caja*-DRB*W1201-restricted activation of $pMOG_{14-36}$ autoantigen-specific $CD4^+$ T cells. The disease course in marmoset monkeys closely resembles chronic MS. Marmoset monkeys immunized with MS-derived human myelin develop a relapsing-remitting or secondary-progressive disease course[83]. Immunization of marmoset monkeys with recombinant human MOG results in the development of a primary progressive disease course, with a broad variation in the onset of clinical signs, ranging between 2 and 16 weeks[79,84]. This variation in length of the asymptomatic period appears to be associated with the extent of MOG-specific epitope spreading[85], a phenomenon also known to be involved in relapse development in mouse EAE[53].

Pathological features of MS and non-human primate EAE brain lesions

Neuropathology of MS brain lesions

The most important pathological hallmark of MS is the presence of demyelinated lesions in the CNS. These lesions are mainly localized in white matter tracts of periventricular regions, cerebellum, brain stem, spinal cord and optic nerves. However, lesions can also be present in the gray matter of the cortex. In inflammatory lesions different infiltrated leukocyte subsets can be found i.e. macrophages (MΦ), dendritic cells (DC), T cells, B cells and granulocytes, which are mostly located in the perivascular space, but these cells can also infiltrate into the brain parenchyma. Furthermore, also resident microglia and astrocytes can be activated (i.e. express HLA-II molecules). Subsequently, these cells are able to produce inflammatory mediators, such as IL-12, IL-23 and nitric oxide (NO). In addition, deposits of antibodies (e.g. anti-MOG)[86] and complement components (e.g. C9neo antigen)[87] provide evidence for humoral autoimmune-mediated reactions in MS brain tissue[30].

Both cellular and humoral autoimmune reactions result in demyelinated areas, with denuded or damaged axons and oligodendrocytes.

Lesion staging systems in MS pathology

In all lesion staging systems, the term 'active' defines the process of active myelin breakdown, as can be demonstrated by myelin products in phagocytosing cells. There are three well documented and frequently used lesion staging systems which were compared by van der Valk and de Groot[88]. These include the staging systems of Lassmann and Brück[89,90], Bö and Trapp[91,92] and the Vienna consensus system[93].

Lassmann and Brück staging system

The Lassmann and Brück staging system is based on the timing of demyelination. Myelin is composed of 20-25% proteins and 75-80% lipids. Myelin proteins are differentially distributed within the myelin sheath. Myelin associated glycoprotein (MAG, 3% w/w of whole myelin) is located in the periaxonal space. MBP (30%) is a cytosolic protein, and PLP (50%) is integrated in the myelin membrane. MOG (0.05%) is located at the outer membrane and can therefore easily be accessed by the immune system. Demyelination of axons appears to be an ordered event, in which MOG and MAG are the first proteins found within MΦ. Luxol fast blue (LFB) staining can be used as a marker for early demyelination. Later on, PLP, MBP and periodic acid-Schiff (PAS; glycolipid staining)-containing MΦ are present within the lesions. According to the sequential myelin degradation, the system of Lassmann and Brück consists of the following stages:

1. *early active*, associated with MRP-14-expressing MΦ containing myelin (by LFB staining) and/or MOG/MAG-positive products
2. *late active*, associated with lysozyme-expressing MΦ ($27E10^{+}MRP14^{-}$) with PAS-positive products and a small amount of LFB-positive products
3. *inactive*, presence of PAS, but not LFB-positive material in MΦ and a limited degree of inflammation
4. *early remyelinating*, defined by the presence of many lymphocytes, MΦ and clusters of thinly myelinated axons
5. *late remyelinating* (shadow plaques), with few MΦ, astrogliosis and thinly myelinated axons

Bö and Trapp staging system

The system of Bö and Trapp is mainly based on cellular criteria and recognizes three different lesion stages:

1. *active (demyelinating)*, hypercellular lesions
2. *chronic active*, lesions with a hypocellular center and a hypercellular rim
3. *chronic inactive*, hypocellular lesions

Vienna consensus
In the Vienna consensus inflammatory criteria (Bö and Trapp) and demyelinating criteria (Lassmann and Brück) have been combined into a basic four-points staging system[93]. A more detailed characterization was recently put forward by Lucchinetti *et al*., who defined four different active demyelinating lesion patterns, based on myelin protein loss, geography and extension of plaques, pattern of oligodendrocyte destruction and immunological evidence of immunoglobulin and complement deposits[94]. The most frequently observed type I and II lesions are localized around small veins and venules and are defined as T cell-mediated or T cell plus antibody-mediated lesions, respectively. In contrast, type III and IV lesions are characterized by ill-defined borders, diffuse spread into the white matter, oligodendrocyte dystrophy and are supposed to be related to virus- or toxin-induced demyelination and not of autoimmune origin. Type III lesions appear predominantly in MS patients with an acute disease course. Type IV lesions are rarely found.

Adapted lesion staging system from Bö/Trapp by van der Valk/de Groot
We have used a staging system described by van der Valk and de Groot, which is a modified version of the system used by Bö and Trapp. This system is also based on both inflammation and demyelination criteria[88,95]. All stages, except the pre-active lesions, have been described in detail by Bö *et al*.[91] and Trapp *et al*.[92]. Van der Valk and de Groot introduced a lesion stage that supposedly precedes the active lesion stages, the pre-active lesion. These pre-active lesions are more extensively described in relation to MRI measurements[96]. It was found that the majority of macroscopically invisible lesions detected by MRI were of the pre-active stage. It is, however, not proven that these lesions are the first stage in MS brain pathology and that all these pre-active lesions will progress into active lesions. Van der Valk and de Groot included this stage since it represents abnormalities in the white matter, which may give insight into the development of active lesions.

Four different lesion stages are recognized:
1. *pre-active*, defined as abnormalities of the white matter with clusters of activated HLA-II-expressing microglial cells and/or perivascular inflammatory cells, but no demyelinated areas, or myelin-breakdown products within MΦ
2. *active demyelinating*, defined by the presence of myelin breakdown products in MΦ, which can be visualized by oil red O, a histochemical staining for neutral lipids
3. *chronic active*, characterized by an active hypercellular rim and an inactive hypocellular center
4. *chronic inactive*, characterized by a hypocellular area with few or no myelin-containing MΦ

Neuropathology of rhesus and marmoset EAE monkeys

Rhesus monkeys immunized with $MOG_{1–125}$ develop an acute EAE. In the brain, large demyelinated hemorrhagic/necrotic lesions are present which contain abundant numbers of neutrophils and extensive axonal damage[79]. In $phMOG_{34-56}$-immunized rhesus monkeys, brain pathology is characterized by a variable degree of demyelination and axonal pathology (B.A. t' Hart, *in prep.*). Lesions contain abundant numbers of T cells, MΦ and few neutrophils. In some animals acute lesions occur, similar to CNS lesions in ADEM patients.

The neuropathological features of myelin-induced EAE in marmosets are highly comparable to MS lesion pathology[83]. In the CNS white matter of marmoset monkeys with EAE, lesions are characterized by focal demyelinated areas, containing cellular infiltrates with activated HLA-II$^+$ CD40$^+$ MΦ, CD154$^+$ T cells, B cells, Th1 and Th2 cytokines, MMP and anti-MOG antibodies[86,97]. The lesions can be classified as type II lesions, described by Lucchinetti *et al*.[94], based on the presence of T cell-mediated inflammation and antibody-mediated primary demyelination. Axonal pathology can vary in EAE lesions of marmoset monkeys, since axonal pathology was not observed in inflammatory inactive lesions, while in inflammatory active lesions damaged axons were present, as demonstrated by the accumulation of β-amyloid precursor protein and reduced phosphorylation of neurofilaments[98]. Moreover, in chronic inactive lesions, severe axonal destruction was observed, accompanied by astrogliosis and remyelination[83,86].

MOG-induced EAE lesions in marmoset monkeys are characterized by predominantly sharply defined demyelinated areas with inflammatory cells. These lesions are categorized as early active lesions based on the Lassmann and Brück staging system[89]. Axonal structures are relatively well conserved in these lesions. In marmoset monkeys, a small number of lesions are chronic inactive and contain substantially damaged axons. The number of these chronic inactive lesions seems to increase with the progression of disease, as determined by serial MRI measurements[84].

Bacterial peptidoglycan, a cofactor in the pathogenesis of autoimmune diseases

Intestinal flora and chronic inflammation

Microbial components may stimulate harmful immune responses against natural antigens (e.g. commensal bacteria and food antigens) in patients with inflammatory bowel disease (IBD) or self antigens in patients with rheumatoid arthritis (RA) and MS. Such components may be derived from infections by bacterial or viral pathogens. In addition, all mucosal, and particularly the mucosal surface of the gastrointestinal tract, serve as a constant major microbial source. From birth onwards the intestinal tract is colonized by maternal-derived and environmental-derived microbes. At least

500 different bacterial species are present within the intestinal tract. One gram of stool contains $1x10^6$ – $1x10^8$ aerobic bacteria and $1x10^9$ - $1x10^{10}$ obligate anaerobic organisms, of which 50% are Gram-positive. Under normal circumstances a symbiosis is present between enteric bacteria and the digestive system. Both the epithelial cell lining and the enteric bacterial cell layer function as an anatomical barrier against harmful agents. Colonization of the gut is not only important as a physical barrier, but also for digestive functions, development of the gut associated lymphoid tissue, limiting intestinal inflammation and minimizing the invasion of pathogens across the single-cell layered epithelial barier.

External changes, such as diet and stress may change the normally stable intestinal bacterial composition. Several studies provide evidence that the composition of the intestinal flora plays a role in the development of the systemic autoimmune diseases IBD and RA[99-101]. Patients with IBD suffer from a chronic inflammation of the gastrointestinal tract and this inflammation appears to result from an inappropriate immune response to the normal intestinal flora. Interestingly, 5-23% of IBD patients additionally suffer from RA[101], with a common direct positive relationship between the activity of both diseases. The intestinal Gram-positive anaerobic bacteria in early RA patients differ significantly from healthy controls[102,103]. Experimental animal models have demonstrated that arthritis can be induced in susceptible animals upon injection of cell wall components of a selected group of commensal Gram-positive strains[101]. Other Gram-positive strains were shown to be nonarthritogenic. One of the major components of Gram-positive bacteria is PG. PG is able to induce inflammatory responses, similar to Gram-negative LPS. It has been proposed previously that PG stimulates chronic inflammation in RA and MS[104,105]. In this thesis we investigated whether bacterial PG may functionally contribute to the disease course in MS, by using animal models.

The bridge between innate and adaptive immunity

The dependency of the adaptive immune response on components of the innate immune system is a well established concept. The innate immune system utilizes serum and cell surface regulatory proteins to efficiently trigger adaptive immune responses. The bridge between innate and adaptive immunity is regulated by cell associated and soluble pattern-recognition receptors (PRR) as well as components of the complement system, as has been thoroughly reviewed recently[106-108]. Upon activation, cells express a distinct repertoire of surface molecules which are involved in the orchestration of subsequent effector functions. CD97 and CD44 are cell surface molecules which cover a wide variety of biological responses e.g. cell activation, migration, adhesion and extravasation into inflammatory sites, and apoptosis. Additionally, these molecules have been indicated to play a substantial role in chronic inflammation, i.e in RA and IBD. In this thesis we assessed whether CD97 and CD44variant isoforms are involved in the immunopathogenesis of MS and mouse EAE. In the following paragraphs an introduction will be given about PG, CD97 and CD44.

Peptidoglycan

PG is a strong, rigid structure formed by three-dimensional cross-linked layers of glycan backbones with peptide bridges. In Gram-positive bacteria, PG contains 10 to 70 layers and accounts for approximately 40-70% of the cell wall mass (Figure 3a). In contrast, PG in Gram-negative bacteria contains 1 or 2 layers and constitutes only 10% of the cell wall mass[109]. PG surrounds the cytoplasmic membrane and functions to withstand osmotic pressures exerted from the cytoplasm[110]. PG is essential for the structure, replication and survival of bacteria. PG is composed of two repeating alternating amino sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), attached to each other by β-1,4-glycosidic bonds. This glycan chain is conserved in all bacteria and is usually *N*-acetylated and sometimes *O*-acetylated. Muramic acid is a unique structural component of PG and can therefore be used as a marker for PG detection by mass spectrometry or high performance liquid chromatography. The glycan chains are cross-linked via attachment of tetra or pentapeptides, the stem peptides of PG. Stem peptides can be attached directly to each other or indirectly via interpeptide bridges. Between different bacteria, interpeptide bridges can vary in length and amino acid composition[111,112]. Some stem peptides are connected with only one glycan chain, and are not cross-linked to other stem peptides.

Teichoic acids (TA) and lipoteichoic acids (LTA) are dispersed through the PG network and may stay in the PG layer after partial digestion. These unique structures for Gram-positive bacteria are water-soluble polymers, which contain chemically modified ribose or glycerol connected by phosphates. Antigenic determinants, such as sugars or D-alanine may be attached to the hydroxyl sites of ribose and glycerol. LTA contains an acyl group and is anchored to the cytoplasmic membrane. In contrast, TA is non-acetylated and is covalently attached to PG strands.

Variation of PG stem peptides

PG can be divided into group A, and the less common group B PG, depending on the position of stem peptide cross-linkage[111]. The connection of group A PG is formed between position 3 of one stem peptide chain and position 4 of another stem peptide chain (Figure 3b). Stem peptides of group B PG are connected at position 2 and 4. The first two amino acids attached to MurNAc of the glycan backbone vary between different bacterial strains. In group A, L-alanine is always the first amino acid attached to MurNAc of the glycan backbone, followed by a D-glutamine in the second stem peptide position. The MurNac-L-ala-D-Glu, called muramyl dipeptide (MDP), is the smallest conserved motif of PG. In PG of group B, the first amino acid attached to MurNAc is a glycine or L-serine, followed by D-glutamine.

PG is further subdivided according to the diamino amino acid of position 3 in the stem peptide. These specific diamino amino acids also vary between different strains[113] and have been show to play an essential role in the recognition of PG by receptors of the innate immune system. Gram-negative bacteria and occasional

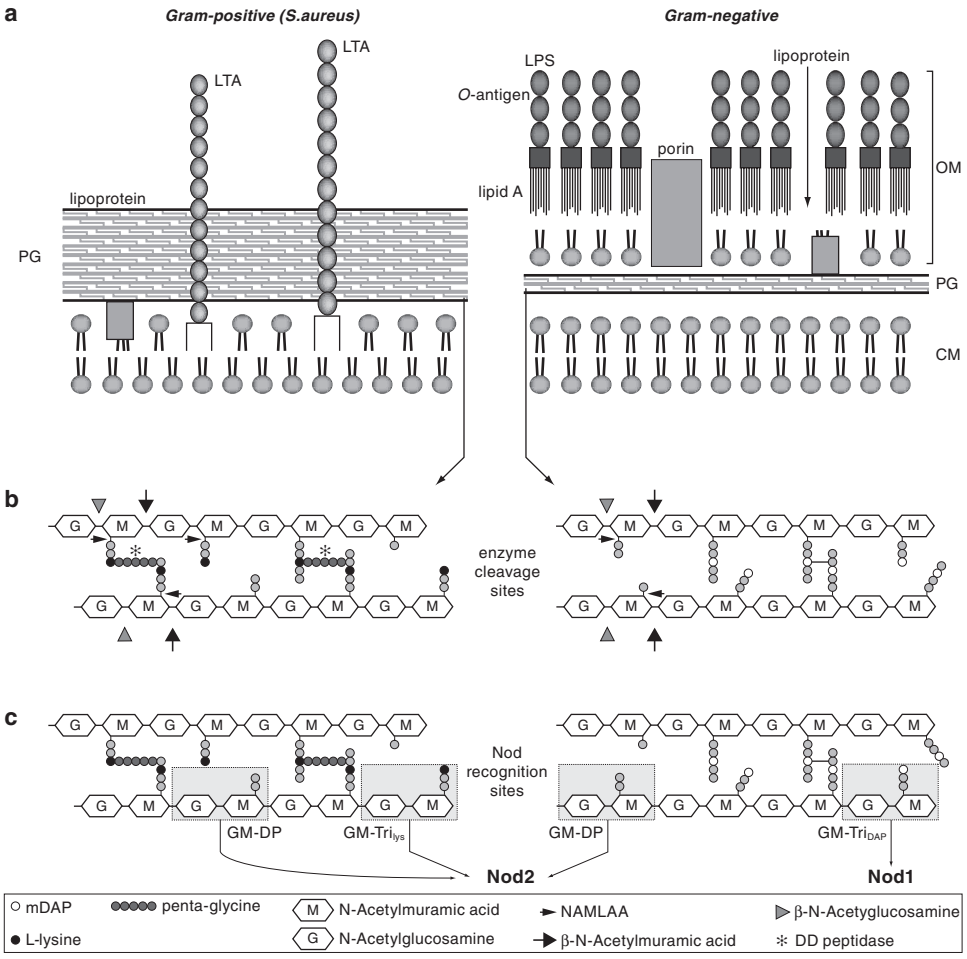

Figure 3. Structural composition of PG in Gram-positive and Gram-negative bacteria.
The architecture of PG differs in Gram-positive and Gram-negative bacteria. (a) PG is a major cell wall component of Gram-positive, but a minor component of the cell wall of Gram-negative bacteria. (b) PG is composed of two alternating sugar residues (G and M), which are connected via stem peptides (circles). Most Gram-positive bacteria contain an L-lysine at position three of the stem peptide (●), whereas mDAP is present at this position in Gram-negative bacteria (○). Cleavage by specific enzymes (arrows) results in the release of PG fragments with different sizes and biological activities. Enzyme cleavage sites have been indicated for the first G-M residue and are identical for the following G-M residues. (c) The recognition of PG by the innate immune receptors Nod1 (Card4) and Nod2 (Card15) depends on the structural motifs of the stem peptides. Nod1 recognizes GM-Tri_{DAP} structures of Gram-negative bacteria. Nod2 specifically detects GM-DP structures, present in both Gram-negative and Gram-positive bacteria. OM: outer membrane; CM: cytoplasmic membrane. Modified from Kaisho and Akira, 2001[339] and Girardin *et al.*, 2003[340].

Gram-positive bacteria (genera *Bacillus* and *Clostridium*) contain *m*-diaminopimelic acid (mDAP) at position 3 (Figure 3b). mDAP is a precursor of L-lysine. mDAP-containing stem peptides are usually directly cross-linked to other stem peptides. Most other Gram-positive bacteria have an L-lysine at position 3 and these stem peptides are mostly cross-linked via interpeptide bridges. Some Gram-positive bacteria contain L-ornithine at this position. Interestingly, 70% of pathogenic bacterial classes contain L-lysine at position 3, while 75% of non-pathogenic bacteria contain mDAP/L-ornithine[114]. These findings correlate well with the natural habitat of bacteria; 86% of L-lysine type bacteria colonize animals, while 75% of mDAP/L-ornithine-containing bacteria live outside living hosts (e.g. in soil).

For our functional studies, we have used purified PG derived from the pathogenic Gram-positive bacterium *Staphylococcus aureus* (Figure 3)(chapter 2 and 3). Together with more than ten other bacterial species, *S. aureus* contains an *O*-acetylation site at the C-6 hydroxyl group of MurNAc. *S. aureus* also contains an L-lysine at position 3 of the stem peptide, which can be connected to other stem peptides through a pentaglycine bridge. Within the structure of *S. aureus*[115], and other bacteria as well, a great variety of stem peptide compositions can be found. PG is insoluble in its native form[116], but can be enzymatically cleaved into smaller soluble portions by specific PG-degrading enzymes or antibiotics. We have used both insoluble and soluble PG (chapter 3).

Peptidoglycan degradation

Bacterial cell wall PG is constantly being synthesized and degraded. During these processes PG components can be released. By preventing the activity of bacterial transglycosidases and transpeptidases, penicillin and other clinically useful antibiotics can inhibit bacterial PG synthesis[112]. This results in the release of large uncrosslinked glycan chains. Three different PG-degrading enzymes, which act as natural antibiotics, are known in humans. The cleavage sites of these specific enzymes are depicted in Figure 3b. Lysozyme (*N*-acetylmuramidase) and *N*-acetylglucosaminidase cleave the β-1-4 glycosidic bond between GlucNAc and MurNAc or MurNAc and GlucNAc, respectively[117,118]. Cleavage by these specific enzymes results in the release of monomeric muropeptides. The third muralytic enzyme is *N*-acetylmuramyl-L-alanine amidase (NAMLAA), which was recently shown to be identical to peptidoglycan recognition protein long form (PGRP-L)[119,120]. NAMLAA breaks the bond between MurNAc and the L-alanine at position 1 of the stem peptide[121]. It is likely that many other as yet unidentified muralytic enzymes exist in humans. In addition, bacteriolytic enzymes can also degrade PG, such as endopeptidases, which cleave PG at the interpeptide bridges.

The sensitivity of PG for degradation by enzymatic activity is dependent on the composition of the cell wall. *O*-acetylated PG is more resistant to degradation than non-*O*-acetylated PG[122,123]. In addition, PG is more resistant to degradation when there is a high degree of cross-linkage between the stem peptides[124]. Also,

polysaccharides, TA and LTA can inhibit muralytic degradation of PG to some extent. Differential persistence of PG within a cell type is thus determined by the structure of PG structure and the spectrum of relevant enzymes and receptors (see below).

Bacterial pattern recognition receptors

The innate immune system can recognize certain viral and bacterial motifs, the so-called pathogen associated molecular patterns (PAMP). PAMP are specifically recognized by a diverse repertoire of PRR, expressed by particular hematopoietic cells (e.g. APC) and non-hematopoietic cells (e.g. epithelial cells). PRR which recognize bacterial PAMP include CD14, members of the TLR family and nucleotide-binding oligomerization domain (Nod) receptors. Such molecules relevant in the studies in this thesis are briefly introduced below.

CD14

CD14 is present as a cell surface receptor expressed by myelomonocytic cells and as a soluble molecule found in serum[125,126]. Cell surface and soluble CD14 are identical in amino acid composition and only differ by the presence of the glycoylphosphatidylinositol (GPI) structure in membrane bound CD14. CD14 functions as a co-receptor for LPS recognition of Gram-negative bacteria[127]. CD14 can also serve as a co-receptor for the detection of Gram-positive cell wall structures, including PG[128,129]. But, LPS and PG differ in CD14 ligation and subsequent induced responses upon CD14 receptor triggering, as described below.

Unlike PG, LPS is bound in serum by LPS-binding protein, which modifies LPS aggregates into single LPS molecules and delivers LPS to membrane bound CD14[130]. In this way, LPS-binding proteins greatly enhance CD14-mediated responses. Also soluble CD14 can transport LPS molecules to cell membranes. These complexes can either induce activation of cells which normally do not express membrane bound CD14 or can enhance responses of membrane CD14-expressing cells[131,132]. By binding of soluble CD14, PG can also enhance responses of CD14-expressing cells[132], but cannot induce activation of CD14-negative cells[133]. Furthermore, CD14 contains discrete binding sites that are partially similar and partially different for LPS and PG[134]. Binding of PG to CD14 is likely dependent on the glycan backbone of PG. Low-molecular weight fragments of PG, such as MDP, disaccharide dipeptides or pentapeptides do not bind CD14, whereas high molecular weight polymeric PG does bind CD14[129,134]. Since CD14 does not contain a transmembrane domain it is unlikely that CD14 by itself can mediate cell activation. Therefore it is generally assumed that CD14 functions as a ligand-binding co-receptor, which delivers and enhances cell activation mediated by other membrane expressed PRR, such as TLR.

Toll-like receptors

TLR are type-I transmembrane molecules that contain extracellular leucine-rich repeats and cytoplasmic TIR domains (Toll/IL-1 receptor). So far 13 mammalian TLR have been identified. TLR play a critical role in the induction of innate immune responses. Bacteria can be recognized and discriminated by TLR2 (+ TLR1 or TLR6), TLR4, TLR5 and TLR9, discussed briefly below.

TLR2 recognizes many different bacterial components, including PG, LTA, lipoproteins, MALP-2, the synthetic PAM3CysK4, lipopeptides and lipoarabinomannan from *M. tuberculosis*. For its functional activation, TLR2 needs to form heterodimers with either TLR1 or TLR6. For example, TLR2 cooperates with TLR6 for the recognition of MALP-2 as was demonstrated by the analyses of knock-out mice.

PG is recognized by TLR2/6 heterodimers[135]. TLR2/6 molecules are recruited into MΦ phagosomes where they recognize intracellular PG[136]. It has been suggested previously that TLR2 directly binds to PG by a sequence of 25 amino acids in the extracellular domain of TLR2[137]. However, the concept that PG stimulates cells via TLR2 has become controversial. Recently, it was demonstrated that PG-induced effects were related to the presence of LTA and TA contaminations in PG preparations[138]. During PG preparation, LTA and TA components are difficult to remove completely. In concordance, LTA signals via TLR2[139,140] and is a potent inducer of proinflammatory cytokines by APC[141,142].

Interestingly, TLR2-induced responses can be enhanced or inhibited by interacting with TLR6 and TLR1, respectively[143]. Furthermore, it was demonstrated that TLR2 might distinguish between different Gram-positive bacteria, since TLR2 transfected CHO cells respond to heat-killed *Listeria monocytogenes*, but not to group B *Streptococci*[144]. TLR2 expression is restricted to phagocytic cells and epithelial cells. In humans, TLR2 is predominantly expressed by monocytes, MΦ, DC and to a lesser extent by neutrophils and a few other cells[145,146]. In contrast, TLR1 and TLR6 are constitutively expressed by many cell types[145]. Taken together, TLR2 plays an essential role in recognition of a variety of bacterial antigens, and may regulate innate responses by cooperating for PAMP binding with different TLR and CD14.

TLR4 functions as the cell signaling receptor for Gram-negative bacterial LPS. LPS is embedded in the outer membrane via its lipid portion (lipid A) and exposes its *O*-polysaccharides (*O*-antigen) outside the bacterial surface. LPS recognition by TLR4 is complex and requires several accessory molecules, such as LPS-binding protein, CD14 and MD2. MD2 is a small protein that lacks a transmembrane region. For the recognition of LPS, TLR4 requires association with MD2[147]. In addition to LPS, TLR4 also appears to mediate inflammatory responses to many different self-ligands (e.g. heat shock proteins) and non-self ligands (e.g. LTA), summarized by

Johnson *et al*.[148]. TLR4 is expressed by a variety of cell types, especially by cells of the immune system, including MΦ and DC[149].

TLR5 is involved in the recognition of flagellin, a highly conserved 55 kDa monomer obtained from bacterial flagella. Flagella are polymeric rope-like subunits, which extend from the outer membrane of bacteria and provide motility. Interestingly, TLR5 is expressed on the basolateral, but not apical surface of intestinal epithelial cells[150]. In this way, the innate immune system can recognize flagellin from pathogenic bacteria that have crossed the epithelial barrier, but ignore flagellin from commensal bacteria, which do not have the capacity to cross epithelial barriers under normal circumstances.

TLR9 mediates inflammatory responses against unmethylated CpG, present in bacterial DNA, viral DNA and synthetic oligodeoxynucleotides[151]. The immunostimulatory capacities of bacterial DNA can be completely abrogated by a single nucleotide substitution or methylation of a cytosine residue[152]. In the mammalian genome, most DNA is methylated and is therefore not recognized by TLR9. Motifs of CpG that optimally stimulate TLR9 differ between mouse and man, due to differences in the extracellular regions of TLR9[153]. This suggests that TLR9 directly recognizes CpG ligands. Interestingly, TLR9 is expressed intracellularly, but not on the cell surface. Therefore, signaling by unmethylated CpG DNA requires internalisation into late endosomal or lysosomal compartments[154].

Nod1 and Nod2 receptors

Nod proteins belong to the nucleotide-binding site leucine-rich repeat (NBS-LRR) family. Nod proteins are intracellular PRR and have been shown to regulate NF-κB activation and apoptosis induction[155]. Two Nod proteins, namely Nod1 (also known as Card4) and Nod2 (Card15) are specifically involved in the recognition of bacterial PG. The functional components of Nod1 are composed of an N-terminal caspase recruitment domain (CARD), an NBS domain and a C-terminal LRR domain[156]. Nod2 contains an additional CARD domain. CARD and NBS domains are crucial for the activation of NF-κB, whereas the LRR might play a negative regulatory role in the activation of NF-κB[157]. Furthermore, the C-terminal LRR domain is involved in the recognition of PG[158,159]. Nod1 is expressed in epithelial cells, whereas Nod2 is expressed in different myeloid cell types, including monocytes, DC and granulocytes[160,161].

It appears that specific parts of the PG structure are responsible for its proinflammatory properties. Interestingly, Nod1 and Nod2 are distinct and non-overlapping in their sensing specificity for PG fragments (Figure 3c). Nod1 specifically detects Gram-negative (mDAP type) PG, composed of the following muropeptide: GlcNAc-MurNAc-L-ala-D-Glu-mDAP (GM-Tri_{DAP})[162,163]. The glycan backbone is dispensable herein, since γ-D-Glu-mDAP is the minimal motif required

for Nod1 recognition[162,164]. This structure has not been identified as naturally occurring bacterial product, in contrast to GM-Tri_{DAP}. The GM-Tri_{DAP} can be seen as a unique bacterial fingerprint, since both D-Glu and mDAP are absent in eukaryotic cells. For Nod1 recognition it is pivotal that mDAP is presented as the terminal amino acid, because cross-linked stem peptides and peptides containing four amino acids are not recognized by Nod1.

In contrast to Nod1, Nod2 recognizes PG from both Gram-positive and Gram-negative bacteria. Of note, Nod2-mediated recognition of PG is dependent on the presence of MurNAc. The minimally required structure for Nod2 recognition is MDP[159,165]. However, MDP is not naturally formed and is inactive in most experimental systems, except for experimental meningitis[166]. GlucNAc-MurNAc-L-ala-D-Glu (GM-DP) is a naturally occurring bacterial product present in all bacteria, which is recognized by Nod2. In this manner, Nod2 can detect PG fragments from both Gram-positive and Gram-negative bacteria. More specific analysis revealed that longer stem peptides need to contain an L-lysine or L-ornithine at position 3 (in Gram-positive bacteria) for recognition by Nod2[164]. In contrast, Nod2 does not recognize MurNAc-Tri_{DAP}. In addition to different recognition specificities for PG, Nod1 and Nod2 also differ in expression. We have used PG derived from *S. aureus*, which does not contain the motif required for Nod1 ligation (GM-Tri_{DAP})[163,167], but contains the motif necessary for Nod2 ligation (Figure 3c)[159,165].

Signaling via TLR and Nod receptors

Activation of intracellular signaling pathways by TLR leads to the transcription of several genes including inflammatory cytokines, chemokines, MHC-II molecules and costimulatory molecules[168,169]. Both TLR and IL-1R depend on signaling via conserved TIR (Toll/IL1R homology) domains. Interestingly, upon specific TLR ligation distinct signaling pathways are initiated, mediated by the use of distinct adapter proteins. So far, four different adaptor proteins have been identified, i.e. MyD88, Mal, Trif and Tram[170]. TLR activation results in the recruitment of the cytoplasmic adapter protein MyD88. MyD88 recruits and autophosphorylates serine/threonine kinases of the IRAK family. IRAK then associates with TRAF-6, which induces activation of TAK1 and MKK6. These kinases induce the degradation of Iκ-B and translocation of NF-κB to the nucleus, and activate MAP (mitogen-activated protein) kinases, such as p38 and c-Jun N-terminal kinases. IRAK-M has been shown to be a negative regulator of TLR signaling[171,172]. TLR-mediated responses can also be mediated by an MyD88-independent pathway and lead to the activation of IRF-3 (interferon regulatory factor 3)[173]. Taken together, a growing body of evidence now indicates that there are different and multiple signaling pathways used by individual TLR.

Nod1 and Nod2 signaling is currently being characterized and these two molecules appear to use similar transduction pathways as TLR. The first step of Nod1/2 signaling involves the oligomerization of Nod[174], followed by recruitment of

the CARD-containing adapter protein Rip2 (also known as RICK)[175]. Subsequently, Rip2 interacts with the regulatory subunit IKKγ (also known as NEMO) of the IKK complex, inducing NF-κB translocation to the nucleus. Nod1/Nod2 signaling is not dependent on the adapter molecule MyD88. In addition to the activation of NF-κB, Nod1/Nod2 oligomerization induces caspase activation. In example, caspase 1 and 9 both contain CARD domains, which can associate through homophilic interactions with the CARD domains of Nod1[157,176]. In addition, activation of Nod1 results in the caspase-1 dependent release of active proinflammatory IL-1β[177]. Taken together, Nod-mediated signaling pathways involve both the activation of proinflammatory gene transcription and activation of pro-apoptotic caspases. These different pathways may interact to form a balance between the activation (i.e. by inducing proinflammatory cytokines, chemokines and costimulatory molecules) and the suppression (i.e. by inducing apoptosis and IL-10 production) of the host defence.

PG distribution and association with chronic inflammation

Immunohistochemical and mass spectrometric analysis revealed that PG is present within peripheral blood leukocytes[178], and within MΦ and DC in spleen[179] from healthy donors. Most bacterial PG in tissues probably originates from absorption of commensal bacteria and bacterial products in the gut. This idea arose by the observation that PG is absent in umbilical vein blood of new-borns[178], which lack gut flora. In accordance, PG is absent in spleen tissue from germ-free and neonatal rats. Upon injection of PG in germ-free rats, or after the age of 10 weeks in conventional rats, PG can be detected in rat spleen sections, by immunohistochemistry[180].

Bacterial products are sampled from the intestine through specialized epithelial M cells or by DC. To this end, DC can penetrate the epithelial barrier with their dendrites and thereby sample the intestinal flora[181-183]. Both M cells and DC can deliver bacterial antigens into the Peyer's patches, as part of the gut associated lymphoid tissue. M cells deliver the antigens to professional APC. The presence of PG is not restricted to lymphoid tissues, since in conventional rats also brain, liver and kidney contain PG[184]. It is assumed that the kidney clears PG from the circulation. This assumption emanated from the observation that small PG fragments are detected in urine after penicillin treatment[185,186].

Intraperitoneal injection of bacterial cell wall components or PG from specific bacterial strains is a widely used model for the induction of experimental arthritis[187,188]. By i.p. injection of PG in rats, it was demonstrated that the spleen and liver serve as natural reservoirs of specific PG subtypes. It was suggested that from these tissues PG is distributed, via the circulation, into the synovium and peri-articular tissues[189,190] and thereby can contribute to the exacerbations of arthritis. Furthermore, mouse studies demonstrated that i.v. injected radiolabeled PG is additionally distributed into the lung, intestine and brain[191]. Recent findings indicate

that the retention and arthritogenic capacity of PG is dependent on its chemical composition. Persistence and arthritogenicity of PG is specifically attributed to the L-lysine at the third position of the stem peptide[192,193].

Taken together, PG may act as a stimulating cofactor for the development and perpetuation of chronic inflammation, even in the absence of infection or bacterial replication. One of the driving ideas of our research is that APC can redistribute biologically active PG from the mucosa to secondary lymphoid organs as well as to sites of chronic inflammation and stimulate autoimmunity at both locations. Indeed, it was shown previously that PG isolated from sterile human spleen was biologically active. Human blood cells produced IL-1, IL-6 and TNF-α upon stimulation with spleen-derived PG[179]. In addition, spleen-derived PG induced $CD4^+$ and $CD8^+$ T cell proliferation, which depended on CD14 binding, proteolytic processing and MHC-I/II presentation of PG by APC[194]. Interestingly, elevated numbers of PG-containing APC are present at sites of chronic inflammation in MS brain tissue[105], RA synovial tissue[104,195], bowel wall tissue of patients with Crohn's disease (CD)[196] and in atherosclerotic plaques[197]. These cells expressed costimulatory molecules, MHC-II and cytokines[104,105].

Cellular events in inflammation; a role for CD97 and CD44variant isoforms

CD97

Class B EGF-TM7 receptors

CD97 molecules are composed of a seven-span transmembrane domain (TM7) and variable numbers of N-terminal epidermal growth factor (EGF) domains (Figure 4). These structural characteristics are specific for members of the long N-terminal class B-TM7 receptor family (LNB-TM7). Family members that exclusively contain EGF domains in the extracellular region are referred to as EGF-TM7 receptors. Six EGF-TM7 receptors have been identified in man: CD97, EGF-like module containing mucin-like receptor protein 1 (EMR1)[198], EMR2[199], EMR3[200], EMR4[201] and EGF-TM7-latrophilin-related protein (ETL)[202]. The mouse genome includes CD97[203,204], EMR1[205,206], EMR4[207,208] and ETL[209], but not EMR2 and EMR3. Mouse EMR1 and EMR4 are also known as the mouse MΦ marker F4/80 and the F4/80-like-receptor (FIRE), respectively.

Expression

CD97 expression is rapidly induced on activated T cells, B cells, monocytes, MΦ, DC and granulocytes, while the molecule is constitutively expressed at low levels by granulocytes, monocytes and resting T and B cells, but not by microglial cells[210-212]. Interestingly, soluble CD97α-subunits (sCD97) can be secreted under inflammatory conditions[213]. sCD97 is not found in normal human serum or plasma,

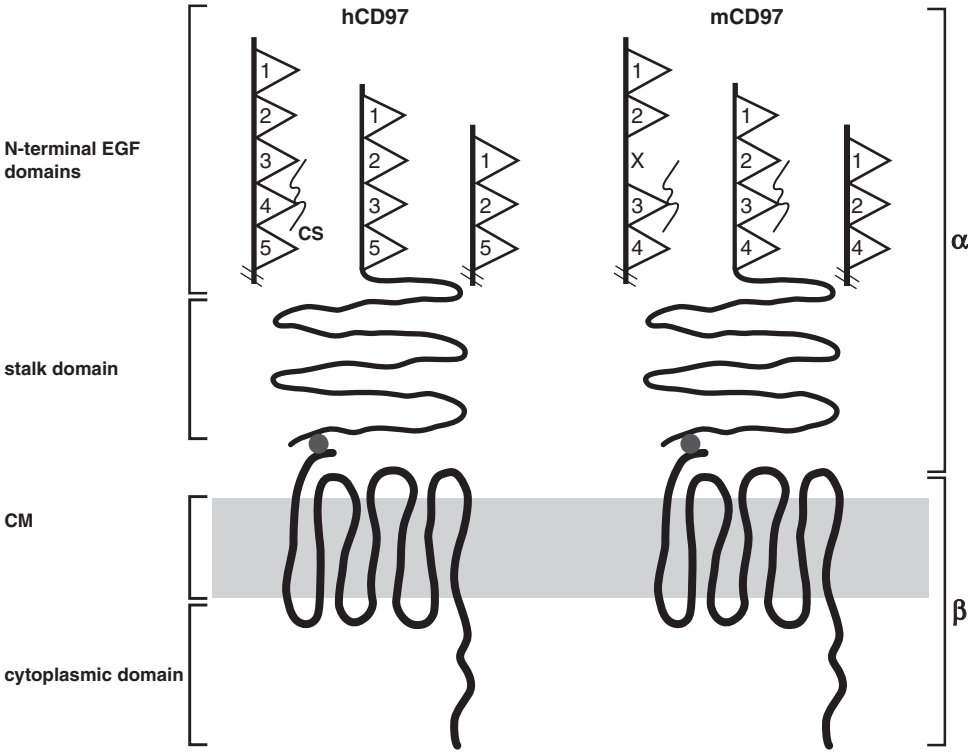

Figure 4. Schematic structure of human and mouse CD97 isoforms.
The CD97 gene is transcribed into one polypeptide, which is proteolitically cleaved at the G-protein-coupled receptor-proteolytic site (●) in the edoplasmatic reticulum. CD97 molecules are expressed as heterodimers at the cell surface, containing an extracellular α-subunit, which is non-covalently linked to an 7-TM β-subunit. Additionally, soluble CD97α isoforms can be secreted under inflammatory conditions. Due to alternative RNA splicing, the α-subunit can be extended by the addition of different numbers of EGF domains (▷). Human EGF4 and mouse EGF3 contain a binding site for chondroitin sulfate (CS). CM: cell membrane, hCD97: human CD97, mCD97: mouse CD97. From Leemans et al., 2004[233].

nor in supernatant of activated T cells[214]. The functional implications of sCD97 are unknown at the moment. Human CD97-EGF4 is expressed by monocytes, DC, MΦ and granulocytes[215]. Lymphocytes express CD97-EGF4 after stimulation with PMA, PHA or anti-CD3 Mab[215]. EMR2-EGF4 expression is restricted to cells of the myeloid lineage, such as monocytes and granulocytes[215]. It was suggested that MΦ and DC express higher levels of EMR2-EGF4 compared to monocytes.

Structural characteristics

Membrane bound CD97 molecules are expressed as heterodimers, consisting of an extracellular α-subunit non-covalently linked with a TM7 β-subunit (Figure 4). The α-subunit is composed of a long stalk region and a variable number of EGF domains, which are formed by alternative RNA splicing. Three different CD97 isoforms can be distinguished, containing three, four or five EGF domains in humans. In mouse CD97, the longest isoform contains four EGF domains with an unidentified additional sequence of 45 amino acids between the second and third EGF domain. Mouse EGF domains 3 and 4 are homologues of human EGF domains 4 and 5, respectively. Human CD97 and EMR2 α-subunits are highly homologous, especially within the EGF domains, which differ by only six amino acids. Figure 4 represents the different isoforms of human (hCD97) and mouse CD97 (mCD97).

CD97 was originally described as a marker for immune activation. It has been postulated that CD97 is involved in signal transduction via the activation of G-proteins. G-protein-coupled receptors activate a sequence of events that results in the influx of intracellular cyclic AMP and Ca^{2+}. Accordingly, EGF-TM7 receptors may induce signal transduction via G-protein independent pathways, as has been reported for other TM7 receptors. By activation of G-proteins, other members of the TM7 family mediate cellular responses via an enormous diversity of signaling molecules with different chemical and functional properties. But, so far, several approaches have failed to support the concept that EGF-TM7 family members induce signaling via G-proteins.

CD97 functions and ligands

EGF domains within CD97 contain Ca^{2+}-binding sites and an RGD (Arg-Gly-Asp) sequences suggest functions associated with cell adhesion and migration. The RDG sequence is located at the COOH-terminal side of EGF5[216]. RGD sequences are attachment sites for a large number of adhesive ECM components and cell surface molecules, such as integrins[217]. *In vitro* assays have indeed shown that CD97 can function as an adhesion molecule, by binding its cellular ligand, CD55[218], or by binding the ECM component chondroitin sulfate B (CS-B), also known as dermatan sulfate[219].

CD55 is also known as decay accelerating factor (DAF) and protects cells from complement-mediated damage by accelerating the decay of complement C3/C5 convertases[220]. Furthermore, CD55 can function as an entry molecule for certain viruses[221,222] and can interact with LPS/sCD14 complexes[223]. CD55 is expressed on a wide variety of cells which are in contact with complement, such as lymphocytes, erythrocytes, epithelial cells and endothelial cells[220]. Lymphocytes and erythrocytes are able to adhere to CD97-transfected COS cells, and this adherence is blocked specifically by Mab CLB-CD97L/1 directed against CD55, and by Mab CLB-CD97/1 directed against the EGF domain 1 of CD97[218]. Binding of CD97 to CD55

is restricted to the interaction between EGF domains 1 and 2 of CD97 and short consensus repeat domains 1, 2 and 3 of CD55[203,224-226]. Accordingly, all CD97 isoforms can bind to CD55, but larger CD97 isoforms (e.g. EGF1-5) have a significant lower affinity for CD55 than smaller isoforms[225]. Although the affinity of CD97-CD55 interactions is low, high avidity cellular interactions might be caused by multiple low affinity interactions, as has also been described for members of the immunoglobulin superfamily[226,227]. Despite the fact that the EGF domains 1, 2 and 5 of EMR2 and CD97 differ only by three amino acids, EMR2 binds to CD55 with an affinity of at least one order of magnitude weaker compared to CD97[199,226].

Recently, CS was identified as the second ligand for CD97, binding to human EGF domain 4 (or mouse EFG domain 3)[219]. CS binding is therefore restricted to the larger CD97 isoforms (EFG1-5 in human and EGF1-4 in mice). Furthermore, CS-B can also bind to EGF domain 4 of human EMR2. CS is a glycosaminoglycan (GAG) abundantly expressed in the ECM and on cell membranes and implicated in many biological processes, such as cell adhesion, proliferation and tissue repair[228,229]. Indeed, it was demonstrated that like CD97EGF1, also CD97EGF4 is involved in cell adhesion. *In vitro* Mab blocking studies against CD97EGF1 and CD97EGF4 revealed that cell binding was reduced to 37% by interference with CD55/CD97 interactions and to 16% by interference with CD97/CS-B interactions[215]. A synergistic effect was demonstrated when both receptor-ligand interactions were prevented, resulting in a complete abolishment of cell binding. GAG, like CS and heparan sulfate (HS), can also function as a collector of growth factors, cytokines, chemokines and protease inhibitors[229]. Thereby, these proteins are locally increased in concentration and can attract and activate other cells[230,231]. Additionally, cellular GAG can function as attachment sites for viruses and bacteria, facilitating entry into host cells. Taken together, alternative splicing regulates the binding of CD97 to its two defined ligands, CD55 and CS-B.

CD97 in inflammation

CD97 is expressed under different inflammatory conditions, e.g. in chronic eczematous dermatitis skin infiltrating T cells expressed high levels of CD97[214]. Previously, an *in vivo* role for CD97 in adhesion/migration was suggested by the co-localization of CD97 and CD55-expressing cells in joints of RA patients. Intimal MΦ expressed CD97 while fibroblast-like synoviocytes in the intimal lining expressed CD55[213]. In addition, sCD97 was present in synovial fluid from RA patients and in pleural fluid containing inflammatory cells or malignant lymphoma cells[213,214]. Furthermore, within the same tumor, scattered tumor cells at the invasion front expressed higher levels of CD97 compared to tumor cells in the solid formation of colorectal and gastric carcinomas[232]. *In vitro* studies indicated that the migration and invasion capacity of colorectal tumor cells correlated with the expression level of CD97[232]. Interestingly, inducing CD97 levels, in a tetracycline-dependent system, could increase migration and invasiveness of fibrosarcoma cells.

Recently, the first evidence for a functional role of CD97 was demonstrated in neutrophil migration/adhesion in two different *in vivo* model systems[233]. First, experimental colitis was induced in mice by administration of dextran sodium sulfate. Mice were subsequently injected with radioactively labeled neutrophils, which were pre-treated with Mab 1B2 anti-mouse CD97(EGF1) or control antibody. Homing of adoptively transferred neutrophils was significantly reduced upon blocking CD97EGF1. In a mouse model for pneumonia, blocking of CD97EGF1 or CD97EGF3 reduced neutrophil infiltration. Furthermore, in a murine infectious model with *Streptococcus pneumoniae*, similar Mab treatment caused impaired neutrophil recruitment to infectious sites and subsequently reduced survival of mice. Collectively, these data led us to the hypothesis that CD97 is involved in the immunopathogenesis of MS and EAE.

CD44

Structural characteristics

CD44 is a type I transmembrane glycoprotein which exists in many different isoforms and ranges in molecular mass from approximately 85 to 250 kDa. The gene is encoded by 20 exons in humans and 21 exons in mice[234-236]. Unlike in mice, exon v1 in humans contains a stop codon and therefore is not expressed. Figure 5 illustrates which exons encode the extracellular, transmembrane and intracellular domains of CD44. The backbone of CD44, present in all CD44 isoforms, is known as CD44 standard (CD44s) or hematopoietic CD44 (CD44H). CD44s consists of 268 amino acids, 248 in the extracellular domain, 23 in the transmembrane domain and 3 or 70 in the cytoplasmic tail. Alternative RNA splicing of ten variant exons (exons v1-v10) generates a diversity of CD44variant (CD44v) isoform molecules. A combination of different variant protein domains, which are present in the extracellular domain, can elongate the CD44s backbone by a total of 381 amino acids. Further diversification is generated by post-translational modifications, such as *O*- and *N*-glycosylation, sulfation and attachment of GAG side chains like CS-A, CS-B, HS and keratan sulfate. The extracellular domain of CD44 can be subdivided into three parts, with different interspecies sequence homology: the amino-terminal domain (80-90% homology), the variable domain (64%), and the membrane-proximal domain (34-45%)[236,237]. The transmembrane and cytoplasmic domains are well conserved, with a sequence homology of 80-90% between different species.

Expression

Recently Cichy and Puré suggested that CD44 proteins may exist in three phases; as a cell surface transmembrane receptor, and as an extracellular molecule in body fluids (soluble CD44) and ECM[238]. CD44s is widely expressed by all mature leukocytes and by many non-hematopoietic cells, e.g. keratinocytes, epithelial cells, endothelial cells and fibroblasts. Resting cells express CD44s at low levels; this expression can be increased by cell activation induced with e.g. T cell receptor-

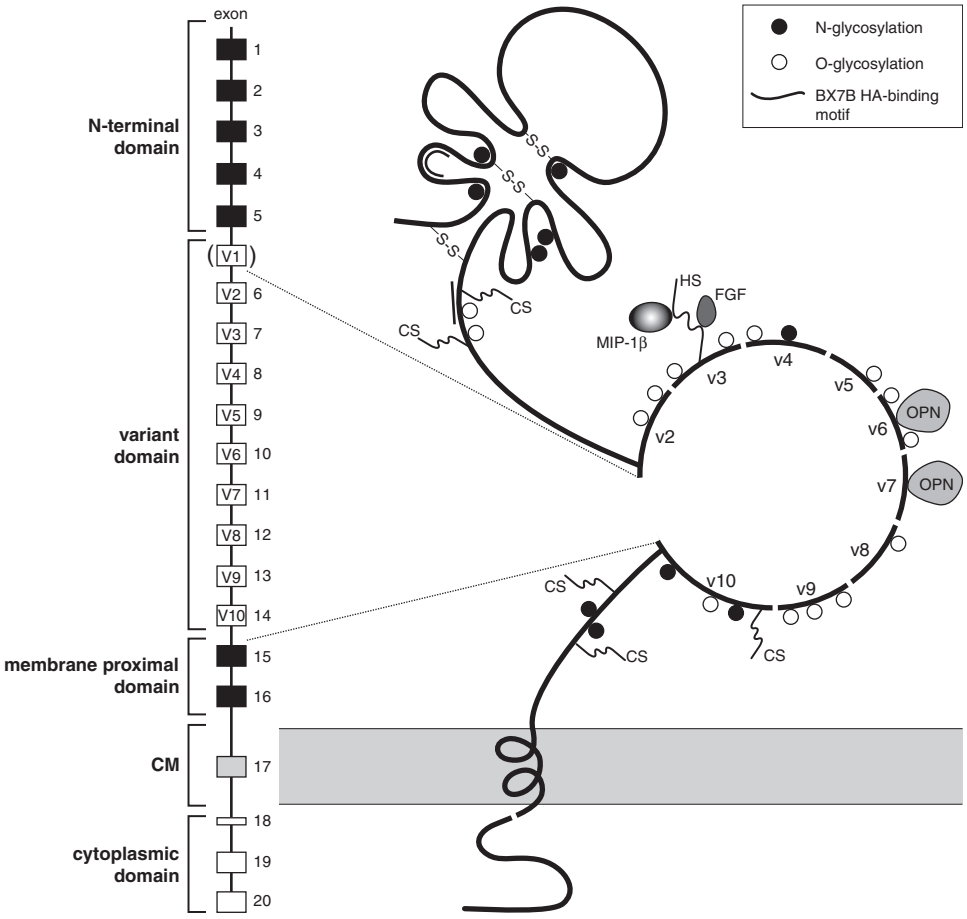

Figure 5. Schematic genetic and molecular structure of CD44.
The extracellular domain of the CD44 backbone (or CD44 standard) is encoded by exons 1-5 and 15-16 (■). A large number of different CD44variant isoforms can be generated by alternative splicing of ten CD44variant exons (v1-v10). CD44v proteins are located in the extracellular domain of the CD44 standard molecule (v2-v10). CD44v1 is not transcribed in humans, because it contains a stop codon. Posttranslational modifications, by *N*-glycosylation (●), *O*-glycosylation (○) and addition of glycosaminoglycan side chains (CS: chondroitin sulfate; HS: heparin sulfate) enlarges variation in CD44 molecules. CD44v3 contains HS side chains, which are docking sites for fibroblast growth factors (FGF) and chemokines (MIP-1β). CD44v6 and CD44v7 contain a binding site for osteopontin (OPN). Like v1-v10, exons 19 or 20 exons are alternatively spliced. Usually exon 20 is expressed, coding for a 70 amino acid cytoplasmic domain. When exon 19 is expressed, the cytoplasmic domain contains 3 amino acids. CM: cell membrane. Modified from T.E.I.Taher, PhD thesis: CD44 & Met: the signaling perspective, 2002.

or cytokine stimulation. In contrast to CD44s, resting leukocytes do not express CD44v isoforms. CD44v isoform expression is mainly restricted to epithelial cells, endothelial cells, activated leukocytes, memory cells and malignant cells[239]. The variation in CD44v isoform composition is likely limited to a hundred different isoforms. The expression levels of these isoforms depends on the cell type and its activation stage. Previously identified isoforms include CD44v4-7 (also known as CD44M or p-Meta1)[240,241], CD44v6-7 (p-Meta2), CD44v8-10 (CD44R1 or CD44E on epithelial cells)[241-243], CD44v10 (CD44R2)[242], CD44v3-10, CD44v3,8-10, CD44v6-10 and CD44v7-10[244,245].

Soluble CD44 molecules, including soluble CD44v isoforms, are likely predominantly generated by proteolytic cleavage of the extracellular domain of surface-bound CD44[246]. Membrane-type 1 and 3 metalloproteinases (MT1-MMP or MMP-14 and MT3-MMP or MMP-16, respectively) are able to cleave or shed CD44[247]. In addition to cleavage, alternative RNA splicing can account for direct synthesis of soluble CD44 molecules[235]. Body fluid levels of soluble CD44 are elevated during immune activation, inflammation, and tumor growth and metastasis[248]. Under physiological conditions, human and mouse serum contains low levels of soluble CD44 (5 and 2 μg/ml, respectively). Levels are increased (3-10 μg/ml) in tumor bearing mice and in autoimmune prone mice, but decreased in immunodeficient mice (0.5 μg/ml). In addition to serum, also lymph fluid, arthritic synovial fluid and bronchoalveolar lavage fluid can contain soluble CD44[248-250]. Cleavage of CD44 has been shown to induce cell migration through hyaluronic acid (HA) *in vitro*, after detachment of the extracellular CD44 domain by MT1-MMP[247]. Interestingly, both CD44 and MT1-MMP were expressed at the leading edge of cells. Thus, CD44 cleavage may allow cells to detach from the ECM and to move to other locations.

CD44 functions and ligands

CD44 has been shown to play a role in a wide diversity of biologicalprocesses, including phagocytosis[251,252], signal transduction[253], cell activation[254], costimulation[255], adhesion and extravasation[256,257], apoptosis[258-261], embryogenesis[262], haematopoiesis[263,264], tissue remodelling[265] and tumor development[266]. The existence of many different isoforms may explain this wide functional variety. The transmembrane domain of CD44 has been suggested to mediate costimulatory responses[255]. CD40 ligation rapidly induces CD44s and CD44v7 expression[255,259]. CD44 can bind diverse ECM components, such as HA[267], laminin[268], collagen I, IV and fibronectin[269,270], which is important for adhesion and transmigration of cells through the ECM[271,272]. Furthermore, GAG (heparan sulphate and CS), gelatinases[273,274] and cytokines (e.g. OPN)[275,276] attach to specific CD44 molecules. In this way, CD44 serves as a docking site, which locally increases the concentration of these factors. CD44 can also function as a co-receptor for several molecules, such as VLA-4[257] and tyrosine kinase receptors (ErbB4 and c-Met)[272,277].

CD44 molecules are associated in lipid rafts and can induce signal transduction and cytoskeletal reorganization[253,278,279]. The role for CD44 as a co-receptor and its function in signal transduction and cytoskeletal reorganization is not described in this introduction, but has recently been reviewed by Ponta, Sherman and Herrlich[272].

(Hyaluronic acid). HA is the most extensively studied ligand for CD44. Hyaluronate is an essential component of the ECM in all tissues, synthesized by fibroblasts, chondrocytes and mesothelial cells. It is composed of disaccharides of glycuronic acid and *N*-acetyl-D-glycosamine and regulates hydration and organization of the ECM. Normal ECM contains HA in a high molecular weight form. In contrast, at sites of inflammation and tissue injury, HA accumulates and is degraded by hyaluronidase, receptor-mediated endocytosis or peroxynitrate, into low molecular weight products with diverse inflammatory capacities[280,281]. Furthermore, HA expression is induced on microvascular endothelial cells after stimulation with proinflammatory cytokines (TNF-α, IL-1) or LPS[281]. HA-CD44 ligation can upregulate endothelial expression of VCAM-1 and ICAM-1[237], molecules which are crucially involved in firm adhesion and transmigration. VLA-4, the functional ligand for VCAM-1, associates with CD44 and mediates extravasation of T cells into inflammatory sites[257]. The specific binding motif of CD44 for HA is confined to the distal N-terminal extracellular region of the CD44s backbone, the Bx7B motif (Figure 5), suggesting that all CD44 molecules can bind HA[282-284]. However, in order to bind HA, CD44 molecules need to be activated, e.g. by T cell receptor stimulation, LPS, cytokines (IL-2, TNF-α) or chemokines (MIP-1β, IL-8)[285-287]. Interestingly, soluble CD44, anti-inflammatory cytokines (IL-14 and IL-10) and hyaluronidase all inhibit CD44-mediated HA binding[288-291]. This binding is dependent on phosphorylation of the cytoplasmic domain. Deletion of this domain abrogates HA binding[292]. The complexity of the CD44-HA interaction is further underscored by the fact that variations in cell type, CD44v isoform numbers, glycosylation, sulfation and GAG attachment also influence the binding affinity for HA[285,293-296].

(Chondroitin- and heparin sulfate). Like CD97, also CD44 molecules can bind the GAG CS, with similar functional implications as described above (CD97 ligands). CS-decorated CD44 molecules can also bind other ECM components, such as collagen and fibronectin. *In vitro* studies have implicated that CS-CD44 interactions are involved in leukocyte rolling under physiological flow conditions[297]. CS is expressed by endothelial cells and may serve as an attachment site for circulating CD44-expressing cells. CD44 can contain HS chains. Modification of CD44 by CS or HS attachment provides additional binding sites for chemokines (e.g. MIP-1β)[298], MMP-9[273] and growth factors (e.g. heparin binding growth factor, basic fibroblast growth factor)[299]. HS GAG attachment is restricted to the variant 3 isoforms of CD44 [298,299].

(Gelatinases). All isoforms of CD44 are associated with MMP-2 (gelatinase A) and MMP-9 (gelatinase B). Upon CD44 activation, pro-MMP-2 is secreted and converted into the proteolytically active form, through cleavage by MT1-MMP[247]. It was recently suggested that MMP-2 plays a role in downmodulating inflammation, by inactivating inflammatory mediators, such as IL-1β[300], MCP-3[301] and fibroblast growth factor receptors[302]. Moreover, genetic deletion of MMP-2 exacerbated antibody-induced arthritis in mice[303]. In contrast, MMP-9-deficiency reduced inflammation and arthritis severity. CD44 contains a docking site for proteolytically active MMP-9, which can degrade collagen type IV. MMP-9 and CD44 are concentrated at the leading edge of migrating cells, the invadopodia[273,304]. Thus, MMP-9 degradation of collagen IV can facilitate cell movement through the ECM. Furthermore, MMP-9 can cleave and activate latent TGF-β, which promotes tumor invasion and angiogenesis[274].

(Osteopontin). By using distinct domains, OPN can bind αvβ1 integrins and CD44v6 and CD44v7 containing isoforms[276]. Binding to CD44v isoforms is likely dependent on cooperative binding to β1-integrins[276]. OPN occurs as an immobilized molecule in mineralized ECM compartments and as a soluble molecule in body fluids. Several cell types can produce OPN, e.g. activated T cells, MΦ and NK cells. OPN exhibits both proinflammatory and anti-inflammatory effects, as reviewed by Denhardt *et al.*[265]. As a proinflammatory mediator OPN induces chemotaxis of T cells and MΦ, induces Th1 polarization, but inhibits Th2 cytokine production. Mice deficient for OPN exhibit impaired Th1 responses upon infection with Herpes simplex and *Listeria monocytogenes*[305]. As an anti-inflammatory mediator, OPN inhibits the production of NO. OPN is also involved in bone remodelling and myelin generation and degeneration[265,306]. Furthermore, it has been demonstrated that OPN-CD44 interactions promote cell survival of mouse bone marrow cells[307].

CD44 in inflammation

CD44 and its variant isoforms have been implicated to play a crucial role in the immunopathogenesis of different inflammatory and autoimmune diseases[258,281,308]. An elevated number of peripheral blood leukocytes expressed CD44v3, v6 and v7 in patients with chronic IBD, RA and systemic lupus erythematosus compared to healthy donors[309-311]. Also at the site of chronic inflammation, CD44v isoforms are expressed by synovial fibroblasts (CD44v3, v6-v10) from arthritis patients and by skin-infiltrating leukocytes (CD44v3 and v10) from patients with the skin-associated autoimmune diseases vasculitis and bullous pemphigoid[312-314].

The functional role of CD44 in chronic inflammatory disease models has been demonstrated by administration of anti-panCD44 Mab. Antagonistic anti-panCD44 Mab binds all CD44 isoforms and can block CD44-HA interactions. Anti-panCD44 treatment reduced disease severity in experimental models for arthritis[315-317],

diabetes[318], cutaneous disease[319] and IL-2 induced vascular leakage syndrome[320]. However, treatment with the same antibody did not affect the development of experimental thyroiditis and Th1-mediated colitis[321,322]. Several studies have indicated that CD44v isoforms exert distinct functional activities. Mab anti-CD44v7 treatment significantly reduced development of TNBS-induced experimental colitis, while Mab anti-CD44v6 did not influence the disease burden[322]. Furthermore, single administration of Mab anti-CD44v3, anti-CD44v6, anti-CD44v7 or anti-CD44v10 reduced Th1-mediated DTH reactions, whereas Mab anti-CD44v6, but not anti-CD44v7 diminished Th2-mediated allergic contact dermatitis[323-325]. Mab blocking of CD44v10 expression in mouse DTH and alopecia areata reduced the cellular infiltration at the site of inflammation[325,326].

Although Mab studies have provided much knowledge about the function of CD44 molecules, results from these studies should be interpreted with some caution. First, it should be noted that Mab treatment efficacy is dependent on the quantity of antibody and time point(s) of administration. Second, upon binding to CD44, Mab anti-CD44 might also induce effects distinct from blocking CD44-HA interactions. Mice deficient for the whole CD44 gene, or specific CD44v exons provide an excellent model to study the role of CD44 in inflammation. For example, CD44-expressing or deficient hematopoietic cells can be separated from non-hematopoietic cells, by generating bone marrow chimeras. In addition, adoptive transfer systems can be used to determine the effect of genetic deletion of CD44 on the effector lymphocyte function, e.g. in EAE development. Despite the fact that CD44 is widely expressed and is involved in many biological functions, genetic deletion of CD44 in mice did not result in major developmental defects[327,328]. This may be due to the fact that other molecules may compensate for the functional loss of CD44. These molecules may include other HA-binding receptors, such as RHAMN, LYVE or TSG-6[281].

Under inflammatory conditions, clear alterations in immune responses were observed in CD44 knock-out ($CD44^{-/-}$) mice compared to wild type mice. Infection of $CD44^{-/-}$ mice with *Cryptosporidium parvum* caused exaggerated granuloma formation compared to wild type controls[328]. In a model for lung injury and inflammation, intratracheal injection of bleomycin caused accumulation of low molecular weight HA fragments and excess numbers of apoptotic neutrophils, resulting in persistent inflammation and even death[329]. These results were in contrast to those found in autoimmune disease models for RA. $CD44^{-/-}$ mice showed a strong reduction in infiltration and disease severity with regard to experimental arthritis[272]. Also in a chronic enterocolitis model (in $IL\text{-}10^{-/-}$ mice), combined deletion of the CD44v6+v7 exons abrogated disease development[259]. Moreover, the single deletion of exon v7 also substantially reduced TNBS-induced colitis. Reduced disease development was associated with decreased levels of IL-12 and increased numbers of apoptotic cells at the inflammatory site in the gut.

Previous studies indicate that CD44s is involved in the immunopathogenesis

of MS and EAE. CD44s was significantly higher expressed by peripheral blood lymphocytes from MS patients compared to control subjects[330]. Additionally, CD44s was abundantly expressed in inflammatory brain lesions of MS patients[331] and EAE-affected mice[332,333]. *In vivo* administration of Mab anti-panCD44 resulted in a reduced EAE development by impairing the recruitment of autoantigen-specific T cells into the CNS[334,335]. Not much is known about the role of CD44v isoforms in MS and EAE development. Previously, we have shown that inflammatory cells in CNS lesions of EAE mice expressed the CD44v10 isoform, but not CD44v6. EAE, induced by $PLP_{139-151}$, could be reduced by early treatment of SJL/J mice with a mixture of antibodies against CD44v6, v7 and v10 isoforms[336]. The mechanism underlying this blockade is unknown. We aimed to dissect the role of CD44v isoforms in order to improve our understanding of the pathogenesis of MS and to open new possibilities for immunotherapy. In line with the hypothesis that specific CD44v are involved in the pathogenesis of MS and EAE, we here determined whether CD44v isoforms are expressed in *post-mortem* MS brain lesions (chapter 5). Furthermore, we specifically assessed the functional role of CD44v7 and CD44v10 in EAE development, by using mice with single deletions for these specific CD44v isoforms.

Aims of the thesis

The general aim of this thesis is to determine whether PG, CD97 and selected CD44v isoforms are functionally involved in MS and EAE development, and to elucidate the underlying cellular mechanisms. In the studies described in this thesis, we have used a combined approach of patient material and animal models, i.e. we analyzed *post-mortem* MS and/or non-human primate EAE brain tissue in order to determine PG presence and expression of CD97/CD44variant isoforms. Futhermore, we have assessed the functional contribution of PG, CD97 and CD44v7/v10 by using different mouse EAE models, including genetic knock-outs.

Several studies have previously provided evidence that bacterial PG contributes to the immunopathogenesis of chronic inflammatory diseases. This concept was underscored by high numbers of immunocompetent PG-containing APC, present at sites of chronic inflammation in patients with MS[105], RA[104,195], atherosclerosis[197] and CD[196]. PG might stimulate processes that contribute to inflammation in autoimmune diseases, in the periphery and/or at inflammatory sites, enhance APC function by inducing expression of MHC-II and costimulatory molecules, and by production of proinflammatory cytokines. The most important PG-degrading enzymes in mammals are lysozyme and the amidase NAMLAA. In joint action, lysozyme and NAMLAA clear PG intracellularly and abrogate its proinflammatory effects.

Although PG is present in MS *post-mortem* brain, little is known about the potential functional role of PG, NAMLAA and lysozyme in MS and EAE development. Therefore, we first assessed whether elevated numbers of PG-containing cells are also present in EAE-affected brain tissue compared to control brain tissue in two different non-human primate species. If so, this would provide new animal models to address PG functionality **(chapter 2)**. Next, we investigated whether the number, location and cellular subtype of PG, NAMLAA, and lysozyme-containing cells in EAE-affected brain and spleen tissue differ between monkeys with an acute disease course (rhesus monkey) and monkeys with a chronic disease course (marmoset). Additionally, we examined whether NAMLAA and lysozyme are expressed in MS and control brain tissue to address whether persistence of PG in tissue is related to the differential expression of the major degrading enzymes. To obtain functional insight into the role of PG in marmoset and rhesus monkey EAE, we assessed whether leukocytes from marmoset and rhesus monkeys are responsive to PG stimulation *in vitro*. **In chapter 3,** we investigated the hypothesis that PG stimulates autoimmune inflammatory responses in MS using EAE. In C57BL/6 mice, we have substituted *M. tuberculosis* by *S. aureus* PG as a single immunostimulatory component in adjuvant. In order to determine whether the structure of PG is important for its proinflammatory function, we have used both soluble and insoluble PG. We examined which cell type(s) may transport PG into secondary lymphoid organs during EAE development. To determine by which mechanisms PG may exert its effect in the development of EAE, we have

explored whether PG contributes to antigen uptake, expression of cytokines and costimulatory molecules and $CD4^+$ T cell polarization *in vitro*.

Upon activation of leukocytes in secondary lymphoid organs as well as inflammatory sites, subsequent events depend on the expression of cell surface molecules for e.g. costimulation, migration, adhesion/transmigration and apoptosis. Both cell-cell and cell-matrix interactions are crucial in the development of local inflammation, as occurring in the CNS of MS patients. CD97 and CD44v isoforms are involved in cell-cell and cell-matrix interactions, and contribute to inflammatory processes. Little is known about CD97 and CD44v isoforms in relation to MS and EAE development, despite abundant *in vitro* and *in vivo* evidence that these molecules codetermine the development of experimental arthritis and colitis. Therefore, we assessed whether CD97 and selected CD44v isoforms are involved in the immunopathogenesis of MS and EAE.

In chapter 4 we determined whether CD97 and its cellular ligand, CD55, are expressed in MS brain lesions and whether this expression is related to the lesion stage. As sCD97 is secreted under inflammatory conditions, we assessed whether sCD97 is present in serum and cerebrospinal fluid of MS patients. We compared sCD97 serum levels in MS with other inflammatory and non-inflammatory neurological diseases and healthy controls. To further test the hypothesis that CD97-CD55 interactions contribute to the immunopathogenesis of MS, we also studied whether early treatment with CD97 Mab against EGF domain 1 affects EAE in SJL/J mice.

Whether CD44v isoforms are involved in the immunopathogenesis of MS and mouse EAE is studied **in chapter 5**. Since CD44v isoforms are upregulated under chronic inflammatory conditions, we explored which of the CD44v isoforms v3 to v10 are expressed in MS brain lesions, and whether this expression is related to the lesion stage. Moreover, we determined the kinetics of CD44v10 expression on different cell subsets in murine CNS tissue during EAE development. Previous mouse studies have indicated that CD44v7 and CD44v10 contribute to the development of Th1-mediated colitis and DTH responses. By using mice with a single genetic deletion for CD44v7 or CD44v10, we analyzed whether these isoforms contribute to the development of EAE, induced by active immunization or adoptive transfer. The EAE course of CD44v7- or CD44v10-deleted mice was reduced compared to wild type mice. To explore which mechanisms account for the reduced EAE development in CD44v7- and CD44v10-deleted mice, we analyzed the CNS tissue of these mice for infiltrates, and assessed the autoantigen-specific T cell proliferation and cytokine production.

In chapter 6 we integrate the data obtained in the previous chapters and discuss them in the context of our hypothesis that PG, CD97 and CD44v isovorms contribute to the immunopathogenesis of MS. Furthermore, based upon the data presented, some lines of research to futher delineate the role of PG, CD97 and CD44v isoforms in MS are suggested.

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CHAPTER

2

Phagocytes carry a disease promoting TLR/Nod ligand into the brain during demyelinating disease in primates

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Abstract

The clinical expression of an autoimmune disease depends on the activation of resident antigen presenting cells (APC) in the target organ(s), being in this study brain and spinal cord. Most evidence comes from rodent models, and it is unclear whether this principle holds in primates (including humans) and which potential APC stimuli reach the brain. We previously demonstrated that APC containing peptidoglycan (PG) are present in *post-mortem* multiple sclerosis (MS) brain. PG is a major cell wall component of Gram-positive bacteria exerting proinflammatory signals via TLR2 and Nod1/2 ligation. In the brain, PG may thus activate both infiltrating and resident APC.

To assess how PG is brought into the brain and to investigate its functional relevance in demyelination, we systematically compared MS to non-human primates, which are genetically and immunologically closely related to humans and are therefore valuable models to analyze the regulation of chronic inflammation in MS. Localization of APC subsets containing PG, as well as their expression of enzymes degrading PG (lysozyme and NAMLAA) were determined in the two main primate EAE models for MS, with acute (rhesus monkey) versus chronic disease (marmoset).

Distinct phagocytic subsets in MS and EAE brain carried PG, including granulocytes, macrophages and dendritic cells. The number of PG-containing cells in brain tissue during both acute and chronic disease was significantly higher compared to control animals, and acute EAE was accompanied by higher numbers of PG-containing cells in brain compared with chronic EAE. PG-degrading enzymes were scarcely expressed in monkey brain, spleen and in MS brain, favouring persistence of intracellular PG in the periphery as well as the CNS. Peripheral blood leukocytes from all three primate species produced IL-12p70 upon stimulation with PG purified from *Staphylococcus aureus*.

The presence of a TLR/Nod ligand in the inflamed brain has major implications, since TLR are widely expressed in the CNS and their ligation potentially promotes inflammation and progression of demyelinating disease.

Introduction

The normal repertoire of humans and non-human primates contains significant numbers of potentially encephalitogenic T cells[1-3]. Under homeostatic conditions these cells are kept dormant by control mechanisms, involving regulatory Tr1 cells. Whether autoreactive Th1 cells or regulatory Tr1 cells are induced critically depends on the maturation state of the antigen presenting dendritic cells (DC) in peripheral lymphoid organs[4-6]. The development of autoimmune encephalomyelitis critically depends on additional activation signals to CNS infiltrating autoreactive

T cells from resident antigen presenting cells (APC). Such additional signals are provided by CpG-TLR9 interaction[10]. Different from mice, neither the myeloid DC in the peripheral compartment nor the resident APC (microglia) of humans[7] or rhesus monkeys (Bajramovic, unpublished) express TLR9. Hence, data obtained in mice on the disruption of tolerance by stimulation with CpG oligonucleotides[8-10] cannot be easily extrapolated to primates (including humans). Hence we have investigated alternative TLR ligands in the primate CNS.

Bacterial peptidoglycan (PG) is a major cell wall component of Gram-positive bacteria and we have shown that PG has strong proinflammatory capacities in animal models of multiple sclerosis (MS)[11]. PG stimulates the innate immune system by binding to the Toll-like receptor (TLR)2/6, although this concept has recently been challenged[12]. Recently identified intracellular PG receptors are the Nod1 (Card4) and Nod2 (Card15) signaling receptors[13-15]. PG can induce signal transduction via TLR2 and Nod1/2 receptors within cells and via TLR2/6 in association with CD14 on the cell surface. Activation of these receptors results in the induction of cell maturation and the production of several chemokines and proinflammatory cytokines such as TNF-α, IL-1 and IL-6[16-18].

PG is a complex structure formed by three-dimensional cross-linked layers of glycan backbones with peptide bridges[19]. The glycan strands of PG in all bacterial species are composed of two alternating sugar residues, *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc)(Figure 1). Stem peptides cross-link these glycan chains and differ in composition between bacteria strains. Stem peptides can be attached directly to each other or indirectly via interpeptide bridges (Figure 1). Between different bacteria, interpeptide bridges can vary in length and amino acid composition[20,21]. PG is digested by enzymes with different substrate specificities of which lysozyme and *N*-acetylmuramyl L-alanine amidase (NAMLAA) are the best characterized. Lysozyme is present in granules of phagocytes[22-24] and partially degrades PG by hydrolyzing the bond between GlcNAc and MurNAc resulting in solubilized PG (Figure 1). We have previously identified and functionally characterized NAMLAA[25-28], which was recently shown to be identical to the PG recognition protein long form (PGRP-L)[29,30](Zhang *et al.*, *submitted*). NAMLAA is expressed by neutrophilic granulocytes, but not by monocytes and resting macrophages (MΦ) under normal conditions. PG is cleaved by NAMLAA/PGRP-L between the MurNAc residue and the first L-alanine of the stem peptide (Figure 1).

Due to its complex structure, PG is highly resistant to degradation under physiological conditions. Recently, it was shown that PG-induced responses were comparable in MΦ from NAMLAA/PGRP-L-deficient and wild type mice[31]. Most likely, several PG-degrading enzymes are necessary for complete PG-degradation. *In vitro* studies clearly show that the combined degradation of PG by lysozyme and NAMLAA reduces the proinflammatory potential of PG[27]. Because MΦ and DC contain lysozyme but lack NAMLAA, incomplete PG degradation may result in the persistence of intracellular bio-active PG. We have previously demonstrated that

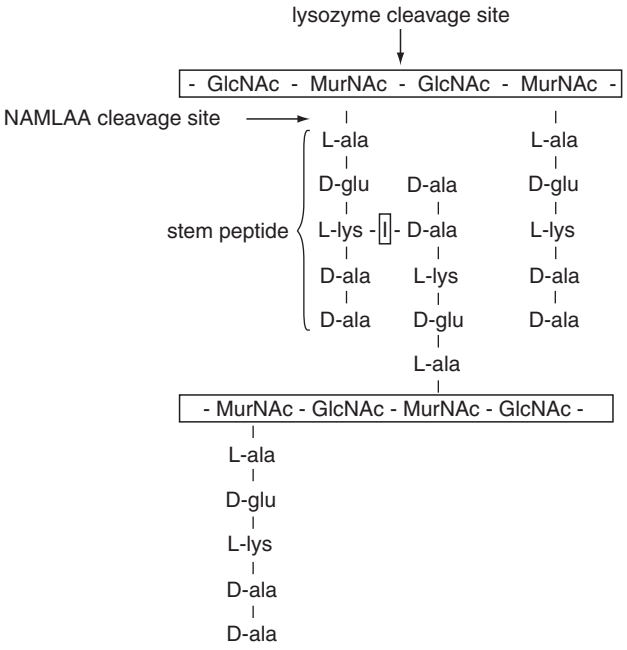

Figure 1. Representative structure of bacterial PG and location of NAMLAA/PGRP-L and lysozyme cleavage sites.
PG is composed of a long glycan backbone which contains two alternating sugar residues, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). Cross-linkage of the glycan backbones is achieved via stem peptide- and interpeptide-bridges, which differ in composition between bacteria strains. The stem peptides in *S. aureus* are composed of L-alanine-D-isoglutamine-L-lysine-D-alanine-D-alanine[87]. The interpeptide bridges in *S. aureus* PG are formed by pentaglycines. NAMLAA hydrolyzes PG by cleaving the bond between the lactic acid group of MurNAc and the first amino acid of the peptide bridge (always L-alanine). In contrast, lysozyme hydrolyzes the bond between MurNAc and GlcNAc, the alternating residues forming the glycan backbone of all PG types. [I] interpeptide bridge.

PG is indeed present in MΦ and DC by immunohistochemical staining of human spleen sections with a monoclonal antibody (Mab) raised against PG isolated from the human faecal flora[32]. PG can also be detected in peripheral blood leukocytes, urine, liver and lymph nodes under normal conditions[32-39]. Furthermore, we have shown that PG derived from sterile human spleen is biologically active, since spleen-derived PG induced proinflammatory cytokine production by peripheral blood leukocytes[39].

We have proposed that PG may act as a costimulating factor for the development of autoimmune disease in the absence of infection or bacterial replication. APC redistribute PG from the mucosa[40-42] to secondary lymphoid organs and to sites of chronic inflammation and may stimulate autoimmune processes locally. We have

previously described PG-containing APC in sites of chronic inflammation in MS brain tissue[43], rheumatoid arthritis synovial tissue[32,44] and bowel wall tissue of patients with Crohn's disease[45]. These cells were fully immunocompetent as determined by the expression of costimulatory molecules, HLA-II and proinflammatory cytokines[43,44]. In rodent animal models, PG has been shown to facilitate development of autoimmune disease. Injection of PG derived from different bacterial strains can induce chronic arthritis and colitis in susceptible rodents[46,47]. Moreover, we have demonstrated that PG induces DC maturation and contributes to the development of mouse experimental autoimmune encephalomyelitis (EAE), an animal model for MS[11].

Mouse EAE models serve as useful tools to assess the functional relevance for PG in MS development. However, non-human primates may better represent the human situation, since differences in TLR2 gene expression and regulation occur between mice and humans[48]. It is likely that this species variation will affect the functional outcome of TLR2-induced responses. In this study, we used EAE in rhesus and marmoset monkeys to further investigate the relevance of PG in autoimmune encephalomyelitis. These non-human primates are outbred and are genetically and immunologically closely related to humans[49]. Upon immunization with myelin components non-human primates develop EAE with a similar pathology and disease course as MS patients. Two well described monkey EAE models are the rhesus monkey (*Macaca mulatta*) and the common marmoset (*Callithrix jacchus*) monkey[50,51]. EAE-affected rhesus monkeys develop large lesions in the brain, which contain abundant numbers of neutrophils[52]. This severe inflammatory necrosis is associated with an acute disease course. In contrast, marmosets develop a relapsing-remitting or primary-progressive disease course with perivascular lesions and demyelinated areas containing mainly MΦ, T and B cells in the central nervous system (CNS). The fundamentally distinct clinical and pathological presentation of EAE might represent different forms of human encephalomyelitis. Rhesus monkey EAE more closely resembles acute disseminated encephalomyelitis (ADEM), while marmoset EAE resembles chronic MS ('t Hart *et al.*, 2005, *submitted*).

To address whether the TLR/Nod ligand PG is carried into primate brain, in this study we assessed whether the presence, location and numbers of PG-containing cells in brain and spleen tissue from rhesus monkeys with acute EAE differs from marmoset monkeys with a chronic disease course. In both models, we determined whether the number of PG-containing cells in the brain was related to the development of EAE. Additionally, we identified the cell types containing PG in both human (primate) and non-human primate brain. Since NAMLAA and lysozyme are enzymes involved in PG-clearance, we assessed whether these PG-degrading enzymes were differentially expressed in MS and non-demented control brain tissues and in EAE-affected versus non-EAE-affected monkey brain and spleen tissue. To obtain functional insight into the role of PG in rhesus monkey and marmoset EAE, we assessed *in vitro* whether leukocytes from rhesus monkey and marmoset monkeys are responsive to stimulation with PG from *S. aureus*, known to precipitate EAE in mice.

Material and Methods

Non-human primate tissues

Spleen and brain tissue were obtained from marmosets and rhesus monkeys raised at the Biomedical Primate Research Centre (Rijswijk, The Netherlands), as described previously[52,54]. Brain tissues used for this study had been neuropathologically well characterized. All brain and spleen tissues were obtained from previous studies, hence no animals were sacrificed solely for the purpose of the current study. All experimental procedures with live animals had been approved by the Institutional Animal Care and Use Comittee. EAE was induced in marmoset monkeys by immunization with 20 mg whole human myelin or 0.1 mg recombinant human MOG emulsified in complete Freund's adjuvant (CFA). As adjuvant controls, two randomly selected monkeys were immunized with 1 mg ovalbumine (Sigma Chemical Co, St. Louis, MO) in CFA, a protocol used for induction of arthritis. EAE was induced in rhesus monkeys by immunization with 0.32 mg recombinant human myelin oligodendrocyte glycoprotein ($rhMOG_{1-125}$), or 0.1 mg MOG_{34-56} in CFA[52]. As adjuvant controls, rhesus monkeys (n=2 monkeys) were immunized with 3 to 5 mg bovine type II collagen in CFA[55]. Animals immunized with myelin antigen were examined daily for clinical symptoms of EAE. According to these examinations, all rhesus monkeys immunized with $rhMOG_{1-125}$ (n=7) developed a hyperacute disease course, with a 24-48 h time period from the onset to full-blown disease[52]. Rhesus monkeys which were immunized with MOG_{34-56} in CFA developed a heterogeneous disease course[50]. The four monkeys which were included in this study were asymptomatic at day 28 after immunization. These monkeys were challenged with MOG_{34-56} in incomplete Freund's adjuvant (IFA)(Table 1, animal 8 and 9) or with an irrelevant peptide MOG_{4-26} in IFA (Table 1, animal 6 and 7). After one or two homologous challenges both monkeys developed acute clinical signs of EAE within 7 days after challenge and were sacrificed. The two rhesus monkeys which were challenged with irrelevant peptide remained asymptomatic.

From the thirty-seven marmoset monkeys which were immunized with myelin antigens twenty-eight developed EAE. In the other nine marmoset monkeys EAE development had been successfully blocked by experimental treatment with new immunotherapeutic reagents. Details regarding immunogens, day of sacrifice and development of EAE for both marmoset and rhesus monkeys are shown in Table 1 and 2, respectively.

Brain tissue samples of MS patients and non-demented control subjects

Human autopsy brain tissue was provided by the Netherlands Brain Bank (Coordinator Dr. R. Ravid, Amsterdam). Information regarding MS patients and non-demented control subjects is shown in Table 3.

Table 1. Rhesus monkey-derived brain and spleen tissues.

animal	immunogen	clinical EAE	day of sacrifice	EAE score
1	collagen II	-	100	0
2	collagen II	-	100	0
3*	collagen II	-	71	0
4*#	collagen II	-	41	0
5*#	collagen II	-	31	0
6	$phMOG_{4-26}$,1x boost $phMOG_{34-56}$	-	138	0
7	$phMOG_{4-26}$,1x boost $phMOG_{34-56}$	-	138	0
8	$phMOG_{34-56}$, 2x boost $phMOG_{34-56}$	+	112	5
9§	$phMOG_{34-56}$, 1x boost $phMOG_{34-56}$	+	35	5
10*#	$rhMOG_{1-125}$	+	24	4
11*	$rhMOG_{1-125}$	+	17	4
12§	$rhMOG_{1-125}$	+	18	4
13	$rhMOG_{1-125}$	+	39	3
14	$rhMOG_{1-125}$	+	32	4
15	$rhMOG_{1-125}$	+	35	2.5
16*#	$rhMOG_{1-125}$	+	20	4

($rhMOG_{1-125}$) recombinant peptide, representing the complete extracellular N-terminal domain of human MOG, ($phMOG_{34-56}$) synthetic peptide, representing an extracellular N-terminal domain of human MOG. (*) spleen tissues which were used for staining for PG and NAMLAA, (#) spleen tissues which were used for double staining, (§) brain tissues which were used for double staining.

Staging of MS brain lesions

As described previously[56], brain lesions were characterized according to an internationally accepted staging system based on inflammation and demyelination criteria[57,58], using three different markers, acid phosphatase (for MΦ, mircroglia), HLA-DR/DQ/DP (for APC) and neutral lipids (for ingested myelin). Pre-active lesions are caracterized by HLA-II-expressing clusters of activated microglia cells and few perivascular inflammatory cells. Pre-active lesion do not contain phagocytic cells containing myelin breakdown products or areas of demyelination. Active lesions are characterized by demyelinated areas. Phagocytic cells in these areas contain myelin breakdown products, which can be visualized by oil red O, a histochemical staining for neutral lipids. In these lesions strong expression of HLA-II is present on perivascular and parenchymal MΦ.

Immunohistochemistry

The immunohistochemical techniques used in this study have previously been described in detail[59,60] with slight modifications. Sections from marmoset and rhesus monkey tissues were fixed for 10 min at room temperature in 4% paraformaldehyde in PBS with 0.02% (v/v) H_2O_2 in PBS to eliminate endogenous peroxidase activity. Human brain tissue sections were fixed at room temperature in fresh acetone containing 0.02% (v/v) H_2O_2. Remaining endogenous peroxidase activity was

Table 2. Marmoset-derived brain and spleen tissues.

animal	immunogen	clinical EAE	day of sacrifice	mean EAE score (range)
1	OVA	-	49	0
2*#	OVA	-	49	0
3*	none	-	n.a.	0
4*#	none	-	n.a.	0
5-8	MOG	-	53-155	0
9-20	MOG	+	44-112	2.3 ± 1 (0-3)
9#§	MOG	+	49	1.8
10#§	MOG	+	49	1.9
12*	MOG	+	67	2.5
19*	MOG	+	67	2.5
20*	MOG	+	44	0
21-25	myelin	-	86	0
26-41	myelin	+	41-93	2.4 ± 1.2 (0-3.5)
36#§	myelin	+	77	0

(*) spleen tissues which were used for staining for PG and NAMLAA, (#) spleen tissues which were used for double staining, (§) brain tissues which were used for double staining, (n.a.) not applicable.

revealed by staining with 4-chloro-1-naphtol-phosphate, which results in a dark blue precipitate (Sigma). Slides were incubated with predetermined optimal dilutions of primary antibodies, overnight at 4°C in humidified atmosphere.

PG-containing cells were demonstrated by staining with Mab 2E9-biotin. The specificity of the murine Mab 2E9, raised against normal human faeces PG-polysaccharides, has been well documented previously[43,61]. Mab 2E9 recognizes PG-containing cell wall fragments, such as lysozyme-solubilized cell walls of different Gram-positive bacteria[43,61]. NAMLAA-containing cells were detected by staining with biotinylated Mab mouse anti-human NAMLAA-biotin IgG1 (AAA4) as described above[28]. Mab AAA4, raised against human serum amidase, specifically recognizes NAMLAA[28](Zhang *et al.*, 2005, *submitted*). Lysozyme-containing cells were detected by staining with polyclonal antibody rabbit anti-human lysozyme (Ab-1, Neomarkers, Fremont, CA), followed by biotinylated donkey anti-rabbit (Amersham Biosciences, Buckinghamshire, England). Incubations with secondary and tertiary reagents were done for 1 h at room temperature. Specific staining for PG, NAMLAA and lysozyme was revealed by peroxidase linked avidin (DAKO, Glostrup, Denmark) and 3-amino-9-ethylcarbazole (Sigma) as chromogen, resulting in a bright red staining.

Nuclei were counter-stained by hematoxylin. As negative controls the primary antibody was omitted and isotype matched control antibodies of irrelevant specificity were used. The controls did not display binding activity in tissue of monkeys and MS patients. Reactive human tonsil sections were included in each staining procedure as positive control tissue. Sections were evaluated by two independent observers.

Table 3. Clinical and neuropathological data of MS patients and controls.

Case	Sex	Age (yrs)	pm time (h: min)	Disease duration (yrs)	Progressive MS	Number of lesions (n) Lesion stage
00/024	F	52	8:25	12	yes	(1) pre-active (1) active demyelinating
00/82	M	34	<42:00	unknown	unknown	(4) pre-active
00/120	F	69	13:20	23	yes	(1) pre-active
01/018	F	48	08:10	9	yes	(2) pre-active (1) active demyelinating (1) chronic inactive
97/123	M	46	03:45	23	yes	(1) pre-active (3) active demyelinating
97/160*	F	40	07:00	11	yes	(2) pre-active (2) active demyelinating
98/176	M	83	07:05	52	yes	(1) pre-active
99/051	F	45	10:55	14	yes	(1) pre-active (1) active demyelinating (1) chronic active
99/066	M	69	16:45	43	yes	(3) pre-active
96/013	F	68	10:30	n.a.	n.a.	non-demented control no lesions
96/078	F	87	08:00	n.a.	n.a.	non-demented control no lesions

From this patient two different tissue samples were stained, (M) male, (F) female, (pm) *post-mortem*, (n.a.) not applicable.

Immunofluorescent double labeling

A double-staining procedure was used to determine which cell types contain PG and NAMLAA. In brief, marmoset spleen tissues were fixed with 4% paraformaldehyde and all other tissues were fixed with acetone, as described above. To reduce autofluorescence, slides were incubated with 0.1% sodium borohydride (Sigma) in MQ and 0.3M glycine (Sigma) in PBS. PG-containing cells were detected with Mab 2E9-FITC and NAMLAA-containing cells with Mab AAA4-FITC. Mabs CD11b (MΦ, microglia, DC, granulocytes)(Leu-15, BD Biosciences, San Jose, CA) and CD83 (DC)(HB15a, Immunotech, Marseille, France) were conjugated to AF594 (Molecular Probes Europe, Leiden, The Netherlands) according to the manufacturer's instructions. Neutrophils were detected by staining with Mab mouse anti-neutrophil elastase (NP57, DAKO) followed by anti-mouse Ig-TRITC (DAKO). Since the available antibodies against human MΦ did not cross-react with rhesus monkey and marmoset MΦ, we were unable to determine the percentage of MΦ within the population of PG-containing cells. A histochemical staining for lipids was performed with 0.3% Sudan black B (BDH Laboratory Supplies, Poole, England) in 70% ethanol, thereby reducing autofluoresence.

Quantitation of PG-containing cells in brain

The number of PG-containing cells in the parenchyma and infiltrates of brain tissue derived from marmoset and rhesus monkeys were quantitated by two independent observers. The surface area of the section was determined using a VIDAS-RT image analysis system (Kontron Elektronik GmbH/Carl Zeiss, Weesp, The Netherlands). Area measurements were performed using a 1.6-fold magnification objective. The frequency of PG-containing cells in the brain parenchyma was calculated by dividing the number of PG-containing cells in the tissue section by the surface area of the section.

Peptidoglycan

Soluble PG (sPG) from *S. aureus* was prepared by gel-permeation chromatography[62]. The content of LPS in sPG was assessed to be < 15 pg/mg (acceptable background values), using a TLR4/MD2 transfected HEK293 cell line, with IL-8 production as a read-out. sPG induced a dose-dependent production of IL-8 by TLR2 transfected HEK293 cells (data not shown). There was no LTA contamination as revealed by HPLC analysis (data not shown). At present, purification and identification of biologically active components in sPG are being performed and may reveal other TLR2 agonists, as described by Travassos *et al.*[12].

Stimulation of peripheral blood mononuclear cells (PBMC) and IL-12p70 ELISA

Blood samples from rhesus monkeys and marmoset monkeys were collected as described previously, using lithium-heparin pre-coated tubes[63]. PBMC were isolated by Ficoll (LSM Lymphocyte Separation Medium, ICN Biomedicals, Aurora, OH) and cells were cultured in 25mM HEPES-buffered RPMI 1640 (Life Technologies, Glasgow, UK) supplemented with 10% FCS (ICN Biomedicals), penicillin and streptomycin (Invitrogen, Paisley, UK), Glutamax (Invitrogen), β-mercaptoethanol (Invitrogen). sPG was sonicated for 20 min and immediately added into the culture. Cells were seeded ($4x10^5$/well in 0.5 ml) with or without sPG (range 1 – 5 - 10 μg/ml) in 48-well plates (BD-Falcon) for 21 h. Supernatants were harvested and diluted 1: 1 in PBS/1%BSA (Sigma) and stored at –20°C for further analysis. According to the manufacturer's instructions, concentration of IL-12p70 was determined by using monkey-specific IL-12 ELISA kits (U-Cytech, Utrecht, The Netherlands).

Statistical evaluation

Statistical evaluation was performed using SPSS 11 software. The Mann-Whitney U test was used to analyze differences. A value of $p < 0.05$ was considered statistically significant.

Results

Presence of TLR/Nod ligand in CNS correlates with infiltration

Brain and spleen tissues of rhesus monkeys and marmoset monkeys were stained for PG, NAMLAA- and lysozyme-containing cells. Animals were divided into different groups, based on immunization procedure and development of clinical EAE (Table 1 and 2). In agreement with previous findings[50,52], large infiltrates were found in rhesus monkey EAE brain tissue compared to smaller infiltrates in marmoset EAE brain tissue (Figure 2A-C). As expected, a positive correlation was found (r=0.67 rhesus monkey and r=0.43 marmoset) between the size of infiltrates and the maximum number of PG-containing cells in infiltrates. The following two sections describe the assessment of PG, NAMLAA and lysozyme in 1) rhesus monkey and 2) marmoset brain tissue.

1) Elevated numbers of cells containing PG and NAMLAA in the CNS of rhesus monkeys with EAE

Infiltrates were absent in brain tissue of rhesus monkeys (n=2) immunized with collagen in CFA, whereas in one of two rhesus monkeys immunized with myelin antigens, but without EAE, one infiltrate (medium/large size) was present in the brain with only a few PG-containing cells (Table 4). In contrast, animals with clinical EAE had many PG- and NAMLAA-containing infiltrates, with high numbers of positive cells in the infiltrates (Figure 2C, 3A, 3B and Table 4). The maximum number of PG-containing cells in these rhesus monkey brain infiltrates was much higher compared to the maximum number found in infiltrates of marmoset EAE brain tissue, which can be explained by the differences in size of the infiltrates (Figure 2D, Table 4). A significantly higher number (p<0.05) of PG-containing cells was found in the parenchyma of EAE brain tissue compared to non-EAE brain tissue (Figure 2E). In contrast to NAMLAA-expression in the infiltrates, only few NAMLAA-containing cells were present in the parenchyma of rhesus monkey brain tissues (data not shown).

Taken together, brain tissue of rhesus monkeys with EAE contains large infiltrates with abundant numbers of PG-containing cells and high numbers of NAMLAA-expressing cells. The number of NAMLAA-expressing cells is consistent with the abundant number of neutrophils present in these lesions. In contrast, only occasionally lysozyme-expressing cells were present near blood vessels (data not shown).

2) Elevated numbers of cells containing PG in the CNS of marmoset monkeys with EAE

One of two marmosets immunized with ovalbumin in CFA had two small infiltrates in the brain, which did not contain PG. In seven of nine marmosets immunized with myelin antigens but without EAE, brain infiltrates (of medium size, number 2.9 ± 2

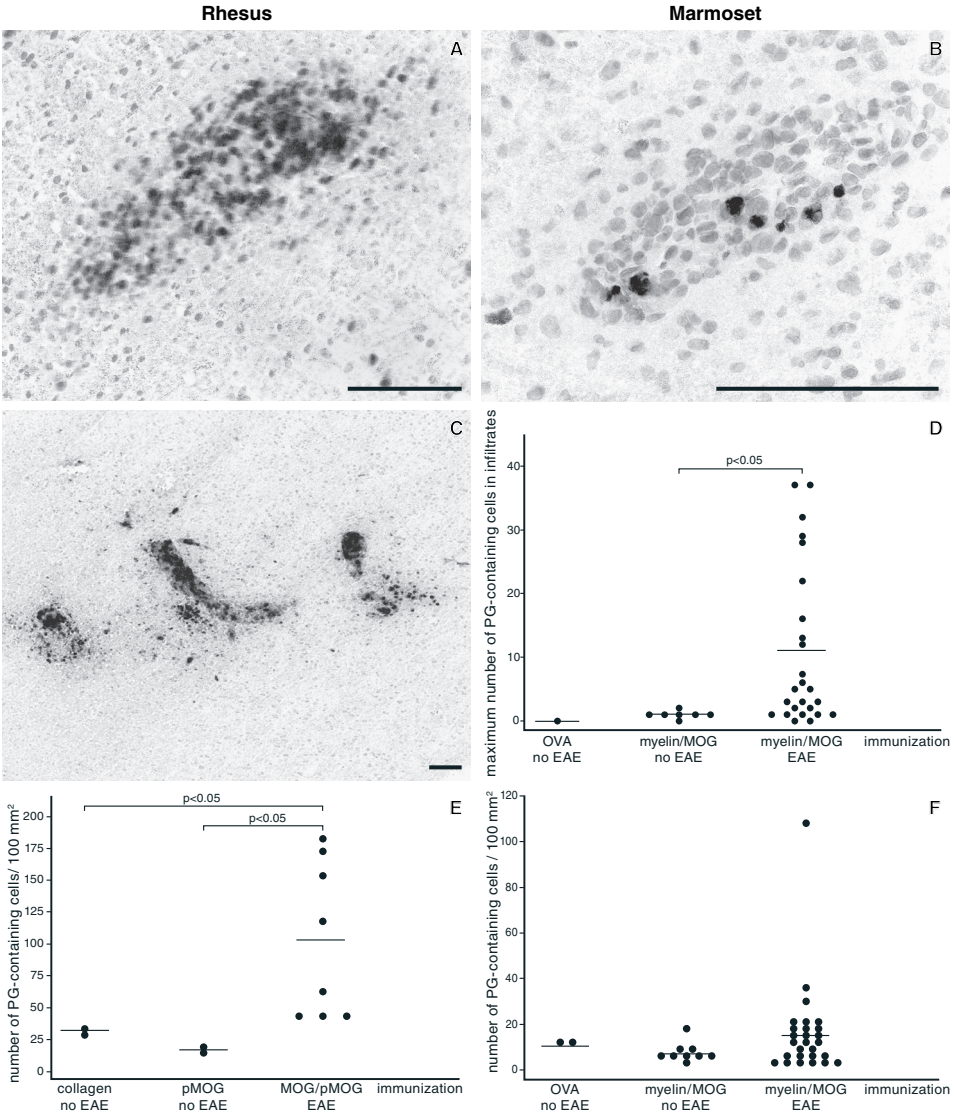

Figure 2. PG-containing cells are present in rhesus and marmoset brain.
Left column: rhesus brain tissue and right column marmoset brain tissue. Many cells in infiltrates of (A) rhesus EAE brain tissue (animal 9) and a modest cell number in infiltrates of marmoset EAE brain tissue (animal 10) contain PG (B, D). Significant higher numbers of cells in infiltrates of marmoset EAE brain tissue (D) contain PG when compared to control brain tissue. Many PG-containing infiltrates are present in rhesus EAE brain tissue (C). Significantly elevated numbers of PG-containing cells are present in the parenchyma of rhesus (E), but not in marmoset EAE brain tissue (F), compared to control brain tissue. Bar = 100 μm. (See Appendix page 221 for a full-color representation of this figure.)

Table 4. High numbers of PG-containing cells in rhesus EAE brain tissue.

				PG-infiltrates		NAMLAA-infiltrates	
animal	immunogen	EAE	infiltrate size	/total infiltrates	PG-cells	/total infiltrates	NAMLAA-cells
1	collagen II	-	-	0/0	0	0/0	0
2	collagen II	-	-	0/0	0	0/0	0
6	*phMOG$_{34-56}$	-	-	0/0	0	0/0	0
7	*phMOG$_{34-56}$	-	++/+++	1/1	2	0/0	0
10	rhMOG$_{1-125}$	+	+	1/1	1	0/0	0
8	#phMOG$_{34-56}$	+	++	6/13	27	1/13	5
11	rhMOG$_{1-125}$	+	++/+++	2/3	50	0/3	0
12	rhMOG$_{1-125}$	+	++	15/22	54	12/22	13
13	rhMOG$_{1-125}$	+	+++	9/9	151	9/9	25
14	rhMOG$_{1-125}$	+	+++	11/11	264	5/11	50
9	#phMOG$_{34-56}$	+	++	32/32	173	25/32	50
15	rhMOG$_{1-125}$	+	+++	10/10	>300	10/10	100

(rhMOG$_{1-125}$) recombinant peptide, representing the complete extracellular N-terminal domain of human MOG, (phMOG$_{34-56}$) synthetic peptide, representing an extracellular N-terminal domain of human MOG, (*) booster with phMOG$_{4-26}$, (#) booster with phMOG$_{34-56}$.
infiltrate size
+ small
++ medium (>30 cells)
+++ large (hundred of cells)

per section) were present with a few PG-containing cells (1 ± 0.8 per infiltrate). In twenty-five out of twenty-seven marmosets with EAE, brain infiltrates (3.3 ± 2.6 per section) were present with a modest number of PG-containing cells (10.5 ± 12.5 per infiltrate). The number of PG-containing cells in infiltrates of marmoset monkeys with EAE was significantly higher (p<0.05) than in animals without EAE (Figure 2B, D). In all marmoset groups a similar frequency of PG-containing cells (13.7 ± 17.5 per 100 mm^2) was present in the brain parenchyma (Figure 2F). Few cells within perivascular infiltrates expressed NAMLAA in EAE and non-EAE marmoset tissues (Figure 3F). No NAMLAA-containing cells were detected in the parenchyma of the brain. In brain and spleen tissue of marmoset monkeys we could not detect lysozyme-containing cells, while positive cells were found in human tonsil tissue. These data indicate that the antibody we used against human lysozyme is most likely not cross-reactive with marmoset lysozyme. In summary, these data show that PG-containing cells are present in a significantly higher number in marmoset brain infiltrates of EAE monkeys compared to non-EAE monkeys, whereas NAMLAA is only scarcely expressed.

Various phagocytic subsets contain PG and NAMLAA in monkey brain tissue

PG is present within MΦ/microglia, DC[43] and granulocytes in MS brain tissue. The cell types containing PG- and NAMLAA in marmoset and rhesus monkey EAE brain tissue were identified by double staining for CD11b (MΦ, microglia, DC, granulocytes),

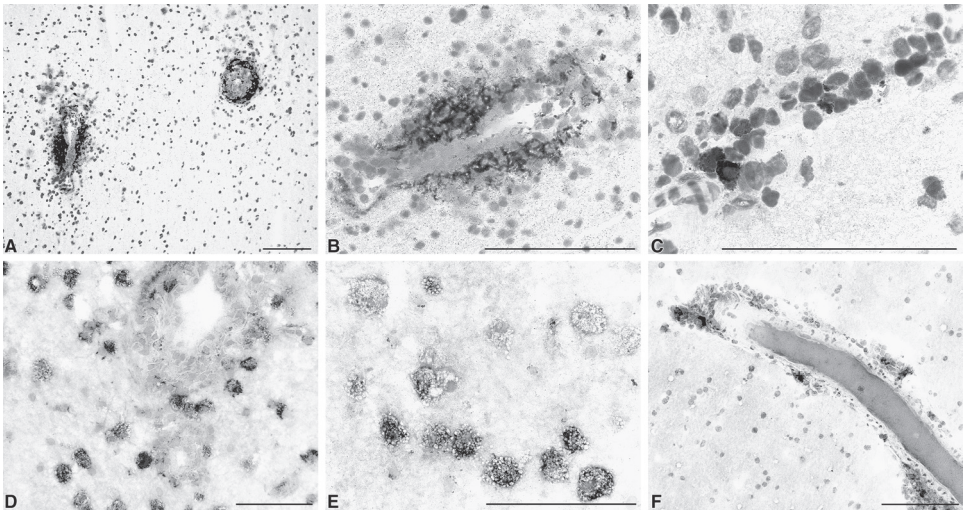

Figure 3. Restricted NAMLAA expression in MS and monkey EAE brain.
In rhesus EAE brain tissue (animal 9) many perivascular infiltrates are present with a moderate to high number of NAMLAA-containing cells (A, B). In marmoset EAE brain tissue (animal 10) some NAMLAA-containing cells are localized near blood vessels (C). Foamy MΦ in active MS brain lesions (sample 97-160) express NAMLAA (D). Expression of NAMLAA is restricted to a certain subpopulation of foamy MΦ (E). Occasionally also perivascular cells express NAMLAA in MS brain tissue (sample 00-082) (F). Bar = 100 μm. (See Appendix page 222 for a full-color representation of this figure.)

CD83 (DC) and neutrophil elastase (neutrophils). EAE-affected brain tissues from marmoset (n=3) and rhesus monkeys (n=2) were selected based on representative numbers of PG- and NAMLAA-containing cells. PG was mostly present in $CD11b^+$ cells (87-100% of PG-containing cells) in both marmoset and rhesus monkey EAE brain tissue. Many neutrophils (62-65%) and also DC (16%) in rhesus monkey brain contained PG. PG was not detected within DC in marmoset brain tissue. The number of PG-and NAMLAA-containing granulocytes in marmoset tissues could not be determined since the available antibodies against human granulocytes did not cross-react with marmoset granulocytes.

In rhesus monkey EAE brain tissue, NAMLAA was mostly expressed by neutrophils (43-80% of NAMLAA-containing cells), $CD11b^+$ cells (25-48%) and by some DC (7-8%). NAMLAA in marmoset EAE brain tissue was occasionally expressed by $CD11b^+$ cells (0.5-1%), but not by DC. Comparing the number of $CD11b^+$ PG-containing cells with $CD11b^+$ NAMLAA-containing cells it can be speculated that PG and NAMLAA are generally present in different cells, i.e. PG is mostly present in cells that express CD11b, whereas NAMLAA is mostly expressed in $CD11b^-$ cells.

Restricted expression of NAMLAA and lysozyme in MS brain

Previously, the number of PG-containing cells was determined in MS and non-MS control brain tissue[43]. To determine whether PG-persistence in MS brain is associated with the expression of PG-degrading enzymes, we assessed whether NAMLAA- and lysozyme-containing cells are also localized at sites of inflammation in MS brain and in control brain tissue. Subsequently, we determined whether expression of NAMLAA and lysozyme in MS brain parallels the expression in marmoset and rhesus brain tissue. In MS brain (n=30 lesions from n=9 patients) with different lesion stages (Table 3), a few to a moderate number of NAMLAA-containing cells were found. These cells had a foamy MΦ appearance, likely due to phagocytosed myelin, and were localized within active lesions (Figure 3D). Within the same active lesions also many NAMLAA-negative foamy cells were present, indicating that myelin-phagocytosing cells do not all or continuously produce NAMLAA (Figure 3E). Occasionally some NAMLAA-containing cells were present near blood vessels (Figure 3F). Pre-active perivascular infiltrates and parenchymal areas of the brain did not contain NAMLAA. NAMLAA-containing cells were absent in non-demented control brain tissues (n=2 patients). Lysozyme-containing cells were absent in all human brain tissues with the exception of occasional intravascular cells. In the positive control tissue, human tonsil, lysozyme-containing cells were present, confirming that the absence of lysozyme in human brain tissue cannot be explained by the failure of the staining. In summary, NAMLAA was expressed by a subpopulation of cells with a foamy MΦ appearance in active MS lesions and lysozyme was not expressed in MS and control brain.

Peripheral PG: Higher numbers of PG-containing cells in EAE-affected rhesus spleen

The priming and perpetuation of autoimmune-mediated reactions take place in secondary lymphoid organs. Activation signals to APC via TLR/Nod-stimulation can potentially influence these priming and effector responses and promote Th1 cell development[64-66]. We have assessed whether EAE development in non-human primates is associated with a different number or localization of the putative TLR2/Nod ligand PG, as well as NAMLAA and lysozyme in spleen. Therefore, we stained spleen tissues from EAE (n=3) and non-EAE (n=3) marmoset and rhesus monkeys (Table 1 and 2).

Table 5 shows the number of PG- and NAMLAA positive cell numbers in rhesus monkey spleen tissue. In all rhesus monkeys, a moderate number of PG-containing cells was present in the red pulp. However, in two EAE monkeys that developed EAE, PG-containing cells were also present in moderate numbers in the marginal zone (Figure 4A, B). No lysozyme-containing cells were found in these spleen sections. A moderate number of NAMLAA-containing cell clusters was present in the red pulp (data not shown) of non-EAE rhesus monkey spleen tissue, whereas individual NAMLAA-containing cells, were occasionally present in spleens of

Table 5. PG- and NAMLAA-containing cells in rhesus spleen.

animal	immunogen	EAE	PG*	NAMLAA cell clusters
4	collagen II	-	+	+
5	collagen II	-	+	+
3	collagen II	-	+	+/-
10	$rhMOG_{1-125}$	+	++	-
16	$rhMOG_{1-125}$	+	++	-
11	$rhMOG_{1-125}$	+	+	-

(-) no positive cells, (+/-) occasional cells, (+) few cells, (++) moderate cell number, (*) marginal zone, ($rhMOG_{1–125}$) recombinant peptide, representing the complete extracellular N-terminal domain of human MOG.

EAE-affected rhesus monkeys. No clear differences in number or localization of PG- or NAMLAA-containing cells were found in EAE-affected and non EAE-affected marmoset spleen (data not shown). In summary, these data suggest that higher numbers of PG-containing cells are present in rhesus monkey EAE spleen compared to non-EAE spleen. These differences were not seen in marmoset EAE spleen. No overt differences were observed in the expression of NAMLAA in marmoset and rhesus monkey spleen tissue.

Various phagocytic subsets contain PG and NAMLAA in monkey spleen tissue

As in brain tissue, we identified the PG- and NAMLAA-containing cells in spleen tissues from rhesus monkey non-EAE (n=2) and EAE (n=2) and marmoset non-EAE (n=2) and EAE (n=3) monkeys. We did not identify PG-containing cells in marmoset spleen, since only few cells contained PG and no differences were found between animals with or without EAE. PG in spleen tissue from both non-EAE and EAE rhesus monkeys was present in comparable numbers of $CD11b^+$ cells (42-

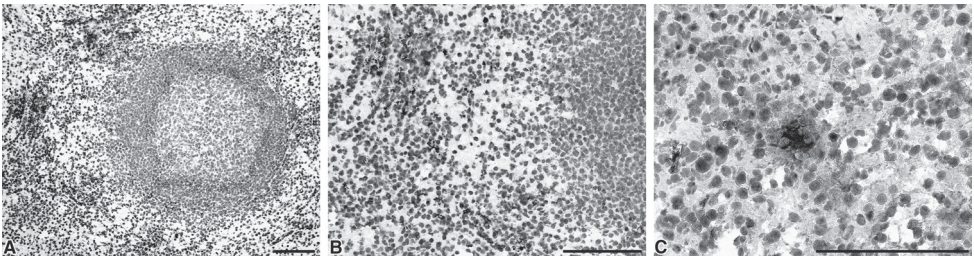

Figure 4. High numbers of PG-containing cells in rhesus EAE spleen.
High numbers of PG-containing cells are present in the marginal zone of rhesus monkeys that developed EAE (animal 10)(A, B). In control immunized rhesus monkeys (animal 5) NAMLAA-expressing cells are present in clusters within the red pulp (C). Bar = 100 μm. (See Appendix page 222 for a full-color representation of this figure.)

50% of PG-containing cells) and neutrophils (40-68%). PG was only occasionally present within DC (~1%) in animals without EAE, whereas in animals with EAE more DC (5-14% of PG-containing cells) contained PG. About half (42-54%) of PG-containing cells in rhesus monkey spleen expressed CD11b, irrespective of disease development. In rhesus monkey spleen, NAMLAA was predominantly expressed by neutrophils (75-91%) and by some DC (2-3%). Also in marmoset spleen tissue NAMLAA-containing cells are most likely granulocytes, whereas NAMLAA was not present within DC. Most NAMLAA-containing cells did not express CD11b (86-99%). Thus, as in brain, splenic PG and NAMLAA are most likely present within different cell types, based on the expression pattern of CD11b.

Proinflammatory S. aureus derived sPG stimulates IL-12p70 production by monkey PBMC

Previously we have demonstrated that sPG, purified from the pathogenic bacterium *S. aureus*, exerts proinflammatory effects on mouse bone marrow-derived DC[11]. Moreover, sPG efficiently induced EAE in mice, when admixed with MOG_{35-55} encephalitogenic peptide in IFA[11]. To further substantiate the pathogenic relevance of PG we determined whether *S. aureus* PG is capable to induce the prototypical Th1 promoting cytokine IL-12 by non-human primate PBMC. IL-12 is an important proinflammatory cytokine in the development of murine and non-human primate EAE[59,67,68]. PBMC from (n=2) marmoset monkeys, (n=2) rhesus monkeys and one human donor, as an intrinsic control, were stimulated with different concentrations of *S. aureus* sPG. As expected, *S. aureus* sPG induced a dose-dependent increase of IL-12p70 upon stimulation of human PBMC (Figure 5). Both rhesus monkeys (n=2) and marmoset (n=1) PBMC also produced elevated levels of IL-12p70 upon sPG stimulation, in a dose-dependent fashion (Figure 5). In conclusion, sPG from *S. aureus* induces a prototypical proinflammatory cytokine thought to be crucially involved in demyelinating disease.

Discussion

Recent studies in EAE models have shown that innate immune responses within the CNS, usually induced by interaction of CpG oligonucleotides with TLR9, are of significant importance in the expression of autoimmunity. The absence of TLR9 on CNS APC in primates hampers direct translation of this principle to MS. We use non-human primate EAE models to bridge the immunological gap between the EAE mouse and the MS patient. Bacterial components can stimulate APC to produce a score of proinflammatory cytokines and chemokines and stimulate the development of antigen-specific Th1 cells. Bacterial PG is a candidate agonist of CNS APC, which stimulates cells via extracellular and intracellular receptors (TLR and Nod). Proinflammatory PG can be redistributed from the periphery into sites of chronic inflammation, such as rheumatoid joints[44] and MS brain[43]. In relation to MS, we

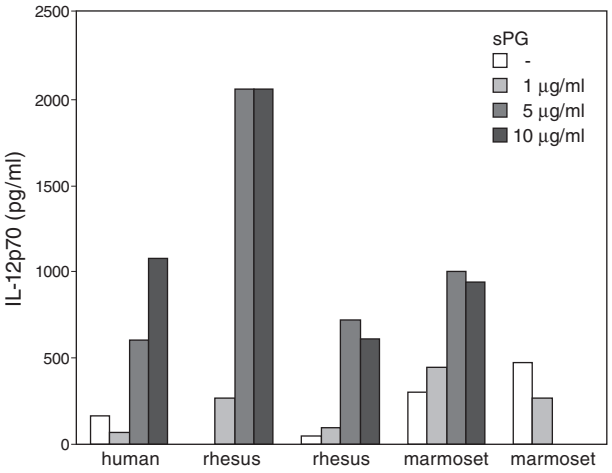

Figure 5. *S. aureus* derived sPG induces IL-12p70 production by rhesus and marmoset APC. PBMC from human (n=1), rhesus (n=2) or marmoset monkeys (n=2) were stimulated with different concentrations of sPG for 21 h. Supernatants were harvested and IL-12p70 was determined by ELISA.

compared EAE-affected rhesus monkeys, which develop an acute disease course with marmoset EAE monkeys, which develop chronic EAE[50]. We show here that in both monkey models for MS, EAE development is associated with a significantly elevated number of PG-containing cells in the brain. EAE-affected rhesus monkeys develop large brain lesions with many PG-containing cells, whereas EAE-affected marmoset monkeys develop infiltrates of medium size with a modest number of PG-containing cells. We hypothesize a causal relation between both features. Both in the CNS and in peripheral lymphoid organs, PG-containing cells may contribute to autoimmunity by producing proinflammatory mediators, induced by TLR/Nod ligation. The persistence of PG in MS and monkey EAE brain tissue might be due to the restricted expression of both NAMLAA and lysozyme. The fact that a TLR/Nod ligand is transported into the brain during autoimmune encephalomyelitis and MS may have major consequences for disease development and perpetuation, as discussed below.

Activation of resident and infiltrated APC by TLR/Nod ligation in the CNS

In MS[43] and rhesus monkey EAE brain tissue, PG is present within MΦ, DC and neutrophils suggesting that CNS infiltrating phagocytes are responsible for the transport of PG into the CNS. In the chronic marmoset EAE model, neutrophils are rarely found within the CNS and PG is detected mostly within MΦ. PG can induce proinflammatory signals through ligation of TLR2[13,14,69] and/or Nod receptors[15,70]. It has been described that different TLR are expressed on resident APC in the MS brain[7]. PG-induced responses in murine astrocytes are TLR2 dependent[71] and

murine microglia produce numerous proinflammatory cytokines and chemokines after PG stimulation[72,73]. These data demonstrate that different resident cell types in the brain produce proinflammatory mediators upon stimulation with PG. Additionally, also blood-derived PG-containing brain infiltrating cells may locally produce proinflammatory mediators via TLR/Nod signaling pathways. Triggering of C-type lectin receptors may abrogate the proinflammatory effects of TLR-induced responses. Hence, it has been proposed that balancing of C-type lectin receptor and TLR-mediated signals to APC might be of importance in maintaining self-tolerance in EAE and MS[74].

Implications of TLR stimulation in tolerance regulation by APC

Under normal conditions autoreactive T cells are controlled to prevent the development of autoimmune disease. Even high numbers of activated autoreactive T cells inside the target organ are not sufficient to induce autoimmune disease[75]. However, autoimmune disease can develop once the autoantigen is presented by resident APC of the CNS to autoreactive T cells in the presence of costimulation by TLR or CD40 ligands[8,75-77]. Both in the periphery and at the inflammatory site, immature APC have a major role in the maintenance of T cell tolerance to self-antigens. For example, introduction of the $PLP_{139-151}$ T cell receptor transgene into B10.S mice did not result in spontaneous EAE development. But when APC from B10.S $PLP_{139-151}$ T cell receptor transgenic mice were activated by systemic TLR ligand administration, 33% of these mice developed EAE[78]. Moreover, TLR-induced activation of CNS APC promoted EAE development in C57BL/6 mice[10]. It has recently been demonstrated that naïve myelin-specific T cells become activated in inflamed CNS by local APC[79] and can cause disease progression by epitope spreading[80,81]. PG can activate local APC and form a possible trigger for the activation of T cells directed against endogenous myelin epitopes, participating in the perpetuation of demyelinating disease.

With respect to the non-human primate EAE models, we here show a dose-dependent induction of IL-12p70 upon sPG stimulation of peripheral blood leukocytes from both marmoset and rhesus monkeys (Figure 5). The p40 moiety of IL-12 is shared by the related family member IL-23, and plays an important role in the development of EAE[82]. It was very recently demonstrated that treatment with Mab anti-IL-12p40 of marmoset monkeys, initiated when MRI detectable lesions first appeared, limited lesion development (‘t Hart *et al.*, 2005, *submitted*). Its importance is furthermore underscored by the fact that mice with an astrocyte-targeted expression of IL-12p70 developed EAE after immunization with CFA and toxin, without specific autoantigen in the emulsion[67]. Thus, PG may contribute to inflammation and disease progression by promoting the production of Th1 stimulating proinflammatory cytokines in the CNS and in secondary lymphoid organs. Interestingly, in EAE susceptible mouse strains, APC have a higher activation state compared with APC from EAE-resistant stains[78]. In accordance, we

are currently testing the hypothesis that the more acute EAE in rhesus monkeys is due to a higher sensitivity of rhesus monkey APC to TLR/Nod-induced activation than marmoset APC. This suggests that TLR/Nod genes may form a genetic susceptibility traint for acute demyelinating disease.

Persistence of PG in the CNS is related to restricted expression of degrading enzymes

Previously, it was shown that radio-actively labeled PG-monomers accumulated over time in brain and intestine of mice[83]. More recently Zhang *et al.*, demonstrated that injected arthritogenic PG was retained for several weeks in spleen, liver and synovial tissue of rats[84]. These data demonstrate that PG can be transferred into and retained at inflammatory sites. In order to understand the persistence of PG in MS and monkey EAE brain tissue we determined the expression of NAMLAA and lysozyme. These enzymes together are capable to abolish the proinflammatory capacity of PG[27].

In the CNS of MS patients and EAE-affected monkeys one might expect many lysozyme-expressing cells, since infiltrates contain many phagocytosing cells and lysozyme is known to be present within granules of phagocytes[22-24]. However, in this study, we could not detect lysozyme-containing cells in infiltrates and in the parenchyma of MS brain tissue. Occasional intravascular cells containing lysozyme were found in non-demented controls, in MS and in rhesus monkey EAE brain tissue, which is in agreement with previous findings in MS brain tissue[85,86]. Also NAMLAA is expressed in a restricted manner in MS and EAE-affected marmoset brain, while in EAE-affected rhesus monkeys many NAMLAA-containing cells are present in the brain. Disease development in marmoset monkeys and MS patients is more chronic compared to rhesus monkeys, which develop an acute encephalomyelitis accompanied by large necrotic brain lesions with many neutrophils and MΦ[50]. This difference in pathology between MS / marmoset EAE and rhesus monkey EAE most likely accounts for the different numbers of NAMLAA- and PG-containing cells. In conclusion, these findings show that intracellular PG persists in MS and monkey brain tissue. Persistence of PG correlates with the absence or low numbers of NAMLAA- and lysozyme-expressing cells. Local conditions in the CNS may restrict expression of these enzymes.

PG can stimulate EAE in secondary lymphoid organs

PG-containing cells may stimulate autoimmune-mediated processes either in the CNS or in peripheral lymphoid organs. We here show that EAE development in rhesus monkeys is associated with a higher number of PG-containing APC in the marginal zone of the spleen. It was shown previously that PG derived from sterile human spleen induced T cell proliferation and proinflammatory cytokine production by peripheral blood leukocytes[39]. Splenic PG may thus be involved in monkey EAE development, possibly by stimulation of peripheral autoimmune-mediated processes.

We recently demonstrated that PG stimulates autoimmune-mediated processes in mouse EAE development[11]. Purified PG from *S. aureus* was added to an emulsion of IFA and MOG_{35-55}. Already at four hours after immunization with this emulsion, PG-containing cells were detected in the draining lymph nodes. At this site, PG stimulated autoantigen-specific T cell expansion and Th1 cell polarization. Subsequently, these mice developed EAE, whereas animals did not develop EAE upon injection with MOG_{35-55} in IFA without PG. Taken together these data show that a single bacterial component, PG, can create an inflammatory environment sufficient to break T cell tolerance. Moreover, in combination with autoantigen presentation, PG is sufficient to induce autoantigen-specific Th1 cell generation and EAE development. Given the costimulatory capacity of PG in EAE development, prevention of PG-trafficking and action may contribute to treat the activity of autoimmune disease in humans.

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CHAPTER

3

Proinflammatory bacterial peptidoglycan as a cofactor for the development of central nervous system autoimmune disease

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Abstract

Upon stimulation by microbial products through Toll-like receptors (TLR), dendritic cells (DC) acquire the capacity to prime naïve T cells and to initiate a proinflammatory immune response. Recently, we have shown that antigen presenting cells (APC) within the CNS of multiple sclerosis (MS) patients contain peptidoglycan (PG), a major cell wall component of Gram-positive bacteria, which signals through TLR and NOD.

In this study, we report that *Staphylococcus aureus* PG as a single component can support the induction of experimental autoimmune encephalomyelitis (EAE) in mice, an animal model for MS. Mice immunized with an encephalitogenic myelin oligodendrocyte glycoprotein peptide (MOG)-peptide in incomplete Freund's adjuvant (IFA) did not develop EAE. In contrast, addition of PG to the emulsion was sufficient for priming of autoreactive Th1 cells and development of EAE. *In vitro* studies demonstrate that PG stimulates DC-mediated processes, reflected by increased antigen uptake, DC maturation, Th1 cell expansion, activation, and proinflammatory cytokine production.

These data indicate that PG-mediated interactions result in proinflammatory stimulation of antigen-specific effector functions, which are important in the development of EAE. These PG-mediated processes may occur both within the peripheral lymph nodes as well as in the CNS and likely involve recognition by TLR on DC. Thus, PG may provide a physiological trigger of DC maturation, and in this way disrupt the normal tolerance to self antigen. As such, PG signaling pathways may serve as novel targets for the treatment of MS.

Introduction

Multiple sclerosis (MS) is considered to be a chronic autoimmune disease of the CNS of unknown etiology critically driven by $CD4^+$ Th1 cells. However, the presence of autoreactive T cells is clearly not the single determining factor for the development of MS, because myelin-reactive T cells are also found in healthy individuals[1,2]. This implies that other factors play an important role in the initiation and perpetuation of pathological autoantigen-specific immune processes.

Bacteria and viruses are prominent cofactors that have been implicated in autoimmune disease initiation and persistence[3]. As such, it has been shown that MS relapses are frequently associated with antecedent infections[4,5], which in some cases are bacterial[6]. In experimental autoimmune encephalomyelitis (EAE), an animal model for MS, a strong adjuvant containing killed whole *Mycobacterium tuberculosis* (complete Freund's adjuvant: CFA) is required for disease induction. When incomplete Freund's adjuvant (IFA) emulsion is being used, animals will not develop EAE[7,8]. Microbial components, such as CpG-DNA

and LPS, are physiological stimuli of EAE development via innate receptors on antigen presenting cells (APC)[9-11]. These data indicate that non-infectious microbial components contribute to disease development in EAE and likely in MS.

Peptidoglycan (PG) is an important bacterial cell wall component implicated in chronic inflammation[12]. We have previously demonstrated that PG isolated from sterile human spleen stimulates T cell proliferation and cytokine production[13]. Furthermore, we have shown that phagocytic cells may distribute PG to sites of chronic inflammation. At these sites, PG-containing dendritic cells (DC) and macrophages (MΦ) are present in high numbers and express costimulatory molecules and cytokines, as revealed by extensive *in situ* analysis of MS brain tissue[14] and rheumatoid arthritis synovial tissue[15,16]. In conclusion, these data support the concept that biologically active PG can be transported by APC to lymphoid tissues and sites of chronic inflammation. Therefore, we hypothesized that PG acts as a supporting factor of autoimmune processes in MS.

As a mechanism of action, PG can activate the innate immune system through binding of extracellular and intracellular receptors[17-19]. Extracellular PG has been claimed to bind Toll-like receptor (TLR)2 in association with CD14, expressed on the cell surface of APC. Some internalized TLR ligands can also engage TLR in phagosomes[20]. In addition, two new intracellular receptors for PG fragments, Nod1/2 (Card4/15) have been described recently[21,22]. Although high numbers of immunocompetent PG-containing APC were found in MS brain tissue by *in situ* analysis, a functional role for PG in the pathogenesis of MS remains to be elucidated. We therefore assessed the role of PG in the development of mouse EAE. In order to achieve this, mice were immunized with autoantigen emulsified in IFA supplemented with PG. We used PG derived from *Staphylococcus aureus*, which does not contain the motif required for Nod1 ligation[22], but contains the motif necessary for Nod2 ligation[23,24]. Mice were assessed for EAE development and draining lymph nodes were analyzed for the presence of PG-containing cells. Because DC play a prominent role in linking the innate and adaptive immune response, we assessed the functional role of PG in antigen uptake, DC maturation, and T cell polarization. Taken together, our data show that proinflammatory PG stimulates the development of autoimmune-mediated processes in EAE.

Materials and Methods

Mice

Female C57BL/6 mice (8- to 12-wk-old) and BALB/c mice (6- to 10-wk-old) were purchased from Harlan (Horst, The Netherlands). $OVA_{323-339}$-specific, MHC class II-restricted, T cell receptor transgenic (OT-II) mice, backcrossed on the C57BL/6 background, were bred within the facility. All animal experiments were performed with approval of the Erasmus University MC committee (Rotterdam, The

Netherlands) for animal ethics. Mice were housed under specified pathogen free conditions and received water and food ad libitum. Paralyzed mice with EAE scores over 2.5 were afforded easier access to food and water.

Peptides, antibodies and PG

Myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ (MEVGWYRSPFSRVVHLYRNGK) was a kind gift from Dr. U. Günthert (Basel University, Basel, Zwitserland). Soluble PG (sPG) was prepared from *S. aureus* by gel-permeation chromatography[25], and insoluble PG (iPG) was prepared with standard methods. The LPS content of both PG fractions was rigorously checked and was < 13 pg LPS/mg PG as measured by *Limulus*-amebocyte lysate assay, which are background values. Absence of LPS was further confirmed by *in vitro* culture with mouse bone marrow-derived DC. Purification and identification of the biologically active components in the sPG fractions are currently being performed, and might reveal other TLR2 agonists as recently described by Travassos *et al*.[26].

PG of all bacterial species is composed of two alternating sugar residues, *N*-acetyl glucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc) forming glycan strands. These strands are connected via stem peptides that differ from one bacterial species to another, and that have the sequence L-alanine-D-isoglutamine-L-lysine-D-alanine-D-alanine in *S. aureus*. In addition, PG from *S. aureus* contains pentaglycine bridges. The complete peptidoglycan network is a highly complex ordered network, the detailed structure of which is subject of debate to this day[27]. The two main hydrolyzing enzymes capable of PG degradation are lysozyme and *N*-acetylmuramyl-L-alanine amidase (NAMLAA), which is identical to PG recognition protein long form (PGRP-L)[28,29]. Lysozyme hydrolyzes the bond between the two sugar moieties, whereas NAMLAA/PGRP-L hydrolyzes the lactate bond between *N*-acetyl muramic acid and the amino group of the first L-alanine.

Induction and clinical evaluation of EAE

C57BL/6 mice were immunized s.c., at four sites (axillar and inguinal), with 200 µg MOG_{35-55} in 0.1 ml PBS emulsified in an equal volume of CFA containing 200 µg *M. tuberculosis* (H37/Ra; Difco Laboratories, Detroit, Michigan, USA). Additionally mice were injected i.p. with 200 ng *Bordetella pertussis* toxin in 0.2 ml PBS on day 0 and 2 after immunization (Sigma-Aldrich, St. Louis, Missouri, USA). Mice were weighed and scored for clinical signs of EAE daily according to the following scoring system: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2, limb weakness and tail paralysis; 2.5, partial limb and tail paralysis; 3, complete hind or front limb paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death due to EAE.

Histology

The immunohistochemical techniques used in this study have previously been described in detail[30,31]. In brief, frozen sections of 6 μm were thaw-mounted on gelatin-coated glass slides and stored overnight in humidified atmosphere. Sections were air-dried for 1 h and fixed at room temperature in fresh acetone containing 0.02% (v/v) H_2O_2. Then the slides were air-dried for 10 min, washed with PBS and incubated with predetermined optimal dilutions of reagents overnight at 4°C in humidified atmosphere. Secondary and tertiary reagents were applied and incubated for 1 h at room temperature. Between each incubation, slides were washed twice with PBS/0.05%Tween20. Horseradish peroxidase (HRP) conversed 3-amino-9-ethyl-carbazole into a bright red precipitate upon incubation for 10 min. Incubation of slides with naphthol-AS-MX phosphate (Sigma-Aldrich) and fast blue BB base (Sigma-Aldrich) for 30 min at 37°C resulted in a in a blue precipitate indicating alkaline phosphatase activity. As a negative control, the primary antibody was omitted. Nuclei were counterstained by hematoxylin.

Four to 7 weeks after immunization, mice were euthanized using CO_2 and brain tissues were snap frozen in liquid nitrogen and stored at -80°C. To determine the cellular infiltration in the CNS, immunohistochemical stainings were performed on frozen sections with anti-mouse B220 (RA-3-6B2, BD Pharmingen, Alphen aan den Rijn, The Netherlands), anti-human cross-reactive to mouse CD3 (DAKOCytomation, Glostrup, Denmark) and anti-mouse MHC-II (M5/114). For CD3, donkey anti-rabbit Ig-biotin (Amersham Biosciences, Buckinghamshire, England) was used as a conjugate. For B220 and MHC-II, rabbit anti-rat IgG-biotin (DAKOCytomation) was used as a conjugate. As a tertiary step, slides were incubated with ABComplex-HRP (DAKOCytomation).

To assess the presence of PG within the draining lymph nodes, we isolated axillary and inguinal lymph nodes at 4 and 96 h after immunization. Lymph nodes were snap frozen in liquid nitrogen and stored at -80°C. A double-staining procedure was used to determine which cell types contain PG. PG-containing cells were detected with Mab 15704-biotin (Gentaur Molecular Products, Brussels, Belgium) raised against *S. aureus* PG, followed by streptavidin-FITC (BD Biosciences, San Diego, California, USA). DC were detected by incubation with anti-mouse CD11c-PE (HL3, BD Biosciences).

Uptake of OVA by DC

DC were prepared as previously described[32]. Briefly, bone marrow was flushed with RPMI 1640 (Invitrogen Life Tachnologies, Paisley, UK) from femurs and tibiae of C57BL/6 mice. Cells were washed, enumerated, and plated in bacteriological 100-mm-diameter petri-dishes. Cell-culture medium (TCM) was RPMI 1640 supplemented with gentamicin (60 μg/ml) (Invitrogen Life Technologies), 2-mercaptoethanol (5 $x10^{-5}$ mol/L) (Sigma-Aldrich) and 5% (v/v) fetal calf serum (Biocell Laboratories, Rancho Dominguez, California, USA). At day 0 of the culture,

the cells were seeded at a concentration of $2x10^6$/dish in medium containing recombinant mouse GM-CSF (200 IU/ml) (kindly provided by K. Thielemans, University of Brussels, Belgium). At day 3, TCM containing 200 IU/ml recombinant mouse GM-CSF was added. At days 6 and 8, half of the medium was collected, centrifuged, and the pellet was resuspended in TCM containing 200 IU/ml of recombinant mouse GM-CSF. At day 9 100 μl TCM containing $1x10^5$ DC were plated in round bottom 96-well plates for 30 min at 4 or 37 °C. DC were pulsed with different concentrations (0, 0.01, 0.1, 1 mg/ml) of OVA-FITC (Molecular Probes, Eugene, Oregon, USA). The endotoxin level of FITC-OVA determined by a *Limulus*-amebocyte lysate assay (Bio Whittaker, Verviers, Belgium) was <0.001 μg, which was previously reported not to affect DCs[33]. DC were stimulated with 10 μg/ml sPG or iPG. Cells were stained for 30 min at 4°C with anti-CD11c-APC (HL3, BD Biosciences) dissolved in PBS containing 0.5% bovine serum albumin and 0.01% sodium azide. Uptake of OVA-FITC was determined on CD11c-allophycocyanin-gated cells by flow cytometry. Dead cells and debris were excluded using propidium iodide.

DC maturation assay

At day 9 of the culture, DC were pulsed overnight with either 100 ng/ml LPS, or 10 μg/ml sPG or iPG. To exclude LPS contamination of the PG preparation, polymyxin B was added to the culture. Cells were centrifuged and supernatant were collected for cytokine analysis (IL-6, IL-10, IL-12p70, TNF-α). The maturation state of DC was determined by staining for 30 min with Mabs anti-CD11c-allophycocyanin, anti-MHC class II-FITC (M5/114.5.2), in combination with anti-CD40-PE (3/23), anti-CD80-PE (16-10A1), and anti-CD86-PE (GL-1) (all obtained from BD Biosciences) diluted in PBS containing 0.5% bovine serum albumin and 0.01% sodium azide. Maturation of DC was determined on CD11c-allophycocyanin-gated cells by flow cytometry. Dead cells and debris were excluded using propidium iodide.

OVA-specific T cell proliferation assay

DC were obtained from bone marrow culture as described above. At day 9 of culture, DC were plated in 24 well plates containing 2 ml of TCM, and pulsed with 10 mg/ml OVA-Worthington (Biochemical Corp, Lakewood, New Jersey, USA) for 24 h. At the dose we used in our experiments, the endotoxin level of OVA measured by a *limulus*-amebocyte lysate assay (Bio Whittaker) was <0.001 μg. Simultaneously DC were stimulated with 100 ng/ml LPS (*Escherichia coli*, strain O26:B6, Sigma-Aldrich), 10 μg/ml sPG, or iPG. OT-II cells were purified as described previously[32]. Briefly, lymph nodes and spleens were collected from OT-II mice. After red blood cell lysis, cells were labeled with CFSE. Cells were enumerated, and dead cells, stained for trypan blue, were excluded. After 24 h DC were washed and $1x10^4$ DC were co-cultured with $1x10^5$ CFSE-labeled OVA T cell receptor transgenic $CD4^+$ OT-II T cells in round bottom 96-well plates containing 200 μl TCM. Cells were centrifuged, and

supernatants were harvested and stored at -20 °C for cytokine analysis. Cells were resuspended and incubated with anti-Vα2-biotin and Vβ5.1/5.2-PE TCR Mabs (eBioscience, San Diego, California, USA) for 30 min at 4 °C. Biotinylated anti-Vα2 was detected using streptavidin-allophycocyanin (BD Biosciences). Percentages of proliferating OVA-specific $CD4^+$ T cells were determined by gating on cells that were double positive for Vα2 and Vβ5.1/5.2. Dead cells and debris were excluded using propidium iodide.

MOG_{35-55}-specific T cell proliferation assay

For evaluation of MOG_{35-55}-specific lymph node cell proliferation, mice were immunized with MOG_{35-55} in adjuvant. Mice were sacrificed and draining inguinal, brachial and axillar lymph nodes were isolated at 33 days after immunization. Lymph node cells were cultured for 4 days in 96-well plates in 200 μl of RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Bio Whittaker). Cells ($4x10^5$/well) were cultured at 37°C and 5% CO_2 in the presence or absence of 10 μg/ml MOG_{35-55}. After 72 h of culture, 1 μCi of [^{3}H]thymidine (Amersham Biosciences) was added for 16 h. Incorporation of [^{3}H]thymidine was measured in triplicate using a filtermat harvester and a beta-plate counter (Perkin Elmer, Wellesley, Massachusetts, USA). Supernatants from MOG_{35-55} proliferation assays were harvested 96 h after culture, centrifuged and stored for further cytokine analysis at -20°C.

Cytokine measurement by ELISA

According to the manufacturer's instructions, concentrations of IL-4, IL-10, IL12-p70, IFN-γ and TNF-α were determined by using OptEIA ELISA kits (BD Biosciences).

Statistical evaluations

Statistical evaluation was performed using SPSS 11 software. The Mann-Whitney U test was used to analyze differences in cytokine production and T cell proliferation. A value of $p < 0.05$ was considered statistically significant.

Results

Both sPG and iPG are potent adjuvants for the induction of EAE

PG is a known proinflammatory stimulator of the innate immune response, but very little is known about the possible role of PG in the development of MS and EAE. To determine whether PG by itself is a sufficient proinflammatory stimulus for the development of EAE, we added highly purified sPG or iPG from *S. aureus* to an emulsion of IFA and MOG_{35-55}. In three independent experiments, standard induction of EAE by immunization of mice with MOG_{35-55} in CFA resulted in an EAE

incidence of 60 to 100%, with a normal EAE course (Figure 1 and Table 1). Mice immunized with MOG_{35-55} in IFA, as a negative control, did not develop EAE, as expected. In contrast, addition of sPG or iPG to IFA/MOG_{35-55} induced EAE with a disease incidence from 40 to 60%. Different dosages of sPG and iPG were used for EAE induction. High dosages of 250 µg sPG and 600 µg iPG did not induce EAE (Table 1). Importantly, however, lower PG dosages induced EAE with an incidence of 40 to 60%. The lowest effective dose for EAE induction with sPG (25 µg/ animal)

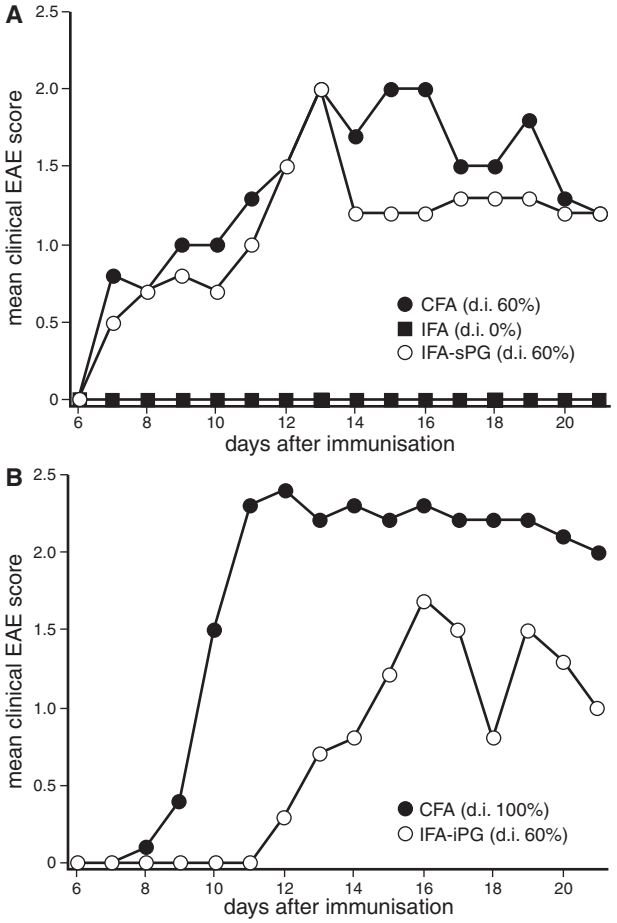

Figure 1. Both sPG and iPG are potent adjuvants for the induction of EAE.
A and B, In two separate experiments C57BL/6 mice were immunized with MOG_{35-55} emulsified in CFA (●). In (A) mice were immunized with MOG_{35-55} in IFA (■), or IFA-25µg sPG (○). In (B) mice were immunized with IFA-400µg iPG (○). Mice were weighed and scored for clinical signs of EAE daily. Five animals were used for each group. Graphs show mean clinical scores of animals that developed EAE. (d.i.) disease incidence.

Table 1. Clinical parameters of EAE induced by sPG or iPG.

Adjuvant	PG (μg/animal)	Disease incidence (%) *n=5*	Onset day[A]	Maximum score[A]	Cumulative score[A] (day 20)
CFA	n.a.	60	8.7 ± 2.9	2.0	20.2 ± 8.8
IFA	0	0	0	0	0
IFA-sPG	25	60	7.0	2.0	15.8 ± 8.8
	250	0	0	0	0
CFA[B]	nd	100	9.5 ± 1.6	2.7 ± 0.5	24.0 ± 4.6
IFA-iPG	25	0	0	0	0
	200	0	0	0	0
	250	40	12.0	2.0	11.0 ± 4.2
	400	60	13.7 ± 1.5	1.7 ± 0.6	9.8 ± 6.7
	600	0	0	0	0

([A]) Represented values are from animals that developed EAE, ([B]) mean values of two separate experiments, (n.a.) not applicable

was ten times lower than with iPG, in keeping with the higher biological activity described for sPG previously. These data demonstrate that PG is a potent adjuvant and can substitute whole *M. tuberculosis* in the induction for EAE.

PG-adjuvant induces classical EAE histopathology

Brain tissue was examined by histology to assess whether the signs of paralysis of mice immunized with PG-containing adjuvant are associated with a characteristic EAE histopathology. In brain tissue of animals that developed EAE, infiltrates were found containing $CD3^+$ T cells, $B220^+$ B cells and MHC-II^+ APC (Figure 2). A comparable number of infiltrates and infiltrating cells were detected in EAE mice injected with CFA and IFA-sPG, while the infiltrate size and number of infiltrating cells in IFA-iPG injected EAE mice was slightly lower (Table 2). These data confirm that paralysis induced with PG-containing adjuvant is associated with a characteristic EAE histopathology.

PG-containing adjuvant promotes MOG_{35-55}-specific Th1 cell polarization

It has been well established that EAE induction by immunization with an encephalitogenic antigen in CFA results in the generation of autoantigen-specific $CD4^+$ IFN-γ producing T cells[34]. To address whether the PG component of bacteria is able to generate MOG_{35-55}-specific Th1 cells, we determined MOG_{35-55}-specific T cell proliferation and IFN-γ, IL-4 and IL-10 production at day 33 after immunization. Lymph node cells from mice immunized with MOG_{35-55} in CFA proliferated dose dependently and produced IFN-γ upon *in vitro* re-stimulation with MOG_{35-55} (Figure 3). Mice immunized with MOG_{35-55} in IFA also showed antigen-specific proliferation, but only low amounts of IFN-γ were produced. Addition of sPG to the immunization mixture resulted in increased antigen-specific proliferation and IFN-γ production compared with immunization with IFA/MOG_{35-55}. IL-4 and IL-10 were undetectable

in any of the groups. These data show that PG creates an inflammatory environment. In combination with autoantigen presentation, PG can effectively induce autoantigen-specific Th1 cells.

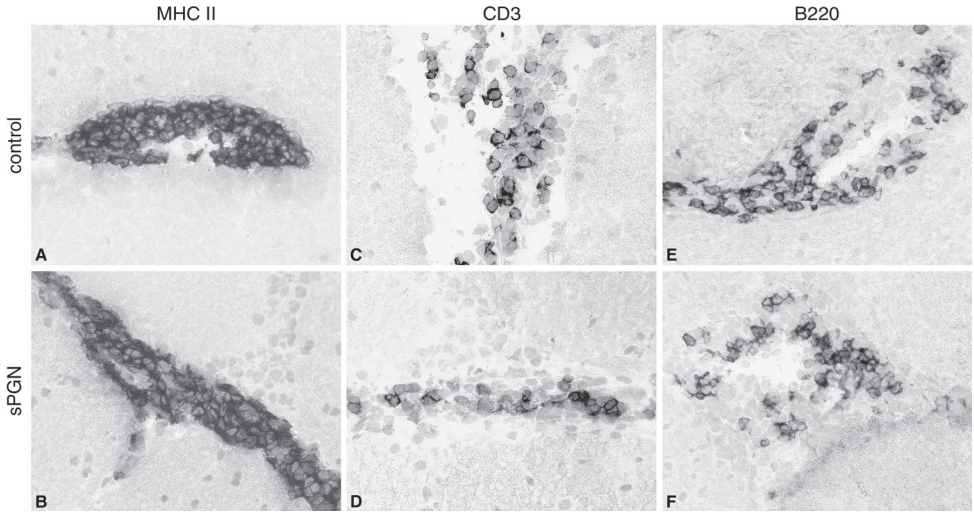

Figure 2. Immunization with PG-containing adjuvant induces classical EAE histopathology. Brain tissues were isolated between 4 to 7 weeks after immunization. Frozen brain sections were stained with hematoxylin in combination with MHC-II (A, B), CD3 (C, D) and B220 (E, F). (See Appendix page 223 for a full-color representation of this figure.)

Table 2. Histological analysis of infiltrates in EAE brain tissue.

Adjuvant	PG (μg/animal)	Number	Size
CFA	n.a.	>10	+
	n.a.	>10	+
	n.a.	4-10	+
	n.a.	4-10	+
	n.a.	>10	+
IFA-sPG	25	>10	+
	25	4-10	+
	25	>10	+
IFA-iPG	250	4-10	-/+
	250	4-10	+
	400	1-3	-/+
	400	1-3	-/+
	400	4-10	-/+

(n.a.) not applicable, (Number) number of inflammatory foci, Size; (-) no infiltrating cells, (-/+) 1-5 cells, (+) 6-20 cells

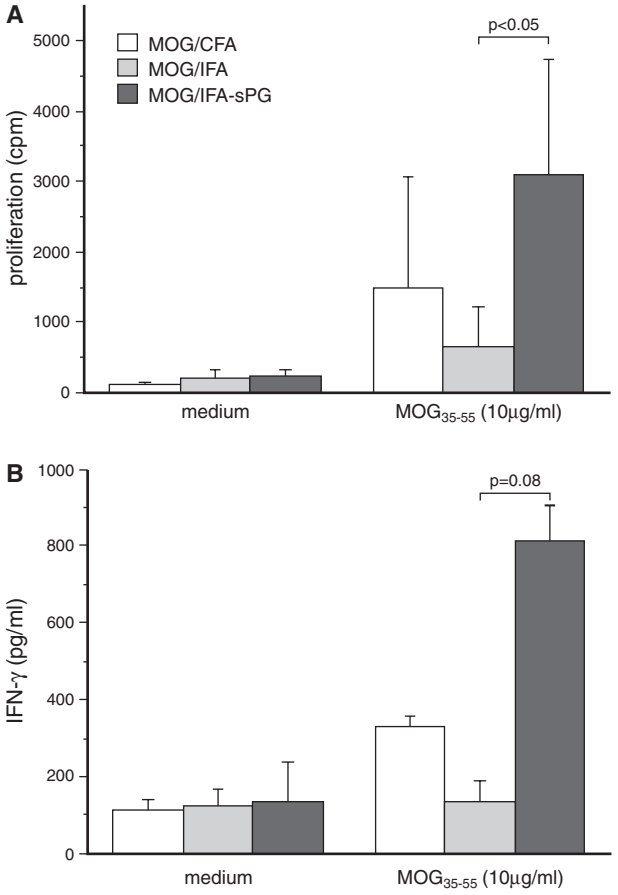

Figure 3. Immunization with PG-containing adjuvant stimulates the development of MOG_{35-55}-specific Th1 cells.
Draining lymph nodes from five individual mice per group were harvested at day 33 after immunization. (A), Cells were stimulated *in vitro* with 10 µg/ml MOG_{35-55}, and proliferation was measured after 96 h of culture by [^{3}H]thymidine incorporation. (B), Supernatants were harvested after 96 h of culture and measured by ELISA for IFN-γcontent. Mean values per group ± SD are shown.

PG is redistributed from the site of immunization to the draining lymph nodes

To determine whether PG is transported from the site of immunization to secondary lymphoid organs during EAE development, we injected PG in combination with IFA and MOG_{35-55} and isolated draining lymph nodes at different time points after immunization. Two mice per group were injected with either 25 µg sPG or 400 µg iPG in IFA/MOG_{35-55} emulsions, dosages of PG optimal for EAE induction. Two

naïve mice were used as negative controls. Axillary, brachial, and inguinal lymph nodes were isolated and frozen at 4 and 96 h after immunization. Per mouse, four lymph nodes were analyzed for the presence of PG-containing cells. In naïve mice individual PG-containing cells were occasionally found in three out of eight lymph nodes (38%), in agreement with previous observations[35,36]. In contrast, we detected several clusters of PG-containing cells in > 90% of the draining lymph nodes from mice immunized with sPG and iPG. The number of PG-containing clusters did not vary between the time points (data not shown). These data indicate that APC can take up and transport PG from the site of immunization to the draining lymph nodes within hours. Double-labeling experiments identified cells in the PG-containing clusters as DC expressing CD11c (Figure 4). PG-containing clusters may thus contribute to the development of a proinflammatory environment to help generate MOG_{35-55}-specific Th1 cells.

Stimulation of DC by PG increases protein uptake and induces DC maturation

At the site of immunization, PG can be recognized by different cell types i.e. DC, MΦ, and granulocytes. Because PG was mainly observed in DC in the lymph

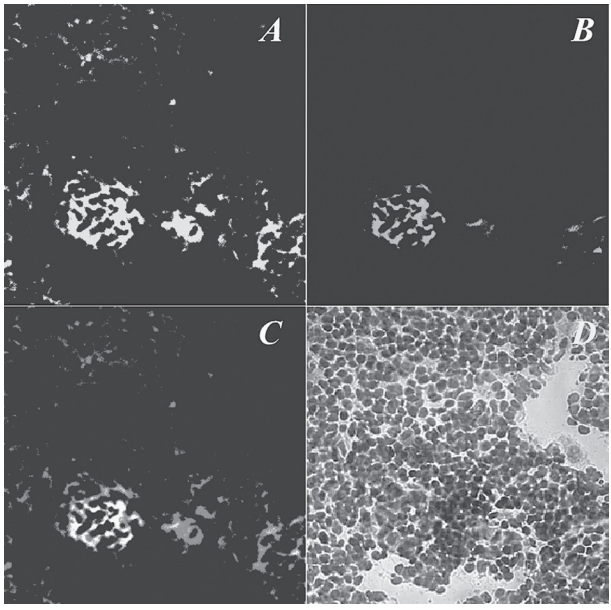

Figure 4. PG is redistributed from the site of immunization to DC in draining lymph nodes.
Draining lymph nodes were isolated at 4 h after s.c. immunization with MOG_{35-55} in IFA-sPG. Frozen sections were stained by immunofluorescence for DC (CD11c in red, A) and for PG (green, B). Overlay in C demonstrates clusters of DC containing PG. D is a lightmicroscopic image of the same area.
(See Appendix page 224 for a full-color representation of this figure.)

nodes, we assessed whether PG stimulates protein uptake, cell maturation, T cell stimulation and polarization by this APC subset crucial for priming of T cells. For these experiments, immature mouse bone marrow-derived DC were used. To examine the effects of PG on protein uptake, DC were incubated with OVA conjugated to FITC in the presence or absence of 10 µg/ml PG at 4˚ or 37˚C. Different concentrations of OVA-FITC (0, 0.01, 0.1 and 1 mg/ml) were applied, and uptake was determined by flow cytometry. Incubation at 4˚C showed a dose-dependent passive uptake of OVA-FITC (data not shown). Addition of PG did not alter the uptake of OVA-FITC at 4˚C (Figure 5A). When cells were cultured at physiological temperature (37˚C), both sPG and iPG stimulated the uptake of OVA-FITC (Figure 5B).

To assess the effects of PG on DC maturation, we stimulated DC with sPG or iPG. As a positive control, DC were stimulated with LPS. Maturation of DC was determined by the expression of MHC-II, CD40, CD80, and CD86 and by the induction of several cytokines. Both sPG and iPG stimulated expression of MHC-II, CD40, CD80 and CD86. Stimulation of DC with sPG efficiently induced expression of MHC-II, CD40, CD80 and CD86 to a similar or higher level as compared to LPS stimulation. These molecules were also induced by iPG, to a similar or lower level as compared with LPS (Figure 6A). Both sPG and iPG induced production of several cytokines, but with a different pattern of production. Whereas sPG induced production of all proinflammatory cytokines measured, iPG induced TNF-α, but not IL-6 and IL-12 (Figure 6B). IL-10 was not produced under these conditions (data not shown). As predicted, addition of the LPS antagonist polymyxin B effectively inhibited LPS-induced maturation marker

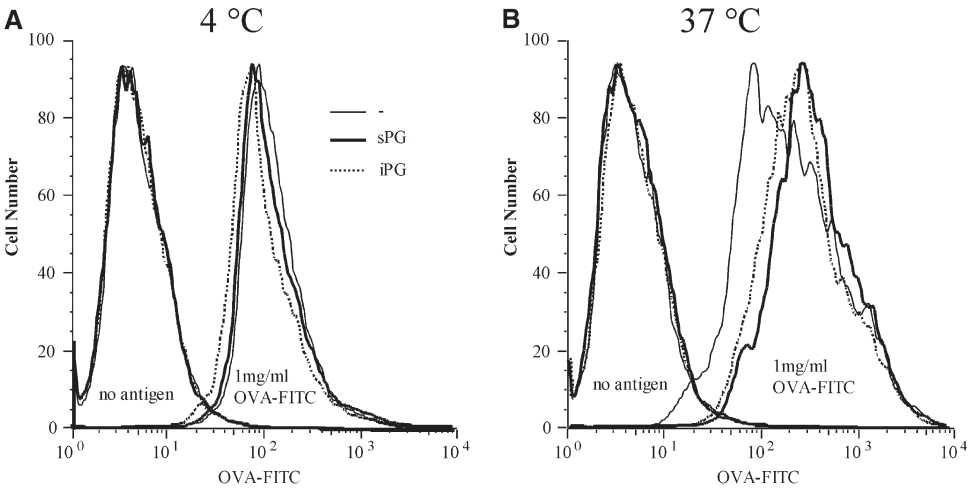

Figure 5. Stimulation of DC by PG increases protein uptake.
Immature DC were cultured in the absence or presence of OVA-FITC. DC were either not stimulated (thin lines) or stimulated with sPG (thick lines) or iPG (dotted lines). Uptake of OVA-FITC was determined at (A) 4°C or (B) 37°C after 30 min of incubation. Histograms represent the number of CD11c-gated cells.

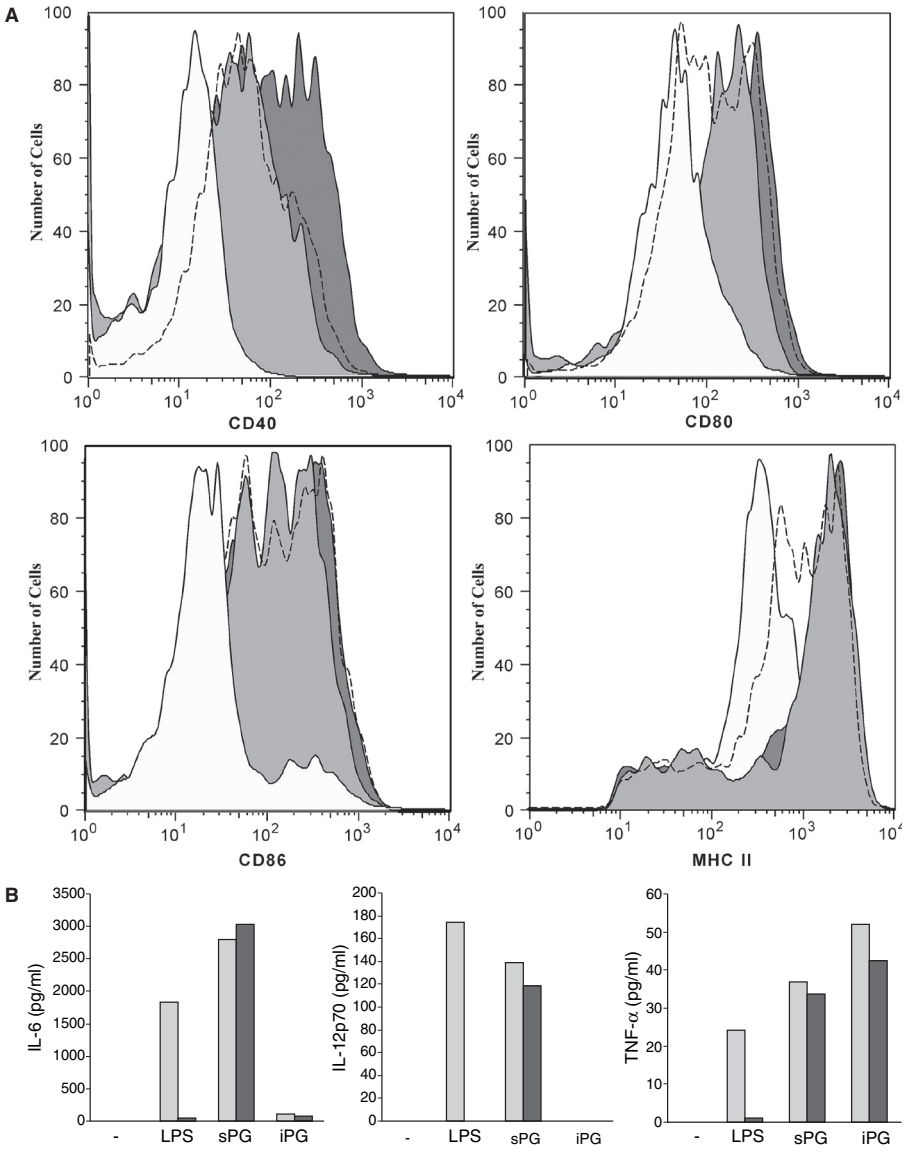

Figure 6. PG induces maturation of bone marrow derived DC.
Immature DC were either not stimulated (white histogram, closed line) or stimulated with LPS (white histograms, dotted line), sPG or iPG (light-gray and dark-gray histograms respectively). A, Maturation of DC was determined by measuring expression of MHC-II, CD40, CD80 and CD86 by flow cytometry. Histograms represent the number of CD11c-gated cells. B, Supernatants were harvested, and production of IL-6, IL-12p70 and TNF-α was determined by ELISA. Dark-gray bars represent cells that were incubated with polymyxin B to exclude LPS contamination.

expression and cytokine production (Figure 6B), whereas no inhibition was seen when cells were stimulated with PG, demonstrating that the effects of PG were not the result of LPS contamination. Taken together, these data demonstrate that both forms of PG can stimulate protein uptake and induce DC maturation *in vitro*.

PG-OVA pulsed DC stimulate T cell proliferation and Th1 cells polarization

After antigen uptake and migration of DC to the draining lymph nodes, mature DC will prime naïve T cells to proliferate and differentiate along a certain pathway, depending on the nature of the stimulus. To address whether sPG and iPG are able to stimulate antigen-specific T cell proliferation and Th1 cell polarization, we used an *in vitro* model system. Bone marrow-derived DC were cultured with OVA in combination with LPS, sPG, or iPG. After 24 h, the DC were incubated with CFSE-labeled naïve $OVA_{323-339}$ T cell receptor transgenic $CD4^+$ T cells. OVA-pulsed DC that were stimulated by LPS, sPG, or iPG, induced an increase in the OVA-specific T cell proliferation as compared to unstimulated cells (Figure 7B-E). Furthermore, both forms of PG induced significantly increased levels of IFN-γ compared with both non stimulated and LPS-stimulated DC (Figure 7F). Only low levels of IL-4 and IL-10 were detected in all OVA-pulsed DC-T cell cultures (data not shown). In summary, these data show that PG stimulates antigen-specific T cell proliferation and Th1 cell development.

Discussion

Bacterial and viral components stimulate innate immune responses which potentially contribute to the development and persistence of autoimmune disease [3,5]. Although it is very difficult to demonstrate mechanistic links between microbial agents and MS, it has been shown epidemiologically that antecedent infections are associated with an increased risk of relapse development[4,5]. Components of bacterial agents may function as physiological adjuvants and stimulate autoantigen-specific adaptive immune responses. In this study, we show that PG, a large bacterial cell wall component, can act as an environmental factor promoting CNS autoimmune disease development via innate immunity.

PG from a vast array of different bacterial species can be derived from different anatomical sites, including all mucosa permanently exposed to the outside world. During bacterial infection, PG can be released, either from bacterial cells upon replication or upon uptake and processing by APC. In the absence of infections, the major bacterial load is located at mucosal sites. At these sites, i.e. the gut, DC may sample bacteria through the intestinal epithelium and subsequently migrate to secondary lymphoid organs[37,38]. Even under normal circumstances, some PG can be detected in the blood circulation, within the liver and within lymphoid tissues, reflecting physiologic processes dealing with exposure to bacterial components[13,39-45].

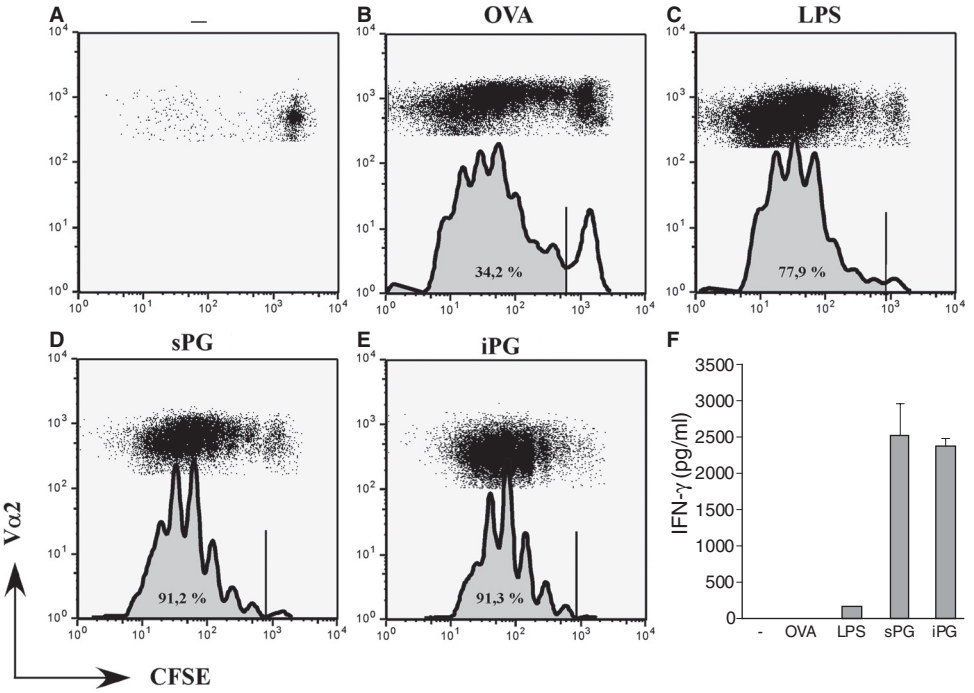

Figure 7. PG stimulates antigen-specific T cell proliferation and Th1 cell polarization.
Immature DC were not stimulated (A) or stimulated with OVA (B) combined with LPS (C), sPG (D) or iPG (E) for 24 h. $OVA_{323-339}$ T cell receptor transgenic $CD4^+$ T cells were incubated with all DC populations. A-E, After 6 days of culture, $OVA_{323-339}$-specific T cell proliferation was assessed by flow cytometry using CFSE. F, Supernatants were harvested, and production of IFN-γ was determined by ELISA. Percentages indicate the number of divided $OVA_{323-339}$-$CD4^+$ T cells. Histograms represent the number of cell divisions.

We therefore hypothesized that bacterial PG can contribute to disease development and progression in MS and EAE in the absence of infection or bacterial replication. To functionally test this hypothesis we determined whether EAE could be induced by immunization of mice with an encephalitogenic peptide (MOG_{35-55}) emulsified in IFA supplemented with *S. aureus* PG, asking whether PG by itself is a sufficient proinflammatory cofactor for induction of EAE.

For our studies, we used two different types of *S. aureus* PG. We observed functional differences between sPG and iPG *in vivo* as well as *in vitro*. Although EAE could be induced with both types of PG, the effective dose for EAE induction was 10-fold lower for sPG compared with iPG. *In vitro* stimulation of DC with iPG only induced TNF-α production, whereas sPG could induce IL-6 and IL-12 in addition to TNF-α. These data implicate that partially degraded PG is more effective in exerting proinflammatory effects than iPG.

High dosages of both sPG and iPG were not able to induce EAE. The reasons for this are not clear but may be related to dose-dependent induction of Th1 versus Th2 responses by administration of TLR ligands *in vivo*[33]. Furthermore, it has been shown that apoptosis of MΦ is induced by PG, as described by Fukui *et al*.[46]. As recently shown, TLR engagement triggers DC apoptosis through up-regulation of the proapoptotic Bcl-2 family member Bim. In this way, both innate and acquired immune system signals cooperate to determine DC lifespan, and hence also the longevity of T-DC interaction[47,48].

L-lysine or diaminopimelic acid (DAP) at position three of the stem peptide are important structural components of PG. DAP-type PG is present in all Gram-negative bacteria, whereas most Gram-positive bacteria contain lysine-type PG[49]. The intracellular PG-sensing molecules Nod1/2 discriminate between lysine-type and DAP-type PG[50]. Nod2 is a general sensor for both Gram-positive and Gram-negative bacteria, through the recognition of muramyl dipeptide, the minimal motif found in all PG[23,24]. In contrast to Nod2, Nod1 specifically recognizes DAP-type PG[21,22]. Bacteria containing L-lysine can induce chronic arthritis, while bacteria with other peptides at this position are nonarthritogenic[51]. Several other studies have also identified the presence of L-lysine as typical for proinflammatory PG[52-54]. Considering the arthritogenic effects of L-lysine at position three of the stem peptide, one might expect similar findings in EAE development. Indeed, we have now shown that EAE can be induced with lysine-type *S. aureus*-derived PG as adjuvant.

Already at 4 hours after immunization, PG-containing clusters could be detected in the draining lymph nodes. These clusters persisted at least for 7 days after immunization. Others have also shown the persistence of PG-containing components until at least 63 days after injection of PG-polysaccharides by muramic acid detection, a specific component of PG, through gas chromatography and mass spectrometry[40]. PG degradation is mainly dependent on the enzymes lysozyme and NAMLAA/PGRP-L[55]. Recently, it was found that the PGRP-L is an amidase[28,29] and is identical to NAMLAA, which we have characterized in detail previously[55,56]. In contrast to granulocytes, MΦ and DC lack NAMLAA/PGRP-L, which may result in incomplete or delayed PG degradation and persistence of intracellular PG. This may explain why DC in MS brain tissue and in EAE lymph node tissues contain PG. The fact that PG is observed both in lymph nodes and in the CNS is important, because PG may act as a physiological proinflammatory adjuvant by stimulating autoantigen-specific immune responses. Indeed, in the current study, we could demonstrate that PG-containing adjuvant enhanced MOG_{35-55}-specific lymph node cell proliferation and IFN-γ production at 4 weeks after immunization.

DC recognize specific motifs on pathogens by expressing a diverse repertoire of pathogen-associated recognition receptors. Upon bacteria-induced stimulation, DC mature, migrate to the circulation, and prime the adaptive arm of the immune response by initiating the development of effector T cells in secondary lymphoid

organs[57]. Because we detected PG within DC in MS brain, rheumatoid arthritis synovium, and EAE lymphoid tissue, we addressed the functional role for PG in EAE development by *in vitro* simulation of the different processes involving DC that occur during immunization for EAE development.

Early events after immunization include uptake of adjuvant and antigen by DC inducing their maturation. These processes can be accelerated by whole *M. tuberculosis*-containing adjuvant as has been demonstrated *in vivo*[7]. Also bacterial components like CpG-DNA are able to increase antigen uptake provided that the antigen is covalently linked to CpG-DNA[58-60]. In concordance with these studies, we now demonstrate that PG increases antigen uptake by DC.

Upon stimulation with TLR ligands or cytokines, DC will mature and migrate to secondary lymphoid tissues to initiate T cell activation. It has been demonstrated that *S. aureus* PG may serve as a maturation signal for murine DC, by inducing the expression of CD86, MHC-II and the production of MIP-2 and proinflammatory cytokines[61,62]. Also, human monocytes secrete several chemokines (IL-8, MIP-1α) and proinflammatory cytokines (TNF-α, IL-1, IL-6 and IL-12) after PG stimulation, as shown by cDNA array analysis[63]. In agreement with these studies, we now show that both forms of *S. aureus*-derived PG induce DC maturation, as demonstrated by enhanced expression of MHC-II, costimulatory molecules, and production of proinflammatory cytokines. These proinflammatory cytokines, induced upon TLR stimulation, can exert direct effects on T cell polarization (i.e. IL-12)[64,65] and T cell regulation (i.e. IL-6)[66] and have been implicated in the pathogenesis of MS as well as EAE[67-69].

Classical EAE induction is dependent on the presence of autoantigen and proinflammatory stimuli from adjuvant components (whole *M. tuberculosis*). A combination of both factors will result in the development of autoantigen-specific $CD4^+$ Th1 cells within the secondary lymphoid organs. Not much is known about the effect of PG on T cell proliferation and differentiation. A recent report proposed that Th cell polarization is dependent on differential TLR ligation[70]. Our *in vitro* studies demonstrate that OVA-pulsed DC did induce T cell proliferation but did not induce Th1 cell development. However, when OVA-pulsed DC were co-incubated with PG, DC were able to induce OVA-specific Th1 cell development, which confirms previous reports[71]. In addition, PG stimulated OVA-DC increased the expansion of OVA-specific T cells, compared with OVA-pulsed DC alone. These data provide evidence that PG stimulates DC-mediated processes and serves as a potent proinflammatory trigger for the development of autoantigen-specific Th1 cells.

In this study, we show that the TLR-Nod2 ligand *S. aureus* PG acts as a proinflammatory factor that is involved in the development of autoimmune disease of the CNS. The contribution of PG on disease development is likely dependent on DC-mediated processes, because PG is located within DC in MS brain tissue and mouse EAE lymph node tissue. We have demonstrated that PG is able to potentiate different DC-mediated processes such as antigen uptake, DC maturation, and

subsequent induction and stimulation of autoantigen-specific Th1 cell development. Targeting PG receptors or their respective signaling pathways may well become a novel therapeutic strategy for the treatment of autoimmune disease in humans.

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CHAPTER

4

Expression of the EGF-TM7 receptor CD97 and its ligand CD55 (DAF) in multiple sclerosis

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Abstract

CD97 is a recently identified seven-span transmembrane (7-TM) protein that is expressed by leukocytes early after activation. CD97 binds to its cellular ligand CD55 (decay accelerating factor), which protects several cell types from complement-mediated damage. The functional consequences of CD97-CD55 binding are largely unknown, but previous data imply that CD97-CD55 interactions play a role in cellular activation, migration and adhesion under inflammatory conditions.

Here we examined the expression of CD97 and CD55 by immunohistochemistry in multiple sclerosis (MS). On the basis of established criteria for inflammation and demyelination, different lesion stages were distinguished in MS *post-mortem* brain tissue. In normal white matter, CD97 expression was not found, but CD55 was expressed with weak staining intensity on endothelial cells. In pre-active lesions, defined by abnormalities of the white matter, many infiltrating T cells, macrophages (MΦ) and microglia expressed CD97. CD55 was highly expressed by endothelial cells. In active lesions with myelin degradation, MΦ and microglia expressed both CD55 and CD97. Furthermore, a sandwich ELISA showed significantly (p<0.05) elevated levels of sCD97 in serum but not in cerebrospinal fluid of MS patients (37%) compared to healthy controls (8%).

Collectively these data suggest that CD97-CD55 interactions are involved in the inflammatory processes in MS. CD55, which is expressed in lesions by vessels to protect against complement-mediated damage, might bind to CD97 on infiltrating leukocytes. This interaction may facilitate cell activation and migration through the blood-brain barrier. In addition CD97-CD55 interactions in the parenchyma of the brain may contribute to the inflammation.

Introduction

One of the hallmarks of MS is the presence of inflammatory demyelinating lesions and axonal loss in the central nervous system (CNS). Lesions contain several blood-derived mononuclear cell types, i.e. infiltrating monocytes, T cells and B cells, and resident microglial cells. The infiltrating cells have passed the blood-brain barrier via mechanisms that are still poorly understood. The cellular infiltration of the CNS is dependent on a complex interaction between adhesion molecules, chemokines, cytokines and proteases. Different effector functions of the inflammatory cells will finally result in impaired nerve conduction[1].

A molecule that may be involved in the inflammatory processes is CD97, a member of the EGF-TM7 family of class B seven-span transmembrane (7-TM) receptors[2-4]. Members of this family, including EMR1, EMR2, EMR3, ETL, and also the mouse microglia marker F4/80, are characterized by an extended extracellular

region with a variable number of epidermal growth factor-like (EGF) domains[5-10]. So far, CD97 is the only EGF-TM7 family member of which a ligand has been identified. Via its EGF domain region, CD97 binds CD55/decay accelerating factor (DAF), a molecule that protects cells from complement-mediated damage by accelerating the decay of C3/C5 convertases[11,12]. Affinity for CD55 differs from CD97 isoforms and, with 86 μM, is highest for the smallest isoform with three EGF domains[13,14]. The consequences of the CD97-CD55 interaction as well as the physiological function of CD97 are still poorly understood.

Receptors of the EGF-TM7 family are expressed by cells of the immune system and by smooth muscle cells. Whereas expression of EMR1, EMR2, EMR3 and F4/80 is largely restricted to myeloid cells[5-7,9,15] and of ETL to smooth muscle cells[8], CD97 is found on a broad array of hematopoietic cells, smooth muscle cells and epithelial tumor cells[2,16,17]. With regard to hematopoetic cells, CD97 is expressed on activated lymphocytes, granulocytes, monocytes and all types of dendritic cells and macrophages (MΦ), except for microglia. Under inflammatory conditions, soluble CD97 (sCD97) is released, whereas sCD97 can be detected neither in normal human serum or plasma, nor in supernatant of activated T cells[4]. The cellular ligand of CD97, CD55, is expressed by several cell types in contact with complement, e.g. leukocytes, erythrocytes, epithelial cells and endothelial cells[12].

Studies on rheumatoid arthritis (RA) suggest a role for the CD97-CD55 interaction in the pathogenesis of autoimmune diseases[4,18]. In the intimal lining of rheumatoid synovial tissue, MΦ express high levels of CD97, while fibroblast-like synoviocytes are strongly CD55-positive. In addition, increased levels of soluble sCD97 were found in synovial fluid of RA patients compared to patients with osteoarthritis or reactive arthritis.

In this study we examined the involvement of CD97 and its ligand in the cellular interactions of MS. To assess whether CD97 and CD55 are involved in MS, expression of CD97 and CD55 was determined by *in situ* analysis in relation to different brain lesion stages, which were distinguished on the basis of established inflammation and demyelination criteria[19,20]. Cell types in MS brain expressing CD97 and CD55 were characterized. In addition, the presence of sCD97 in CSF and in serum of MS patients was determined by ELISA.

Material and methods

Patient samples

Human autopsy brain tissue from MS patients and patients with non-neurological disorders was provided by the Netherlands Brain Bank in Amsterdam (Coordinator Dr. R. Ravid). Cerebrospinal fluid (CSF) samples (n=37), provided by the Department of Neurology, Erasmus MC in Rotterdam, were obtained from MS patients only. Serum samples were obtained from untreated MS patients (n=54;

including the patients from whom CSF was taken), from patients with other inflammatory neurological diseases (OIND), consisting of Guillain Barré syndrome, meningitis, myasthenia gravis and sarcoidosis (n=28), and from patients with other neurological diseases (OND), consisting of stroke, Parkinson disease and epilepsy (n=24). Serum samples (n=38) were also obtained from healthy blood bank donors.

Immunohistochemistry

The immunohistochemical techniques used in this study have previously been described in detail[21-23]. In brief, frozen sections of 6 μm were thaw-mounted on gelatin-coated glass slides and stored overnight in humidified atmosphere. Sections were air-dried for 1 h and fixed at room temperature in fresh acetone containing 0.02% (v/v) H_2O_2. Then the slides were air-dried for 10 min, washed with PBS and incubated with predetermined optimal dilutions of reagents (Table 1) overnight at 4°C in humidified atmosphere.

Incubations with secondary and tertiary reagents were done for 1 h at room temperature. Between each incubation, slides were washed twice with PBS/ 0.05%Tween20. Horseradish peroxidase (HRP) activity was revealed by incubation for 10 min at room temperature with 3-amino-9-ethyl-carbazole, which resulted in a bright red precipitate. Incubation with naphthol-AS-MX phosphate (Sigma, Saint Louis, MO) and fast blue BB base (Sigma) for 30 min at 37°C was used to reveal alkaline phosphatase (AP) activity, resulting in a blue precipitate. For double staining, different combinations of antibodies were used. For CD3 and laminin detection in blue, goat anti-rabbit Ig-AP (DAKO, Glostrup, Denmark) in combination with rabbit anti-goat IgG-AP (Southern Biotechnology Associates Inc., Birmingham, AL) were used as conjugates. For CD55 and CD97 detection in blue, goat anti-mouse Ig-AP (DAKO) in combination with rabbit anti-goat IgG-AP were used. In addition, for CD55 in blue, horse anti-mouse IgG-biotin (Vector Laboratories, Burlingame, CA) in combination with avidin-AP (Sigma) were used. For CD55 and CD97 detection

Table 1. Antibodies and reagents used for immunohistochemistry.

Marker	Cellular specificity	Clone designation
CD3	all T cells	n.a.
CD55	lymphocytes/ activated endothelial cells	CLB-CD97L/1
CD97	activated lymphocytes/ granulocytes/monocytes/ MΦ/dendritic cells	CLB-CD97/1
HLA-DR	MHC class II: MΦ/dendritic cells/ microglia	L-243-biotin
Laminin	basement membrane of blood vessels	n.a.
Acid phosphatase	MΦ/microglia	n.a.
oil red O	myelin neutral lipids in phagocytic cells	n.a.

Abbreviations: (n.a.) not applicable. Suppliers: CLB-CD97/1, CLB-CD97L/1 (CLB, Amsterdam, The Netherlands); CD3 antiserum, L-243-biotin, laminin antiserum (ICN Pharmaceuticals, Aurora, OH).

in red, horse anti-mouse IgG-biotin in combination with ABComplex-HRP (DAKO) were used. HLA-DR-biotin was combined with ABComplex-HRP. Staining for AP activity was performed prior to staining for peroxidase activity. As a control, single stainings were performed in parallel with all the incubation steps of the double staining procedure, but omitting the first antibody incubation. Cells expressing one marker were stained red or blue, and cells expressing both markers were stained violet. All cell-specific reagents used are shown in Table 1. Human reactive tonsil was used as positive internal control tissue. As negative controls, sections were incubated with isotype-matched primary antibodies of irrelevant specificity, or the primary antibody was omitted.

Staging of MS lesions

MS brain lesions were staged on the basis of internationally accepted inflammation and demyelination criteria, described earlier[19,20], using three different markers, i.e. acid phosphatase, HLA-DR and neutral lipids. Acid phosphatase, an enzyme, which is present in lysosomes of cells of the monocytic lineage, is a useful marker to detect infiltrating monocytes, MΦ and microglial cells. Breakdown of myelin, i.e. active demyelination, was demonstrated by the detection of neutral lipids in antigen presenting cells by staining with oil red O. It should be noted that in a single brain section normal white matter and different lesion stages could all be present. Immunoreactivity of CD97 and CD55 was scored on a five-point scale based on positive cell numbers[24].

ELISA for sCD97

A capture ELISA was used, essentially as described previously, to determine sCD97 levels in CSF and serum, by using two monoclonal antibodies (Mabs) recognizing distinct epitopes on CD97. Mab CLB-CD97/1 is directed against the first EGF domain of the molecule, while Mab CLB-CD97/3 is directed against the stalk region of CD97[18]. Briefly, flatbottom microtiter plates (Nunc Maxisorb, Roskilde, Denmark) were coated with Mab CLB-CD97/3 (5 μg/ml in carbonate buffer, pH 9.5) overnight at 4°C. Plates were then blocked for 2 h with PBS, 2% full-fat milk and incubated overnight at 4°C with one in two dilutions of CSF or serum in high performance ELISA buffer (CLB). Subsequently, plates were incubated with biotin-conjugated Mab CLB-CD97/1 in high performance ELISA buffer for 1 h, and with poly-HRP (CLB) in high performance ELISA buffer for 1 h. Plates were washed with PBS, 0.05% Tween-20 between all incubations. The color reaction was measured at OD450, using 3,3',5,5'-tetramethylbenzidine and 0.02% H_2O_2 as the substrate (KPL, Guildford, UK). To calculate sCD97 levels, each ELISA included a serial dilution of a positive control, a fusion protein that has been generated by ligation of the total extracellular part of CD97 immediately upstream to the CH3-CH2-hinge region of human IgG1 (CD97-Fc3). Undiluted, the positive control contained 100 U/ml by definition and a 1:100 dilution gave a half maximal OD450 value. Each ELISA

also included a negative control, a fusion protein containing the EGF domain region of CD97 without the stalk region (CD97-Fc1). The detection limit of the ELISA was 0.01 U/ml. All samples containing more than 0.01 U/ml sCD97 were considered positive. The OD450 readings of the negative control were always smaller than, or equal to, the buffer control.

Statistical analysis
Serum sCD97 levels in the MS, OIND, OND, and healthy control groups were compared with the Kruskal-Wallis and Mann-Whitney test, using Stata 6 software. A p value of < 0.05 was considered statistically significant.

Results

Expression of CD97 and CD55 in human MS brain
To investigate whether CD97 and CD55 are expressed in different lesion stages of MS brain tissue, immunohistochemistry was performed. Fifteen staged brain tissue samples from eight MS patients were tested for expression of CD97, and eight staged brain tissue samples from six MS patients for expression of CD55. In addition, expression of CD97 and CD55 was assessed in normal white matter from two patients with non-neurological diseases and in normal white matter from three MS patients. In normal white matter, no CD97 expression was found. The ligand CD55 was occasionally expressed, with low staining intensity, on endothelial cells, but not on other cell types (Figure 1B). Pre-active lesions are characterized by clusters of activated microglial cells (HLA-DR positive), few perivascular inflammatory cells and no demyelination. In these lesions, few to moderate numbers of CD97-expressing cells were found, depending on the size of the infiltrate (Figure 1C). It was also found that parenchymal cells in pre-active lesions expressed CD97 (Figure 1A). Unlike CD97, CD55 was highly expressed by endothelial cells in pre-active lesions and in all other lesion stages (Figure 1D and Figure 1F; arrows). Occasionally, CD55 was expressed by infiltrating cells and parenchymal cells in pre-active lesions (Figure 1D). Active lesions are characterized by the presence of phagocytic cells, which contain myelin degradation products like neutral lipids and myelin proteins. Many of these phagocytosing CD97-expressing cells were found in active lesions (Figure 1E; red). In these lesions, a moderate number of cells also expressed CD55 (Figure 1F; red). Most of the cells in chronic inactive lesions, which are defined as hypocellular, expressed CD97. In all lesion stages, the number of CD97-positive cells exceeded the number of CD55-positive cells. Table 2 provides an overview of the expression of CD97 and CD55 in all the four distinct lesion stages. These findings indicate that in all lesion stages CD97 and its ligand CD55 are expressed under inflammatory conditions in the CNS of MS patients.

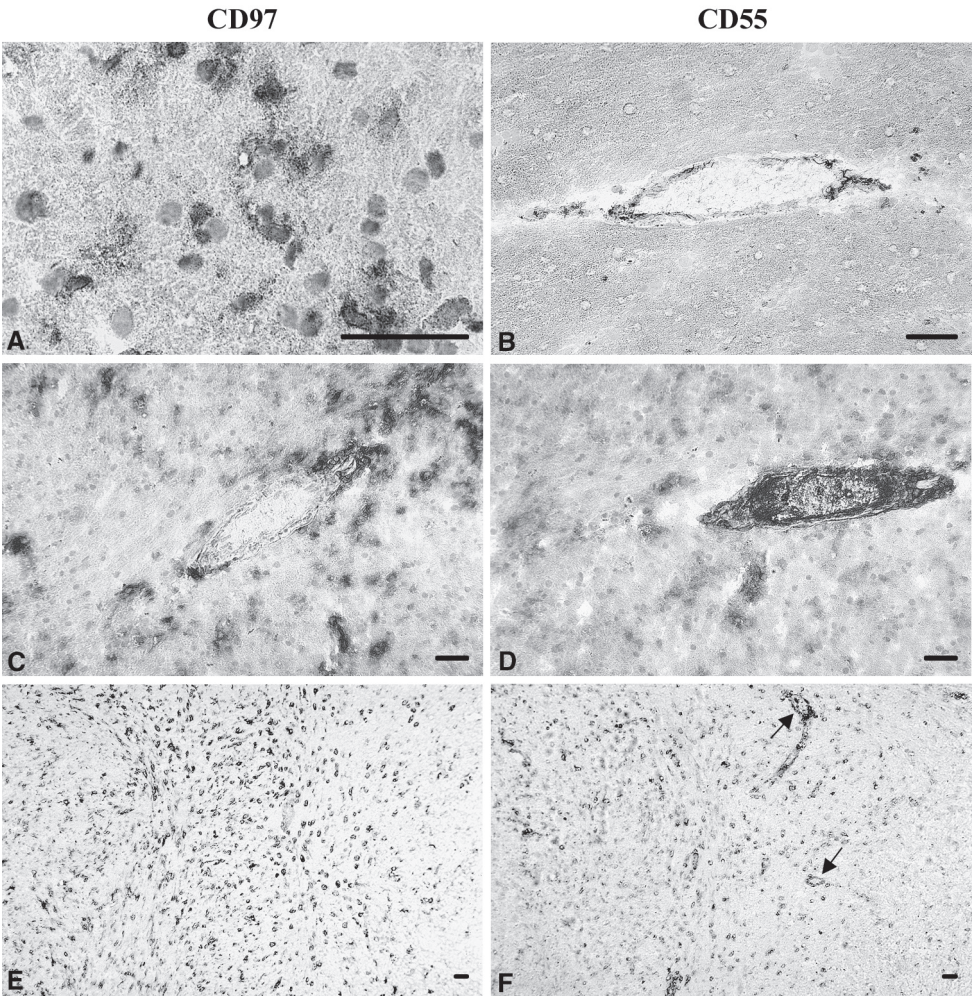

Figure 1. Expression of CD97 and CD55 in human MS brain.
Left column, CD97 expression; and right column, CD55 expression. In pre-active lesions, parenchymal cells as well as infiltrating cells expressed CD97 (A and C, respectively). In normal white matter, CD55 was expressed at low levels by endothelial cells (B), while in pre-active lesions, CD55 was expressed at high levels by endothelial cells and by some infiltrating cells (D). In active lesions, in which phagocytic cells contain myelin debris, numerous cells expressed CD97 (E) and also a moderate number of cells expressed CD55 (F). Bar = 45μm. (See Appendix page 225 for a full-color representation of this figure.)

Identification of cellular subsets which express CD97 and CD55

Double-labeling was applied to identify cell types which express CD97 and CD55. In pre-active lesions, vessels are surrounded by clusters of infiltrating cells. Laminin, a

Table 2. CD97 and CD55 expression in MS brain tissue.

Lesion stage	CD97 (mean score)	CD55 (mean score)
Normal white matter	-	-
pre-active	+/++	±
Active demyelinating	++/+++	+/++
Chronic active		
Hypercellular border	+++	++
Hypocellular centre	±/+	-/±
Chronic inactive	+	-/±

Positive cell number: (-) no positive cells, (+/-) occasional cells, (+) few, (++) moderate number, compare to Figure 1C , (+++) abundant, compare to Figure 1E.

component of the vessel basement membrane, was used as a marker for endothelial cells. In Figure 2A, double-labeling of a pre-active lesion is shown for CD97 in red and laminin in blue. No expression of CD97 by endothelium was found, while perivascular leukocytes did express CD97.

Via double labeling of CD97 and CD3, we identified many infiltrating T cells expressing CD97 (Figure 2C). Furthermore, a moderate number of CD97-expressing infiltrating cells expressed HLA-DR and acid phosphatase, indicating activated MΦ/microglia (Figure 2E). In addition, in close vicinity to these perivascular cells, parenchymal cells expressed CD97 and co-expressed HLA-DR and acid phosphatase, implying that they are activated MΦ/microglia. In contrast to CD97, CD55 was co-expressed with laminin (Figure 2B). Only a few infiltrating T cells and MΦ or microglial cells expressed CD55 in pre-active lesions (Figure 2 D and F). In active lesions, CD97 and CD55 were both expressed by numerous activated MΦ or microglial cells (data not shown).

In summary, these data show that both CD97 and CD55 are expressed in MS lesions by infiltrating T cells, MΦ and resident microglial cells. In addition, CD55 is also expressed by endothelial cells in MS lesions.

sCD97 detection in CSF and serum

To assess whether sCD97 can be used as an activation marker for disease in MS patients, we tested serum and CSF of MS patients for the presence of sCD97. Despite the abundant expression of CD97 in brain lesions of MS patients, sCD97 was not detected in any of the CSF samples (n=37) from MS patients. The possibility that CSF contained a factor that disturbed the ELISA was excluded by testing CSF samples to which recombinant CD97-Fc1 or natural sCD97 from synovial fluid had been added (data not shown). Significantly elevated sCD97 levels ($p<0.05$) were however found in more serum samples of MS patients (37%) than healthy controls (8%) (Figure 3). However, these elevated levels were not MS specific because serum of a number of patients with OIND (25%) or OND (25%) also contained elevated sCD97 levels. Within the MS patients, no specific correlation was found between serum levels of sCD97 and clinical parameters, such as disease course, duration, onset, sex, or age.

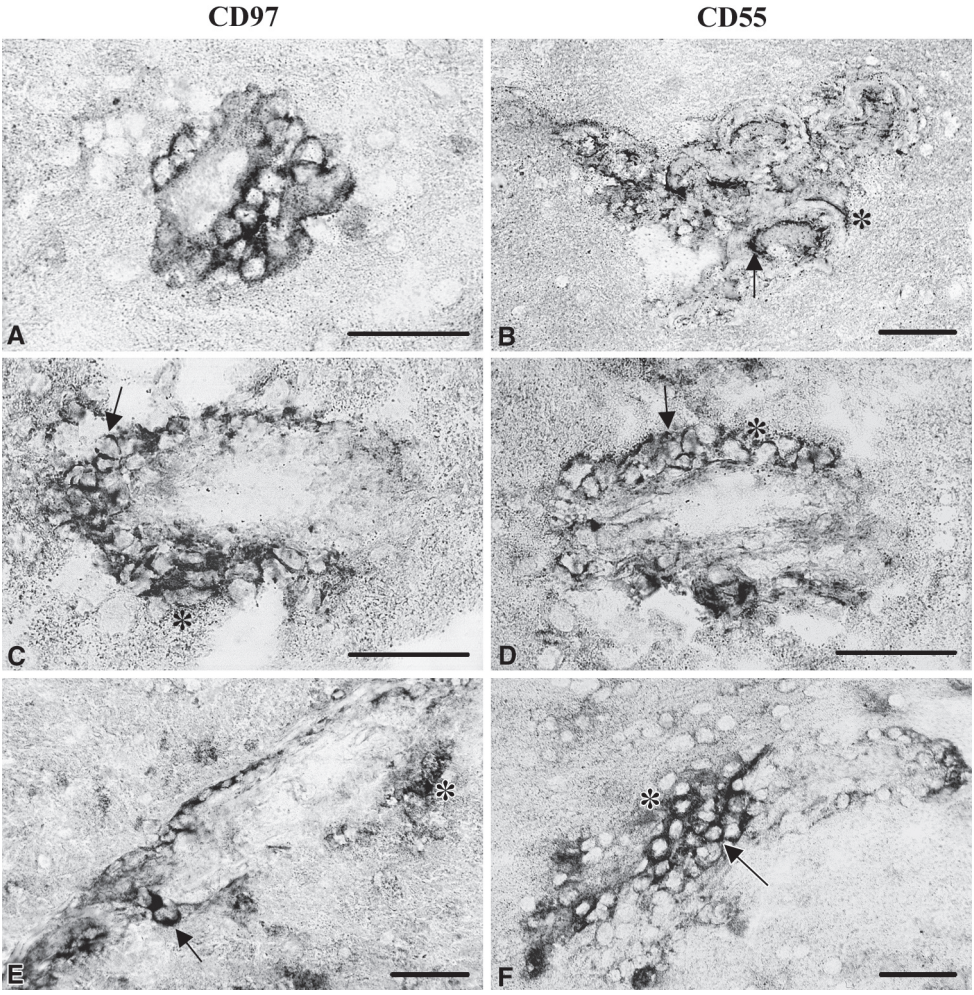

Figure 2. Identification of CD97- and CD55-expressing cell types in pre-active MS brain lesions. Left column, CD97 expression; and right column, CD55 expression. CD97 (red) is not expressed by endothelial cells (laminin in blue)(A). In contrast, double labeling showed co-expression (arrow) for CD55 (red) and laminin (blue; asterisk)(B). Many infiltrating $CD3^+$ T cells (blue) expressed CD97 (arrow)(C), and some expressed CD55 (arrow)(D). A moderate number of HLA-DR positive (red; asterisk) infiltrating MΦ and resident microglial cells co-expressed CD97 (arrow)(E), while only a few co-expressed CD55 (arrow)(F). Bar = 45μm. Asterisks indicate single expression (red or blue). Arrows indicate double expression (purple). (See Appendix page 226 for a full-color representation of this figure.)

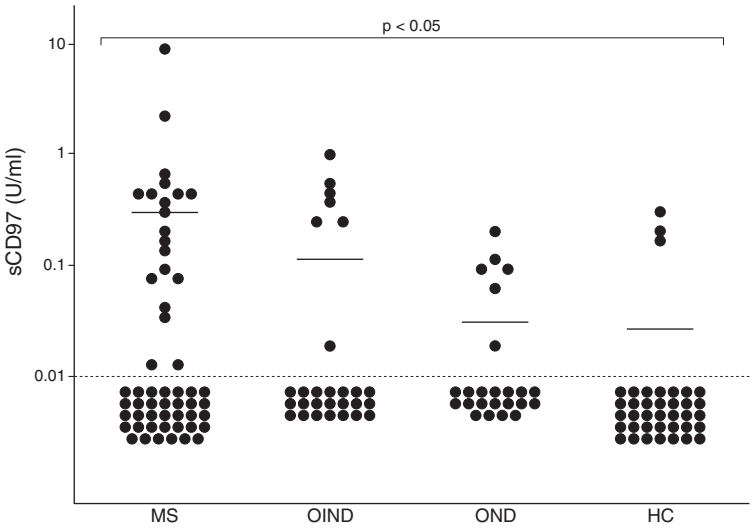

Figure 3. Levels of sCD97 in human serum.
MS: multiple sclerosis; OIND: other inflammatory neurological diseases; OND: other neurological diseases; HC: healthy controls.

Discussion

In this study, we analyzed expression of the EGF-TM7 receptor CD97 and its cellular ligand CD55/DAF in MS brain lesions. In agreement with previous findings we found that CD97 is not expressed by microglia under normal conditions[16]. However, microglial cells in MS in all lesional stages strongly express CD97. In addition, CD97 expression was found on infiltrating MΦ and T cells. In pre-active lesions, CD55 was only occasionally expressed by T cells and MΦ or microglial cells, while in active lesions, many MΦ/microglial cells co-expressed CD55 and CD97. Strikingly, high expression of CD55 was found on endothelial cells in both pre-active and active lesions, whereas in normal white matter only modest expression by endothelial cells was found.

These findings are reminiscent on the situation related to vessels in human tissue outside the brain, where CD55 is expressed on perivascular reticular cells, and CD97 on lymphoid cells and monocytes/MΦ within the vascular lumina and the surrounding tissue[16]. In inflamed RA joints, CD55 is found on fibroblast-like synoviocytes of the intimal lining and on endothelial cells, while CD97 is expressed by MΦ of the intimal lining and sublining[18].

The perivascular location of CD97-expressing T cells and MΦ suggest that these cells recently entered the CNS from the peripheral blood. In parallel, CD55

expression on the vessel walls was increased in all lesion stages. Given the binding properties of the CD97-CD55 complex, this expression pattern may suggest that leukocyte CD97 binding to endothelial CD55 contributes to adhesive and/or migratory events at the blood-brain barrier. The recent data of Boulday *et al*. argue against a role for CD55-CD97 interaction in static adherence of CD97-expressing leukocytes to CD55 transgenic porcine endothelial cells, but in this study, functional assays for migratory events were not performed[25]. It is possible, but yet unproven, that next to classical adhesion pathways via lymphocyte function-associated antigen-1 /intracellular adhesion molecule-1 and very late activation antigen-4 /vascular cell adhesion molecule-1[26], additional pathways such as via CD97-CD55 interaction contribute to activation and migration of the invading cells.

An increase in endothelial CD55 expression probably functions primarily to reduce complement deposition. CD55 expression can be increased by the proinflammatory cytokines TNF-α and IFN-γ, and also by the membrane attack complex[27]. It is tempting to assume that circulating Th helper cells and activated monocytes secreting these cytokines contribute to the enhanced CD55 expression on the brain endothelium. In such a scenario, basically beneficial CD55 expression may also have deleterious side effects because of the capability to facilitate the entry of activated CD97-expressing leukocytes into the CNS. Next to its major role in protecting hematopoietic and endothelial cells from complement-mediated damage[27], CD55 itself is also involved in cellular activation and signal transduction of different cell types[28-32]. Therefore, it is possible that binding of CD97 to CD55 leads to reverse signaling of the CD55-expressing cell. The physiological consequences of this reverse signaling are poorly understood.

sCD97 levels were found by ELISA in serum, but not in CSF of a number of MS patients. It is currently unknown by what mechanism(s) sCD97 is generated. We presume this is a result of proteolytic cleavage of the extracellular part of the membrane-expressed receptor, because we have found no evidence for alternatively spliced mRNA that could generate a soluble variant (unpublished observations). The interaction of CD97 with CD55 may be the triggering event for release of sCD97, in analogy with other molecules[33]. sCD97 has been found *in vivo* at inflammatory sites and in B cell lymphoma, but is absent in normal serum or plasma[4,18]. In this study, we were not able to detect sCD97 in CSF of MS patients. In contrast, we detected significantly elevated sCD97 levels in serum of a number of MS patients compared to healthy controls. Levels of sCD97 were also elevated in serum of a number of patients with OIND and OND, indicating that sCD97 is not a useful marker for MS. Within the MS patients, no specific correlation was found between serum levels of sCD97 and clinical parameters, such as disease course, duration, onset, sex or age.

The discrepancy between high levels of sCD97 at the site of inflammation in RA (synovial fluid) and the absence in the CSF of MS patients is not without precedent. In general, ratios of activation markers in synovial fluid versus blood are above 1.0

under inflammatory circumstances[34]. In contrast, CSF concentrations of activation markers in MS patients most often are lower than in the blood, with CSF-blood ratios ranging from 0.1 to 0.01, sCD27[35], sCD30[36], sCD62L[37], sCR-1[38], sFas[39], sIL-6R and sgp130[40]. Still, levels of such activation molecules are often elevated when compared with CSF concentrations of control groups. It is possible that in MS, CSF sCD97 concentrations are relatively elevated, but we failed to demonstrate this because the concentrations were below the threshold of our ELISA.

In addition to sCD97, soluble CD25[35] and soluble MHC class II[41] are also only found in the serum, but not in the CSF of MS patients. MHC class II is abundantly expressed by MΦ/microglia in MS brain tissue. This indicates that membrane expression and soluble presence of certain molecules, like MHC class II and CD97, are not necessarily correlated. The mechanism(s), sites of release, and redistribution of sCD97 need to be further investigated.

In conclusion, this study introduces CD97-CD55 as a candidate receptor-ligand pair that plays a role in cell migration and costimulation during MS. CD55 expressed on brain endothelial cells may serve a dual role, i.e. protection of vessels from complement-mediated damage and functional ligation of CD97 on leukocytes. Furthermore CD97-CD55 interactions in the brain parenchyma might contribute to the chronic inflammation. The functional role of CD97 will be further elucidated in mouse EAE by treatment with blocking Mab or genetic deletion of CD97.

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CHAPTER

5

Genetic deletion of CD44v7 and CD44v10 reduces the severity of mouse experimental autoimmune encephalomyelitis

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Manuscript in preparation

Abstract

CD44variant isoforms (CD44v) are transmembrane molecules that have been suggested to play an important role in the development of various autoimmune disorders. Ten CD44variant exons (v1-v10) can be alternatively spliced and inserted into the CD44 standard (CD44s) backbone.

Here we determined the functional role of CD44v isoforms in multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE), a mouse model of MS. Both CD44v3 and CD44v10 were expressed on glial cells and on perivascular infiltrating cells in *post-mortem* analyzed brain lesions of MS patients. In the EAE model, brain infiltrating CD44v10$^+$ leukocytes preceded the onset a of EAE and paralleled EAE disease scores. Genetic deletion of CD44v7 or CD44v10 isoforms reduced clinical EAE burden, and the number of inflammatory infiltrates in the spinal cord. Furthermore, adoptive transfer experiments showed that CD44v7 expression on effector T cells and antigen presenting cells both participate in the development of EAE. CD44v7/v10 expression may contribute to the development of MS and EAE by increasing the longevity of auotantigen-specific CD4$^+$ T cells.

In conclusion, we here show that the specific CD44v isoforms CD44v7 and CD44v10 are critically involved in the pathogenesis of EAE and MS. Targeting these CD44v isoforms might therefore reduce inflammatory processes and clinical symptoms in MS.

Introduction

Multiple sclerosis (MS) is a common chronic and disabling autoimmune disease of the CNS and often affects young adults. No efficient and long lasting therapies are available yet. Therefore, there is an urgent need for further elucidation of the mechanisms underlying the pathogenesis of MS. So far, current evidence suggests that MS is mediated by inflammatory attacks against components of the myelin sheath of nerve axons in the CNS.

Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS and greatly advanced our knowledge on the pathogenesis of autoimmunity affecting the CNS. Together with clinical observations, data from animal experiments suggest that an initial CD4$^+$-mediated autoreactive T cell response initiates a cascade of inflammatory events including complement activation, production of reactive oxygen and nitrogen species and digestion of myelin by macrophages (MΦ) that finally result in impaired nerve conduction, demyelination, axonal loss and neurological impairment[1-3]. The formation of mononuclear cell infiltrates in the CNS as well as the activation of T- and B- cell mediated effector mechanisms require intercellular communication between antigen presenting (APC) and effector cells. Thus, various chemokines and cytokines, as well as specific interactions

through accessory/activation molecules on the cell surfaces regulate the initiation and progression of autoimmune responses.

Several adhesion molecule interactions, i.e. VLA-4/α4 integrin and LFA-1/ICAM-1, have been implicated to play a crucial role in the development of MS and EAE[4-6]. CD44 and its variant isoforms (CD44v) are also important adhesion molecules. Ten CD44v isoform exons (v1-v10) can be alternatively spliced and inserted into the CD44s backbone, potentially forming over a thousand different CD44v isoforms. Interestingly, CD44 and its isoforms are involved in various biological processes such as lymphocyte activation, migration, adhesion, extravasation into inflammatory sites and protection against apoptosis[7-11]. Furthermore, MMP-9, involved in the degradation of type IV collagen, can bind specific glycans of CD44 and in addition may activate latent TGF-β[12]. Additionally, osteopontin, a proinflammatory cytokine, can bind to CD44v6/v7 isoforms[13]. Both these ligands have been shown to promote the progression of MS and EAE[14-16]. CD44 plays not only an essential role in EAE[17,18] but also in other autoimmune disease models such as rheumatoid arthritis[19,20], and diabetes[21]. In these models, treatment with an anti-panCD44 Mab, which recognizes all CD44 molecules, completely abrogated disease development.

Physiologically, CD44s is ubiquitously expressed on various cells, while the expression of CD44v isoforms is restricted to activated leukocytes and epithelial cells[22]. Recently CD44 has been shown to associate with VLA-4 on T cells extravasating to inflammatory lesions[9]. Targeting CD44v isoforms, which are specifically upregulated on activated leukocytes, may therefore reduce inflammatory responses and refine therapeutic approaches. In fact, blocking a selected number of CD44v isoforms reduces or prevents autoimmune disease development. In TNBS-induced colitis, an animal model for inflammatory bowel disease, treatment with Mab anti-CD44v7, but not with Mab anti-CD44v10 prevents disease[23]. Accordingly, mice with a targeted deletion for CD44v7 develop only transient and minimal disease[8,24,25]. Corresponding lesions contain high numbers of apoptotic cells, indicating that absence of CD44v7 might protect from autoimmunity by mediating apoptosis of inflammatory cells.

So far, very little is known about the role of different variant isoforms of CD44 in MS and its animal model EAE. Increased expression of CD44 on astrocytes in active lesions in MS brain could be associated with the pathogenesis of the disease[26]. In an adoptive transfer mouse EAE model, it has been shown that CNS infiltrated MBP-specific $CD4^+$ T cells, representing the effector/memory phenotype, specifically expressed CD44 and CD45RB(low)[27]. Previously, we have shown panCD44 and CD44v10 expression on mononuclear cells infiltrating the CNS of mice with EAE, whereas CD44v6 was not expressed.

In order to determine the functional involvement of selected CD44v isoforms in EAE and MS we assessed the expression of CD44v3-v10 in *post-mortem* MS brain tissue. Furthermore, we specifically addressed the role of CD44v7 and CD44v10 in EAE development using mice with single deletions for these specific CD44v isoforms.

Materials and methods

Patients and brain tissue samples

Human *post-mortem* brain tissue was provided by the Netherlands Brain Bank (Coordinator: Dr. R. Ravid, Amsterdam). Information regarding MS patients and non-demented control subjects is shown in Tables 1a and b, respectively.

Histological staging of MS brain lesions

Brain lesions were quantified by immunohistology. To determine the stage of the MS lesions, a generally accepted staging system was used[28]. An additional stage, the so called pre-active lesion had been described in combination with MRI measurements[29]. Despite the fact that it is not proven that pre-active lesions progress into active lesions, they reflect abnormalities in the white matter, that

Table 1a. Clinical and neuropathological data of MS patients.

Case	Sex	Age (yrs)	Disease duration (yrs)	Number of lesions	Lesion stages
93/089	F	71	23	3	pre-active
93/319	F	39	3	1	chronic active
95/148	F	56	6	4	pre-active
96/102*	F	74	24	1	pre-active
96/232[a]	F	40	4	4	3 pre-active and 1 active demyelinating
				3	pre-active, active demyelinating and chronic inactive
96/234	F	81	49	0	
96/352	F	53	13	2	active demyelinating and chronic inactive
97/024#	F	62	25	1	pre-active
97/189	F	82	15	3	pre-active
97/202	M	50	17	2	pre-active* and chronic active

* Not analyzed for CD44v10 expression
\# Not analyzed for CD44v3 expression
[a] Two different tissue samples

Table 1b. Clinical and neuropathological data of non-demented controls.

Case	Sex	Age (yrs)	Neuropathology
94/104	M	79	Many HLA-II-expressing cells in one area of the tissue.
94/110	M	83	No abnormalities.
96/016	M	63	Individual HLA-II-expressing cells throughout the tissue.
96/030	F	68	Some clusters and individual HLA-II-expressing cells throughout the tissue.
96/132	F	90	Small clusters of HLA-II-expressing cells in one area of the tissue.
96/238	F	87	Many HLA-II-expressing cell clusters throughout the tissue.
96/249	F	78	Many HLA-II-expressing cell clusters throughout the tissue.

might preceed the development of active lesions and were therefore included in our study.

Pre-active lesions include the presence of HLA-II-expressing clusters of activated microglia cells, few perivascular inflammatory cells, but no phagocytic cells containing myelin breakdown products or areas of demyelination. Active lesions are represented by the additional presence of demyelinated areas. Phagocytic cells in these areas contain myelin breakdown products, which can be visualized by oil red O, a histochemical staining for neutral lipids. In these lesions, HLA-II is strongly expressed by perivascular and parenchymal MΦ. To evaluate cell numbers in lesions we used a previously published 5-point scale system[6].

Immunohistological staining for CD44v isoforms in human brain tissue

Immunohistological staining was performed as described[30,31]. In addition, the Tyramide Signal Amplification (TSA) Biotin System (Perkin Elmer, Boston, MA) was used, according to the manufacturer's instructions. Antibodies of the isotype IgG2a (Table 2) were applied with 25% normal human serum to prevent binding to Fc-receptors. Horseradish peroxidase conversed 3-amino-9-ethyl-carbazole into a bright red precipitate. Nuclei were counterstained by hematoxylin. Reactive human tonsil sections were included in each staining procedure as positive control tissue.

Generation of CD44v10-deficient mice

The mouse CD44variant region was isolated from a 129SV genomic library. Two 34 bp loxP sites were inserted in direct repeats into a single BstEII site 5' of exon v10 and at the 3' end of the neo^r cassette, which was then inserted into the single BstXI site 3' of exon v10. For electroporation the targeting vector was linearized with PvuI and 20 μg were transfected into 10^7 R1 embryonic stem cells. Embryonic stem

Table 2. Antibodies against human CD44v isoforms.

Marker	Isotype-label	Clone designation	Supplier/ Reference
CD44v3	IgG2b	3G5	R&D Systems, Oxon, UK
CD44v4	IgG2a-biotin	FW11.10	22
CD44v5	IgG1-biotin	VFF-8	Bender Medsystems, Vienna, Austria
CD44v6	IgG2a-biotin	FW11.9	22
CD44v7	IgG1	VFF-9	Bender Medsystems
CD44v7	polyclonal	n.a.	Chemicon International, Hampshire, UK
CD44v7/v8	IgG1-biotin	VFF-17	Bender Medsystems
CD44v9	IgG1	FW11.24	22
CD44v10	IgG1	VVF-14	Bender Medsystems
Isotype control	IgG1	11711.11	R&D Systems
Isotype control	IgG2b	MPC-11	BD Biosciences, San Jose, CA
Isotype control	IgG2a	20102.1	R&D Systems
Isotype control	IgG2a-biotin	20102.1	R&D Systems

(n.a.) not applicable

cells were maintained on a feeder layer of embryonic fibroblasts in the presence of leukemia inhibitory factor. After selection with G418 (300 μg/mL) 391 clones were analyzed by Southern blotting using a 3' external probe (HindII-BamHI). BamHI digests revealed that 2 clones showed homologous recombination. Positive clones were injected into C57BL/6 blastocysts and chimaeric male offspring was mated with C57BL/6 cre deleter females. Offspring was genotyped by PCR using exon v10 flanking oligos and analyzing for deletion of the loxP targeted region.

Mice

Female CD44v7$^{-/-}$ and CD44v10$^{-/-}$ mice were bred in the mouse facility at the Institute of Medical Microbiology, Basel or at the Erasmus MC, Rotterdam and backcrossed for ≥ 10 generations on the appropriate C57BL/6 or SJL/J background depending on the experiment. All mouse experiments were performed in accordance with Swiss and Dutch law and were approved by institutional ethics comittees and the local authorities. Mice were housed under specified pathogen free conditions and received water and food *ad libitum*. Paralyzed mice with EAE scores over 2.5 were afforded easier access to food and water.

Induction and clinical evaluation of EAE

C57BL/6 mice were immunized s.c., at four sites (axillar and inguinal), with 200 μg MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) in 0.1 ml PBS emulsified in an equal volume of CFA containing 100 μg *Mycobacterium tuberculosis* (H37/Ra; Difco Laboratories, Detroit, MI). All mice were injected i.p. with 200 ng of *Bordetella pertussis* toxin in 0.2 ml PBS on days 0 and 2 (Sigma-Aldrich). SJL/J mice were immunized with 50 or 100 μg $PLP_{139-151}$ (HSLGKWLGHPDKF) in 0.1 ml PBS emulsified in 0.11 ml of CFA containing 110 μg *M. tuberculosis*. For adoptive transfer, lymph nodes (SJL/J) or lymph nodes and spleens (C57BL/6) were isolated 10 days after immunization. Crude cell suspensions ($4x10^6$/ml) were cultured at 37°C and 5% CO_2 in the presence of 10 μg/ml MOG_{35-55} (C57BL/6) or 10 μg/ml $PLP_{139-151}$ (SJL/J) for 4 days, washed and then injected into recipient mice.

Mice were weighed and scored for clinical signs of EAE daily according to the following internationally accepted scoring system: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 1.5, limb weakness without tail paralysis; 2, limb weakness and tail paralysis; 2.5, partial limb and tail paralysis; 3, complete hind or front limb paralysis; 3.5, paraplegia; 4, quadriplegia; 5, death due to EAE. Cumulative EAE scores were calculated by adding up all the scores per animal over the indicated time points after EAE induction, representing a total disease load.

Histopathology of mouse CNS

Mice were euthanized and brain or spinal cord tissues were snap frozen in liquid nitrogen. Frozen sections were fixed in acetone and stained with hematoxylin.

Isolation of mononuclear brain cells

Mononuclear cells were isolated from brain tissues as described[32]. Briefly, brains were removed and single cell suspensions were prepared by passage through a wire mesh. After washing, cells were resuspended in 7 ml of 80% Percoll (Pharmacia, Uppsala, Sweden), and then overlaid with 8 ml of 40% Percoll to form a discontinuous gradient in a 15-ml centrifuge tube. The gradient was centrifuged at 400 *g* for 40 min at 21ºC, and the cells at the 40–80% interface were harvested.

Flow cytometry

Immunofluorescence analysis of cells was performed on a FACScan™ (Becton Dickinson, Mountain View, CA). $PLP_{139-151}$-restimulated lymph node cells and mononuclear brain cells were incubated with the specific Mabs for 30 min at 4°C in staining buffer (PBS with 2% FCS and 0.02% sodium azide). The following antibodies were used: CD4 (clone GK1.5, rat IgG2b), CD8 (Ly-2)(clone 53-6.7, Rat IgG2a), CD45R (B220)(clone RA3-6B2, rat IgG2a) and CD11b (Mac-1)(clone M1/70, rat IgG2b), isotype control (clone R35-95, rat IgG2a), all purchased from PharMingen (San Diego, CA) and CD44v10 (clone K926, rat IgG2a)[33]. The goat anti-rat IgG secondary antibody used to detect CD44v10-expressing cells was obtained from Southern Biotechnology, Birmingham, AL. As a negative control for CD44v10 an isotype matched control antibody of irrelevant specificity was used. Dead cells and debris were excluded using propidium iodide.

MOG_{35-55}- and $PLP_{139-151}$-specific T cell proliferation assay

Lymph node cells ($4x10^5$/well) were cultured in 96-well plates in 200 μl of RPMI 1640 supplemented with 10% heat-inactivated FCS (Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Bio Whittaker) in the presence or absence of MOG_{35-55} or $PLP_{139-151}$. After 72 h of culture, 1 μCi of [^{3}H]-thymidine (Amersham Biosciences, Buckinghamshire, England) was added for 16 h. Incorporation of [^{3}H]-thymidine was measured in triplicate using a filtermat harvester and a beta-plate counter (Perkin Elmer, Wellesley, MA). Alternatively, supernatants from MOG_{35-55} proliferation assays were harvested 96 h after culture, centrifuged and stored for further cytokine analysis at -20°C.

Establishment of autoantigen-specific cell lines

After the first *in vitro* restimulation with 10 μg/ml MOG_{35-55} (C57BL/6) or $PLP_{139-151}$ (SJL/J) for 4 days, lymph node cells were washed and diluted 1:1 in complete RPMI. After a resting period of 10 days, cells were restimulated ($1x10^6$ cells/ml) with 1 μg/ml peptide for 4 days. Conditions of each following restimulation cycle were identical to the second restimulation.

Cytokine measurement
IL-4, IL-10, IFN-γ and TNF-α levels were measured using OptEIA ELISA kits (BD Biosciences) according to the manufacturer's instructions. Small volumes of supernatants were tested with the Cytometric Bead Array Th1/Th2 Cytokine kit (BD Biosciences) for the content of IL-2, IL-4, IL-5, IFN-γ and TNF-α by FACS analysis according to the manufacturer's instructions.

Statistical evaluation
Statistical evaluation was performed using SPSS 11 software. The Mann-Whitney U test was used for the evaluation of differences in the onset of EAE, maximum and cumulative EAE severity scores. For analyzing differences in EAE incidences a χ^2 test was used. For analyzing statistical differences in proliferation, the stimulation index was used (cpm of antigen-stimulated cells divided by cpm of non-stimulated cells). Proliferation responses and cytokine levels were compared using ANOVA and the t-test. A p value of < 0.05 was considered statistically significant.

Results

CD44v3 and v10 are expressed in MS brain lesions
To determine whether CD44v isoforms are expressed in MS and non-demented control brain tissues, we assessed the presence of CD44v3, v4, v5, v6, v7, v7/v8, v9 and v10 in *post-mortem* frozen brain sections by immunohistochemistry (Table 2). Frozen sections of human tonsil were used as positive control tissue for the expression of CD44v isoforms.

We did not observe specific staining for CD44v7 and CD44v7/v8 in reactive human tonsil and brain tissue. In accordance with these finding, it has been shown that immuno-staining with both Mab anti-CD44v7 (VFF-9) and anti-CD44v7/v8 (VFF-17), by flow cytometry and immunohistochemistry resulted in false negative results[34]. By using transfected cell lines it was shown that Mab anti-CD44v7 (VFF-9) does not detect unglycosylated CD44v7 molecules. Glycosaminoglycan side chains can modulate the conformation of CD44 molecules[35], and can expose the CD44v7 epitope for Mab binding. Most likely, CD44v7 molecules are expressed in MS and reactive human tonsil, since this epitope is specifically upregulated at sites of chronic inflammation, in patients with Crohn's disease and rheumatoid arthritis[36,37]. Therefore, we have functionally assessed the role of the CD44v7 molecule in the development of mouse EAE, as described below.

In contrast to epithelial cells in human tonsil tissue, we could not detect CD44v4, v5, v6 and v9 expressing cells in MS and non-demented control brain tissue. CD44v3 and v10, on the other hand, were expressed in MS brain tissue samples (n=9 patients) containing different lesion stages (Table 1a and Table 3). CD44v3-expressing cells were sporadically observed in perivascular infiltrates of pre-active

lesions (Table 3). In active lesions, CD44v3 was expressed by many of the foamy MΦ. CD44v10 was expressed on endothelial cells in brain tissue from MS patients and non-demented controls (Figure 1C). CD44v10 was also expressed on cells with a glial morphology in pre-active (Figure 1B, C) and active MS lesions, as well as in areas also containing HLA-II-expressing parenchymal cells from six out of seven non-demented control brain tissues (Table 1b). The number of v10-expressing cells was higher compared to the number of HLA-II-expressing cells (Figures 1 A, B). In 78% of pre-active perivascular infiltrates (n=72 infiltrates), few leukocytes expressed CD44v10 (Figure 1C). In addition, CD44v10 was also expressed by occasional to moderate numbers of foamy MΦ (Table 3 and Figure 1D).

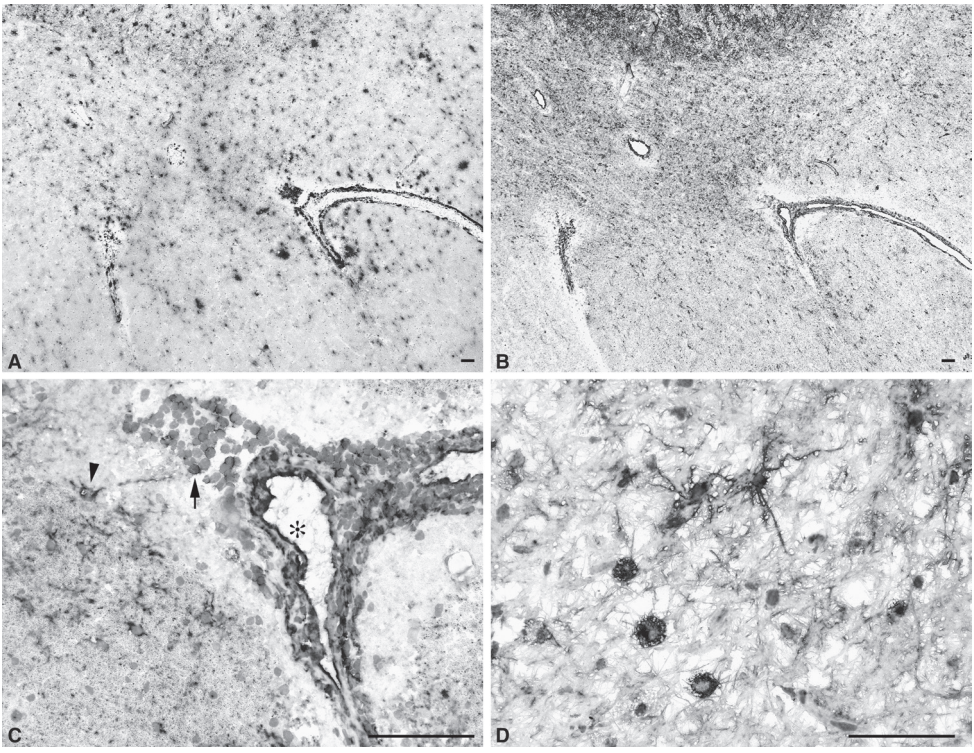

Figure 1. CD44v10 is expressed in MS brain tissue.
Pre-active lesions are characterized by HLA-DP/DQ/DR-expressing activated MΦ/microglia (A), which are located in perivascular infiltrates or in clusters in the brain parenchyma (A). An abundant cell number expressed CD44v10 in the parenchyma within pre-active lesions (B). These parenchymal v10-expressing cells had a fibrillary glial morphology (arrowhead) (C). Besides glial cells also few perivascular cells (arrow) and endothelial cells (asteriks) expressed CD44v10 (C). In active lesions a moderate to an abundant number of foamy MΦ expressed CD44v10 (D). Bar = 100 μm.
(See Appendix page 227 for a full-color representation of this figure.)

Table 3. Expression of CD44v3 and v10 in MS brain lesions.

CD44v isoform		v3	v10[#]
lesion type	area		
pre-active	parenchymal	-	+++[*]
	perivascular	±	+
active demyelinating		++/+++	+
chronic active	hypercellular border	++	++
	hypocellular rim	±	±
chronic inactive		±	±

[*] glial cells
[#] endothelial cell expression

positive cell number:

- none
± occasional
+ few
++ moderate, compare to perivascular CD44v10-expressing cells in Figure 1C
+++ abundant, compare to parenchymal CD44v10-expressing cells in Figure 1B

In conclusion, CD44v isoforms were differentially expressed in MS and non-demented control white matter brain tissue. In normal white matter, glial cells did neither express CD44v3 nor CD44v10. However, in activated (HLA-II-expressing) areas, in both MS and non-demented control brain, fibrillary glial cells expressed CD44v10. In pre-active and active MS lesions, but neither in normal white matter from MS or non-demented controls, perivascular infiltrating leukocytes and foamy MΦ expressed both, CD44v3 and CD44v10.

CD44v10 expression correlates with EAE development

In accordance with our findings in MS lesions, we have previously shown that CD44v10 is also expressed by infiltrating cells in mouse EAE spinal cord tissue. Moreover, it is known that the combined treatment with Mab against the CD44v6, v7 and v10 isoforms reduces EAE severity[38]. To more specifically assess the functional role of CD44v10, we asked whether EAE development in C57BL/6 mice correlates with CD44v10 expression on specific brain cell subsets.

Brain cells were isolated and evaluated for the expression of CD44v10 on different cell subsets by flow cytometry at different time points after immunization. Analysis of mononuclear brain cell suspensions from both, CFA and CFA/MOG_{35-55} immunized mice, revealed CD44v10 expression on $CD4^+$ and $CD8^+$ T cells, $CD11b^+$ MΦ and $B220^+$ B cells (Figure 2A-D). Thus, the presence of inflammatory cells is not dependent on encephalitogenic immune responses but rather correlates with the non-specific inflammatory response. However, the maximum number of all $CD44v10^+$-expressing cells peaks earlier in CFA/MOG_{35-55} immunized mice than in CFA immunized controls (Figure 2A-E). Furthermore, the number of $CD4^+$ CD44v10-expressing cells was 2-fold higher in mice immunized with CFA/MOG_{35-55} compared with CFA

immunized mice at days 11 and 15 after immunization (Figure 2A). In addition, CD44v10-expressing $CD4^+$ T cells persisted in low numbers in CFA/MOG_{35-55} immunized mice, whereas a decrease back to baseline levels at day 54 after immunization was found in CFA immunized mice (Figure 2A).

In relation to the EAE course, elevated numbers of CD44v10-expressing cells were found in CFA/MOG_{35-55} immunized mice as early as 5 days after immunization (Figure 2E). Maximum numbers of CD44v10-expressing cells were detectable at day 11 after immunization, and preceded the peak of disease between days 17-22 after immunization. During the remission phase (days 29 and 54 after immunization) CD44v10 cell numbers also decreased.

Taken together, induction of non-specific inflammation results in the accumulation of CD44v10-expressing T cells, B cells and MΦ in the brain. Development of EAE is associated with a significant increase of infiltrating $CD44v10^+CD4^+$ T cells in CFA/MOG_{35-55} immunized mice. These CD44v10-expressing cells persisted in the brain of EAE mice for up to 54 days after immunization.

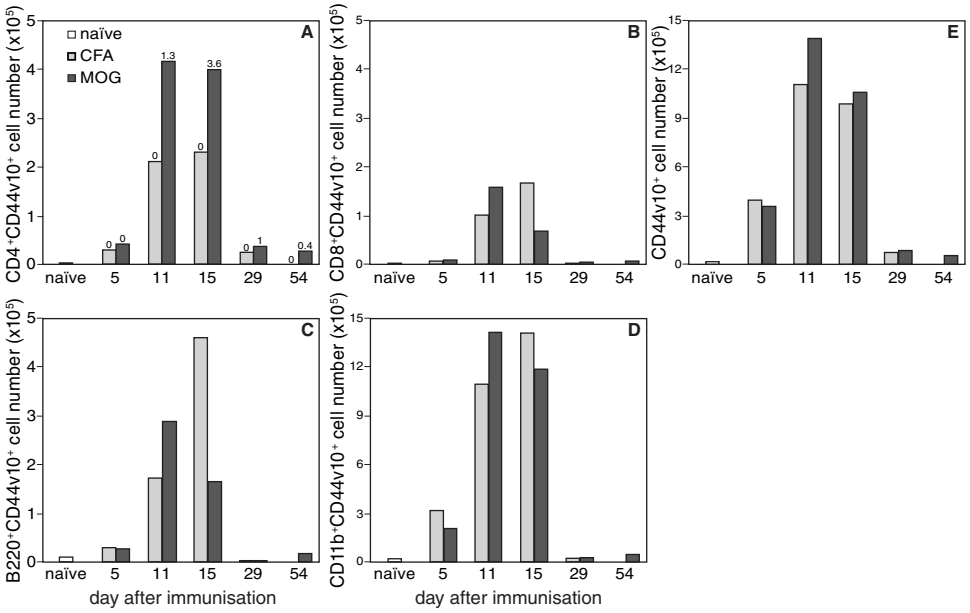

Figure 2. Increase in brain infiltrating $CD44v10^+$ cells upon CFA immunization.
C57BL/6 mice were naïve (open bars) or immunized with CFA alone (gray bars) or MOG_{35-55} with CFA (black bars). Brain cells were isolated at different time points after immunization. Bars represent the absolute number of CD44v10-expressing $CD4^+$ T cells (A), $CD8^+$ T cells (B), $CD11b^+$ MΦ (C) $B220^+$ B cells (D) and total brain mononuclear cells (E). Numbers above the bars indicate mean EAE scores (n=4 mice). Note the higher scales for D and E, reflecting higher number of MΦ.

Autoreactive CD4⁺ T cells express CD44v10

Next, we addressed the capacity of CD44v10⁺ CD4⁺ T cells to mount autoreactive, encephalitogenic responses. First we determined the numbers of CD44v10-expressing CD4⁺ T cells in the draining lymph nodes of C57BL/6 mice after immunization with MOG_{35-55}/CFA and in SJL/J mice immunized with $PLP_{139-151}$ at day 10 after immunization. Whereas CD44v10⁺ CD4⁺ T cells were present in lymph nodes of immunized C57BL/6 mice, they were detectable in SJL/J mice only after *in vitro* restimulation with $PLP_{139-151}$. After two rounds of restimulation with $PLP_{139-151}$, CD4⁺ T cells proliferated dose-dependently (Figure 3A) accompanied by a dose-dependent upregulation of CD44v10 and CD25 (IL-2R, T cell activation marker)(Figure 3B). After three rounds of *in vitro* restimulation CD44v10 was upregulated on 86-97% of the CD4⁺ T cells.

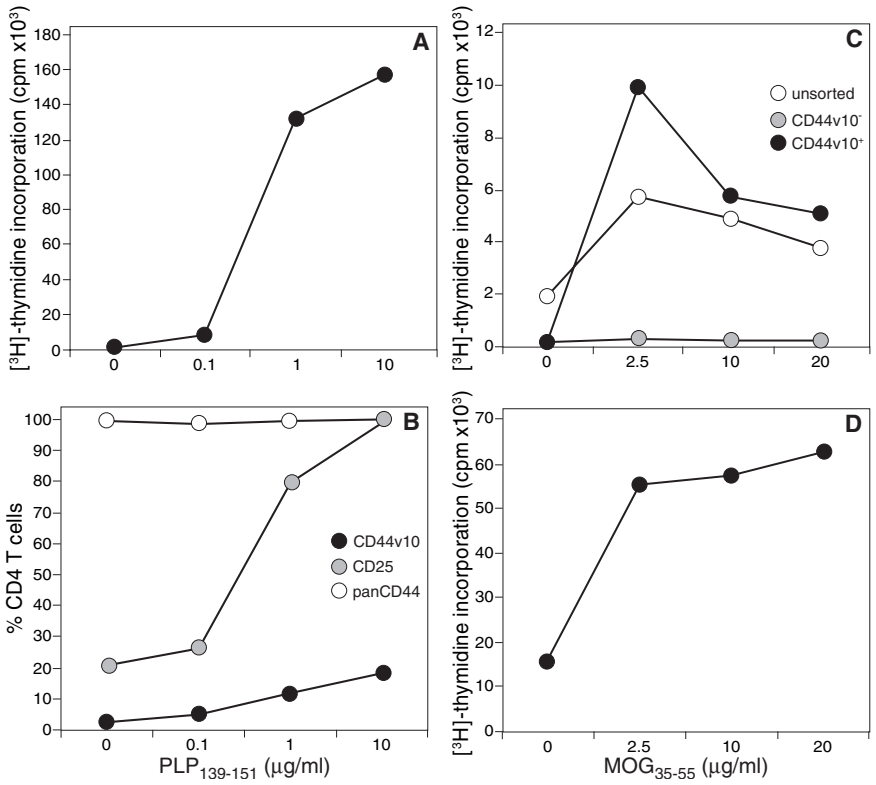

Figure 3. CD44v10⁺ CD4⁺ T cells respond specifically to autoantigen.

CD4⁺ T cells were restimulated and proliferated dose-dependently to $PLP_{139-151}$ (A). Upon restimulation, CD44v10 was dose-dependently upregulated on $PLP_{139-151}$-specific CD4⁺ T cells (B). Ten days after MOG_{35-55}/CFA immunization CD4⁺ T cells were sorted for CD44v10 expression. CD4⁺CD44v10⁺ T cells responded specifically to MOG_{35-55} (C) and survived thirteen rounds of restimulation (D).

To determine whether CD44v10 is important for APC cell proliferation, lymph node cells were isolated 11 days after immunization of C57BL/6 mice with MOG_{35-55}/CFA and $CD4^+$ T cells were sorted for CD44v10 expression. As shown in Figure 3C, $CD4^+CD44v10^+$ T cells expanded *in vitro* on MOG_{35-55}-pulsed irradiated APC. In contrast, $CD4^+CD44v10^-$ T cells did not proliferate. An intrinsic defect of $CD4^+CD44v10^-$ T cells to respond to activation was excluded by the observation that these cells showed strong proliferation upon ConA stimulation (data not shown). In addition, $CD4^+CD44v10^+$ T cells survived thirteen rounds of *in vitro* restimulation with MOG_{35-55} (Figure 3D).

Taken together, these data show that antigen-specific *in vitro* expansion of $CD4^+$ T cells is restricted to the CD44v10-expressing population.

Genetic deletion of CD44v7 and CD44v10 protects from EAE

To test the hypothesis that CD44v7 and v10 are crucial for the development of EAE, we determined how genetic deletion of CD44v7 or CD44v10 affects EAE susceptibility.

As shown in Tables 4a, 5, and Figure 4, disease severity was significantly reduced by deletion of CD44v7 in both SJL/J and C57BL/6 mice compared to wild type mice. Genetic deletion of one CD44v7 allele ($CD44v7^{+/-}$) did not affect EAE (Table 5). Histopathological analysis of SJL/J spinal cord tissues obtained at different time points after immunization revealed a delayed development of infiltrates in CD44v7-deficient mice (Figure 5). As expected, a correlation was found between delayed infiltration and delayed onset of EAE development (Table 5, experiment 1).This delay in onset of EAE between $CD44v7^{-/-}$ and wild type mice was observed in 2 out of 6 experiments, suggesting that deletion of CD44v7 does not per se lead to a delayed influx of inflammatory cells into the spinal cord.

Table 4a. Clinical parameters of actively-induced EAE in C57BL/6 mice.

Genotype	Number of mice	Disease incidence (%)	Disease onset# (day)	Maximum score#	Cumulative score# (day)
wild type	6	100	11.7 ± 0.5	3.1 ± 0.3	26.3 ± 9.3 (29)
$CD44v7^{-/-}$	7	71	10.4 ± 1.9	1.9 ± 1.2	12.5 ± 8.2* (29)
wild type	16[a]	100	11.7 ± 1.4	3.4 ± 0.6	44.0 ± 7.8 (28)
$CD44v7^{-/-}$	13[a]	92	14.7 ± 2.8**	3.1 ± 1.0	31.4 ± 13.7* (28)
wild type	13	77	10.3 ± 1.3	1.7 ± 1.1	23.0 ± 24.4 (55)
$CD44v7^{-/-}$	12	67	18.0 ± 10.3	1.5 ± 1.4	14.5 ± 17.0 (55)
$CD44v10^{-/-}$	14	43	12.5 ± 1.4*	0.4 ± 0.5**	4.6 ± 7.7* (55)
wild type	6	100	10.2 ± 1.3	2.0 ± 0.5	4.8 ± 2.4 (12)
$CD44v7^{-/-}$	6	33*	10.5 ± 0.7	1.5 ± 0.7*	1.1 ± 2.0* (12)
$CD44v10^{-/-}$	6	0.0**		0.0**	0.0** (12)

mean values ± SD

[a] 1 mouse died due to EAE (score 5), and was excluded from calculations after that time point

* $p < 0.05$ compared with wild type mice

** $p < 0.005$ compared with wild type mice

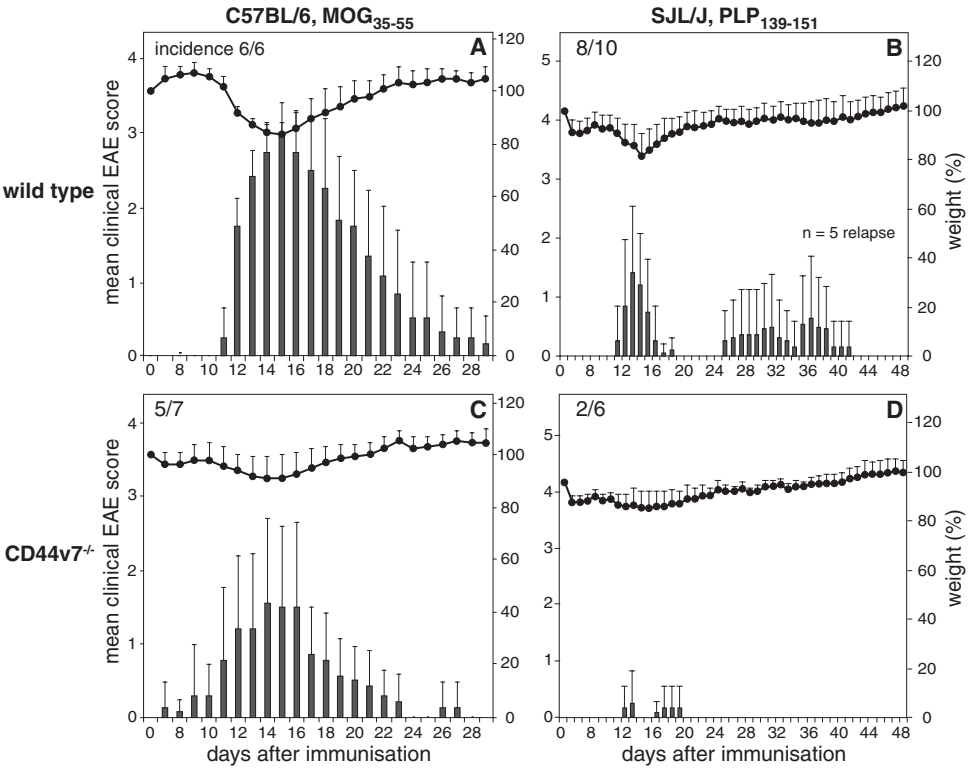

Figure 4. CD44v7 deletion in two different mouse strains results in reduced EAE burden, induced by active immunization.
C57BL/6 were immunized with MOG_{35-55} (A, C) and SJL/J mice were immunized with $PLP_{139-151}$ (B, D). Cumulative scores of wild type mice (A, B) were significantly higher ($p<0.05$) compared to CD44v7$^{-/-}$ mice (C, D). Mice were weighed and scored for clinical signs of EAE daily. Bars represent the mean clinical scores, lines the mean weight.

Fifteen and 20 days after immunization, spinal cord infiltrates were reduced in number and size in six out of eight CD44v7$^{-/-}$ mice compared to wild type mice. These findings correlated with the absence of EAE development or reduced EAE severity in CD44v7$^{-/-}$ mice (Figure 5). In contrast, no differences in the number and size of infiltrates were observed in brain tissue derived from wild type and CD44v7$^{-/-}$ mice 5, 10, 15 and 20 days after immunization (data not shown).

Similar to CD44v7$^{-/-}$ mice, CD44v10$^{-/-}$ C57BL/6 mice also showed significantly reduced cumulative EAE scores compared to wild type controls after MOG_{35-55}/CFA immunization (Table 4a). Histological analysis of brain and spinal cord tissues 12 days after immunization revealed a similar extent of infiltrates in the brain tissue of CD44v10$^{-/-}$ and wild-type mice (Table 4b). However, analysis of spinal cord tissues

Table 4b. Histological analysis of CNS infiltrates in C57BL/6 mice.

Genotype	Cumulative score	Infiltrate number		Infiltrate size	
		brain	sc	brain	sc
wild type	4.5	10	6	++	++
wild type	6.0	7	8	+	++
CD44v7$^{-/-}$	5	4	10	+	++
CD44v7$^{-/-}$	0	2	1	±	±
CD44v10$^{-/-}$	0	7	0	++	-
CD44v10$^{-/-}$	0	7	0	++	-

sc spinal cord

Infiltrate size

-	no infiltrating cells	
±	1-50 cells	small
+	51-500 cells	medium
++	> 500 cells	large

revealed no infiltrates in CD44v10$^{-/-}$ mice compared to wild type controls. Accordingly, CD44v10$^{-/-}$ mice did not develop clinical signs of EAE, 12 days after immunization.

In summary, both CD44v7- and CD44v10-deficient mice develop reduced clinical EAE scores compared to wild type mice. Strikingly, this observation is paralleled by reduced inflammatory infiltrates in the spinal cord, but not in the brain tissue of CD44v7- and CD44v10-deficient mice.

Table 5. Clinical parameters of actively-induced EAE induced in SJL/J mice.

Genotype	Number of mice	Disease incidence (%)	Disease onset# (day)	Maximum score#	Cumulative score# (day)
wild type	10	70	11.1 ± 0.7	1.6 ± 1.5	7.4 ± 5.4 (20)
CD44v7$^{-/-}$	9	33	13.0 ± 1.0*	2.0 ± 1.4	1.8 ± 3.9* (20)
wild type	10	80	12.1 ± 0.8	1.6 ± 1.1	10.7 ± 13.2 (48)
CD44v7$^{+/-}$	5	80	12.5 ± 1.7	1.6 ± 1.2	12.3 ± 17.0 (48)
CD44v7$^{-/-}$	6	33	13.5 ± 1.5	0.4 ± 0.2*	1.0 ± 1.4* (48)
wild type	10	70	11.7 ± 0.8	0.8 ± 1.1	10.2 ± 10.6 (35)
CD44v7$^{+/-}$	10	40	13.0 ± 1.4	0.6 ± 0.8	6.5 ± 12.2 (35)
CD44v7$^{-/-}$	10	20*	12.5 ± 0.7	0.3 ± 0.7	1.1 ± 2.3* (35)

mean values ± SD

* $p < 0.05$ compared to wild type mice

Distinct roles for CD44v7 and CD44v10 isoforms expressing antigen-specific T cells

As previously described, CD44v isoforms are involved in APC-T cell interactions and modulation of T cell proliferation and cytokine production[33,39-41]. In order to address the mechanisms explaining the reduced EAE severity in CD44v7$^{-/-}$ and CD44v10$^{-/-}$ mice, we asked whether autoantigen-specific lymph node cell proliferation

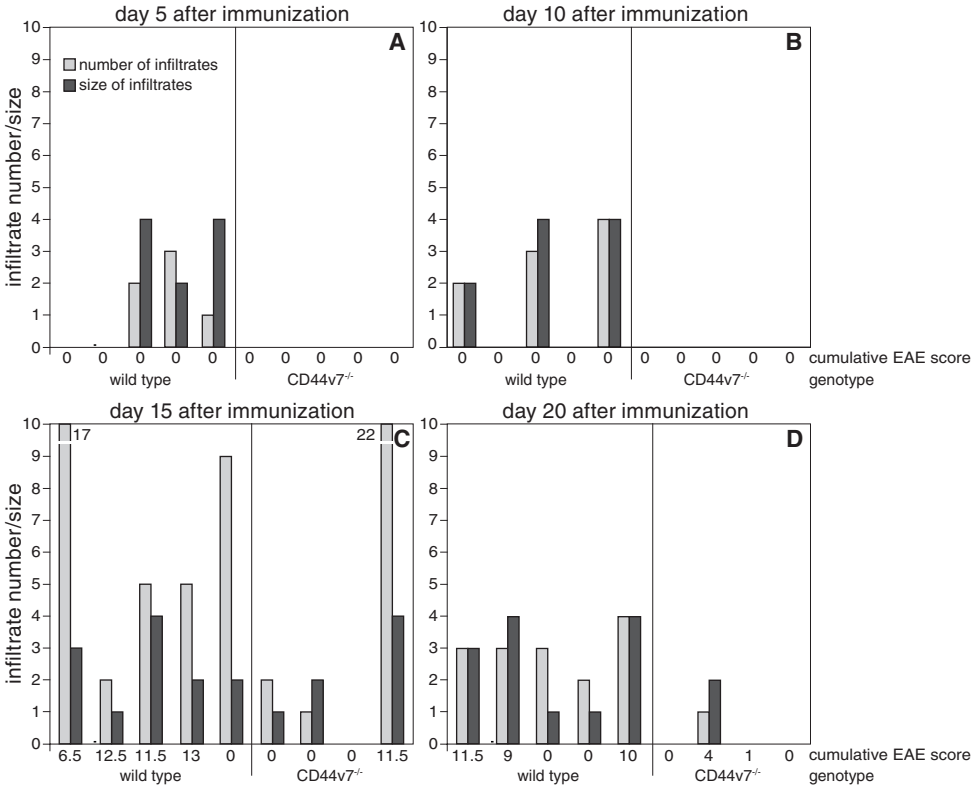

Figure 5. Reduced infiltration in spinal cord tissue from CD44v7$^{-/-}$ SJL/J mice.
Wild type and CD44v7$^{-/-}$ SJL/J mice were immunized with $PLP_{139-151}$ in CFA. The number and size of infiltrates was determined in spinal cord tissues, which were isolated at the indicated time points. Bars indicate the number and size of infiltrates in individual mice.

size of infiltrates

0	no infiltrating cells
1	1-20 cells
2	21-50 cells
3	51-101 cells
4	>101 cells

and cytokine production is altered.

Results from three independent experiments showed that in SJL/J and C57BL/6 mice, CD44v7-deficiency resulted in moderate time-dependent effects on autoantigen-specific T cell proliferation (Figure 6A-C). At day 5 after immunization, lymph node cells from CD44v7-deficient SJL/J mice (n=5 mice) showed a variable proliferative response against $PLP_{139-151}$, which was significantly increased (p<0.05)

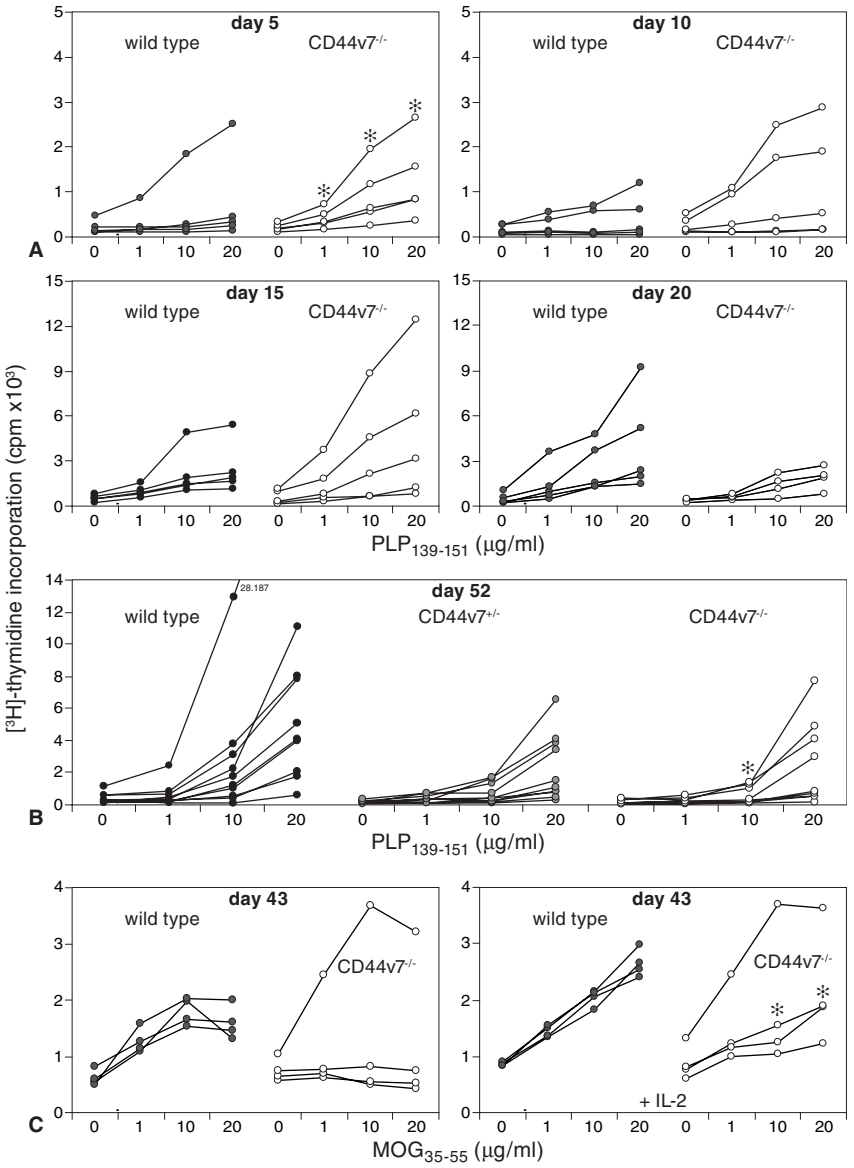

Figure 6. Time-related effects of CD44v7 deletion on autoantigen-specific T cell proliferation. At different time points after immunization with $PLP_{139-151}$ (A, B) or MOG_{35-55} (C) in CFA, draining lymph node cells were isolated and restimulated *in vitro* with the respective antigens for 4 days. Autoantigen-specific T cell proliferation of CD44v7$^{-/-}$ or CD44v7$^{+/-}$ cells was compared to wild type cells. Lines represent cpm values of individual mice. (∗) $p<0.05$

compared with wild type cell proliferation (Figure 6A).

Twelve days after immunization, C57BL/6 CD44v7$^{-/-}$ and CD44v10$^{-/-}$ lymph node cells proliferated equally well as wild type cells upon *in vitro* restimulation with MOG_{35-55} (data not shown). Similar results were found with SJL/J mice at days 10 and 15, respectively (Figure 6A). No overt differences were observed in IFN-γ levels between C57BL/6 CD44v10$^{-/-}$ and wild-type lymph node cells (wild type: 2413 ± 84 pg/ml, CD44v10$^{-/-}$: 2806 ± 78 pg/ml) with 10 μg/ml MOG_{35-55}. Upon restimulation with 10 μg/ml MOG_{35-55}, CD44v10$^{-/-}$ cells produced higher levels of IL-10 (356 ± 2 pg/ml) compared to wild type cells (114 ± 6 pg/ml). In both, groups the levels of TNF-α and IL-4 were below the detection limit of the ELISA. In SJL/J mice IFN-γ, IL-4 and IL-10 levels did not differ between wild type and CD44v7$^{-/-}$ mice (data not shown).

To assess whether differences were present in the T cell or APC compartment, we purified CD4^{+} T cells from wild type and CD44v7$^{-/-}$ mice at day 10 after immunization. CD44v7$^{-/-}$ and wild type CD4^{+} T cells were co-cultured with CD44v7$^{-/-}$ and wild type irradiated spleen cells as APC and restimulated with different concentrations of MOG_{35-55}. In all combinations, T cells proliferated equally well in a dose-dependent response upon restimulation with MOG_{35-55} (data not shown). Furthermore, levels of IFN-γ, TNF-α, IL-2, IL-4 and IL-5 did not differ (data not shown).

In the remission phase of EAE, 20 days after immunization, a modest decrease in T cell proliferation of SJL/J CD44v7$^{-/-}$ lymph node cells compared to wild type cells was observed with 20 μg/ml $PLP_{139-151}$ (Figure 6A). Also at day 52 after immunization, CD44v7$^{-/-}$, but not CD44v7$^{+/-}$, lymph node cells (n=10 mice per group) proliferated significantly less (p<0.05) upon restimulation compared to wild type cells. More pronounced effects of CD44v7 deletion were observed in C57BL/6 MOG_{35-55}-specific T cell proliferation responses at day 43 after immunization. While autoantigen-specific T cell proliferation was observed with cells from all wild type mice (n=4), CD44v7$^{-/-}$ cells did not respond to MOG_{35-55} restimulation, with the exception of 1 animal (Figure 6C). Addition of IL-2 only partially restored proliferation, since a significant decrease (p<0.05) in T cell proliferation compared to wild type cells was still observed in CD44v7$^{-/-}$ mice (n=4).

These data indicate that CD44v7$^{-/-}$ lymph node cells were capable to proliferate in an autoantigen-specific manner, however the capacity to expand upon antigen challenge is reduced during the remission phase of EAE (day 20-52).

CD44v7 deficiency affects both autoreactive T cells and the non-T cell compartment

To determine how CD44v7 affects the capacity of T cells to transfer EAE in recipient mice, C57BL/6 wild type mice were injected with MOG_{35-55}-specific, either wild type or CD44v7-deficient CD4^{+} T cells (Table 6). Compared to wild type cells, MOG_{35-55}-specific CD44v7$^{-/-}$ cells did not induce EAE after transfer in C57BL/6 wild type recipients. CD44v7 deletion in recipient mice also resulted in a significantly reduced severity of EAE compared to wild type mice, upon injection with wild type cells.

Table 6. Clinical parameters of EAE induced by adoptive transfer.

Strain cell number[a]	Genotype cells – mice	Number of mice	Disease incidence (%)	Disease onset[#] (day)	Maximum score[#]	Cumulative score[#] (day)
C57BL/6	wt – wt	5	100	8.2 ± 1.6	2.5 ± 0.3	17.8 ± 5.9 (33)
$4x10^6$	wt – $v7^{-/-}$	5	60	7.0 ± 0.0	1.8 ± 0.5*	6.3 ± 5.7* (33)
	$v7^{-/-}$ – wt	5	0***		0.0**	0.0** (33)
SJL/J	wt – wt	4	100	7.5 ± 0.9	3.1 ± 0.4	42.3 ± 19.0 (37)
$5x10^6$	wt – $v7^{-/-}$	4[b]	100	6.5 ± 0.5	3.9 ± 0.7	65.0 ± 17.9 (37)
	$v7^{-/-}$ – wt	4	100	12.8 ± 3.9*	2.5 ± 0.8	25.3 ± 16.9 (37)
	$v7^{-/-}$ – $v7^{-/-}$	4	100	10.8 ± 2.5	2.5 ± 0.6	17.6 ± 8.5 (37)
SJL/J	wt – wt	6	100	7.2 ± 1.0	2.5 ± 0.6	41.1 ± 3.6 (30)
$15x10^6$	wt – $v7^{-/-}$	6	83	9.8 ± 3.1	1.4 ± 0.9	7.3 ± 4.4*** (30)
	$v7^{-/-}$ – wt	6	83	7.0 ± 0.9	2.5 ± 0.4	33.4 ± 13.1 (30)
	$v7^{-/-}$ – $v7^{-/-}$	6	100	7.8 ± 1.9	2.1 ± 1.1	16.6 ± 8.7*** (30)

wt wild type
$v7^{-/-}$ $CD44v7^{-/-}$
[#] mean values ± SD
[a] number of injected *in vitro* reactivated auotantigen-specific cells
[b] 1 mouse died due to EAE, and was excluded from calculations after that time point
mice – cells donor cells injected into recipient mice
* $p < 0.05$ compared to wt - wt
** $p < 0.01$ compared to wt - wt
*** $p < 0.005$ compared to wt - wt

In SJL/J mice, both wild type and CD44v7-deficient effector cells induced disease in wild type mice (Table 6). Two individual adoptive transfer experiments in SJL/J mice were performed in separate laboratories (Table 6, Figure 7). Injection of 5 or 15 million wild type cells into wild type recipients showed similar cumulative disease scores, however in contrast to our expectation, the maximum EAE score was higher in the experiment where 5 million cells were injected. In both SJL/J experiments wild type and CD44v7-deficient effector cells could induce disease efficiently in wild type mice (Table 6). Disease onset was delayed upon injection of 5 million, but not 15 million $CD44v7^{-/-}$ cells compared to wild type cells. A significant reduction in cumulative EAE scores occurred upon injection of 15 million cells into $CD44v7^{-/-}$ recipient mice, but not in the experiment where 5 million donor cells were injected (Figure 7). However, using 5 million cells, a pronounced reduction in total EAE burden was also found when both donor and recipient cells were deficient for CD44v7 (Figure 7A).

Taken together, these data show in two different mouse strains that CD44v7 deficiency affects both the autoreactivity of $CD4^+$ T cells and susceptibility of the recipient.

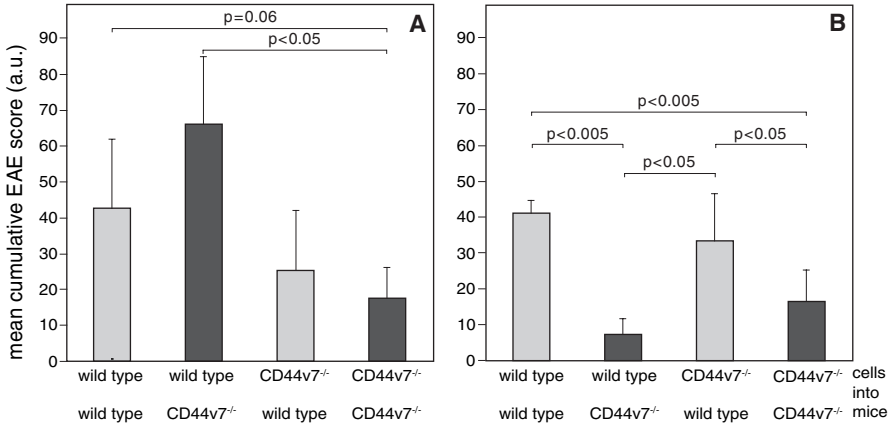

Figure 7. CD44v7-deficiency reduces EAE severity, induced by adoptive transfer.
CD44v7$^{-/-}$ or wild type donor mice were immunized with $PLP_{139-151}$ in CFA. Draining lymph node cells were isolated at day 10 after immunization and restimulated for 4 days with 10 μg/ml $PLP_{139-151}$. Wild type or CD44v7$^{-/-}$ recipient mice were injected with 5 or 15x10^6 wild type or CD44v7-deficient $PLP_{139-151}$ reactivated lymph node cells (A and B, respectively). Mice were weighed and scored for clinical signs of EAE daily. Bars represent mean cumulative EAE scores. The cumulative EAE score is a measure for the severity of the disease. This was calculated by adding up all the scores per animal over the indicated time points after EAE induction, representing the total disease load.

Discussion

This study shows that CD44v3 and CD44v10 are expressed in inflammatory brain lesions in MS patients. In agreement with previous findings where combined Mab treatment against CD44v6, v7 and v10 reduced EAE disease burden[38], we here moreover show that absence of CD44v7 or CD44v10 by genetic deletion reduced the severity of EAE, which was associated with reduced infiltration of the spinal cord.

A role for CD44v3 and v10 in MS

It has been shown previously that panCD44 is expressed constitutively by normal white matter astrocytes under normal conditions and expression is upregulated in MS brain lesions[26,42-45]. In contrast, CD44v isoforms are not expressed by glial cells under normal conditions[45]. However, upon *in vitro* stimulation, mouse astrocytes do express CD44v6/v7- and CD44v10-containing isoforms[46]. In agreement with these findings we describe here that glial expression of CD44v10 isoforms is restricted to activated areas (HLA-II-positive) of the brain, in non-demented controls and in MS patients. CD44v3-v6 and CD44v9 were not expressed in these activated regions. We could not assess the expression of CD44v7 and CD44v7/v8 isoforms, which may partly be explained by the incapability of the Mabs to stain unglycosylated

CD44v isoforms, as previously described[34].

Furthermore, CD44v10 was expressed by endothelium and perivascular infiltrating cells in MS brain tissue and may well participate in the migration and adhesion of cells, as such functions of CD44v10 have been described previously[47,48]. Additionally, CD44v3 is involved in extravasation of leukocytes as described in mouse delayed-type hypersensitivity[49], and may as well participate in leukocyte extravasation in MS. Under inflammatory conditions, such as TNF-α stimulation, human monocytes differentiate into dendritic cells and express CD44v3[50]. This finding may explain why CD44v3 is expressed only occasionally by perivascular infiltrating cells (including monocytes) in pre-active lesions, but by many foamy MΦ in active MS brain lesions. CD44 can be post-translationally modified by the attachment of sulfate groups[51,52]. Sulfated proteoglycans are binding sites for growth factors, chemoattractants and cytokines[53,54]. CD44v3- and CD44v10-expressing cells in MS brain can potentially carry heparin and chondroitin sulfate side chains and as such may function as a collector of inflammatory mediators. Additionally, CD44v3-expression may stimulate the production of proinflammatory cytokines, since blocking CD44v3 in Th cell and APC interactions by Mab treatment reduced the production of IL-2, IL-12 and IFN-γ[49].

How might CD44v10 deletion impair the development of EAE?

A minority of mouse lymph node cells expresses CD44v10 under normal conditions[55]. However, upon *in vitro* or *in vivo* stimulation CD44v10 expression is upregulated by T cells, B cells and MΦ[49,55]. In agreement with these findings, we here show that CD44v10 expression is specifically upregulated on *in vitro* restimulated encephalitogenic $CD4^+$ T cells. CD44v10 is also expressed by brain infiltrating leukocytes and CD44v10 expression parallels the development of EAE. Extravasation of CD44v10-expressing leukocytes to brain tissue is autoantigen independent, since these cells were also detected in brain tissue of CFA immunized control mice. This observation is not surprising, because it is well known that activated or resting memory T cells with irrelevant antigen specificity can migrate to the CNS[27,56-58]. MOG_{35-55}-specific $CD4^+CD44v10^+$ sorted T cells proliferate upon MOG_{35-55} restimulation, while $CD4^+CD44v10^-$ cells do not respond. As described previously, treatment with Mab anti-CD44v10 did not affect the proliferation of ConA stimulated spleen cells[55]. According with these findings, we demonstrate here that $CD44v10^{-/-}$ and wild type lymph node cells proliferate equally well upon restimulation with MOG_{35-55}. Therefore it can be excluded that the reduced EAE burden in $CD44v10^{-/-}$ mice is the result of a lack of proliferation.

Whereas proliferation was similar comparing wild type and $CD44v10^{-/-}$ cells, $CD44v10^{-/-}$ lymph node cells produced IFN-γ and elevated levels of IL-10 upon *in vitro* restimulation with MOG_{35-55}. It has been described very recently that antigen-specific regulatory T cells producing IL-10 and IFN-γ develop during Th1 polarized responses[59]. The fact that MOG_{35-55}-restimulated $CD44v10^{-/-}$ lymph node cells

produced elevated levels of IL-10 compared with wild type cells may indicate that regulatory T cells are elevated or induced in CD44v10$^{-/-}$ mice and may explain the reduced EAE burden. Indeed, it was recently demonstrated that upon stimulation more CD4^{+}CD25^{+}CTLA-4^{+} T regulatory cells are present in CD44v7$^{-/-}$ mice[60]. Further evaluation of MOG_{35-55}-restimulated CD44v10$^{-/-}$ cells may demonstrate expression of the transcription factor Foxp3, which is known to be specifically expressed by regulatory T cells[61].

How might CD44v7 deletion impair the development of EAE?

Here we show in two mouse strains that the severity of EAE, induced by active immunization or adoptive transfer, was significantly reduced in CD44v7-deficient mice compared to wild type controls. Similar, but more pronounced effects of CD44v7-deficiency were observed in adoptive transfer EAE and T cell proliferation of C57BL/6 mice, compared with SJL/J mice. CD44v7 deficiency reduced EAE burden by modulating APC and/or T cell effector functions, since CD44v7 deficiency affected both compartments in adoptive transfer EAE. Proliferation of autoantigen-specific cells was gradually regulated by CD44v7, since modest differences were observed in proliferation of CD44v7$^{-/-}$ cells before the onset and in the remission phase of EAE, compared to wild type cells. IL-2 could partially restore T cell proliferation defects of CD44v7$^{-/-}$ C57BL/6 cells, which is in agreement with the fact that CD44 can enhance T cell proliferation and IL-2 production, as described[62,63]. In conclusion, these findings may imply that early after sensitization CD44v7-deficient cells proliferate faster, but eventually also exhaust faster, i.e. by a higher susceptibility to apoptosis, than wild type cells.

Interestingly, reduced EAE burden in CD44v7$^{-/-}$ and CD44v10$^{-/-}$ mice is associated with a reduced number and size of infiltrates in spinal cord, but not in brain tissue. This finding may imply an impaired migration into the spinal cord or an increase in apoptosis of infiltrated cells. Accordingly, it was previously suggested that CD44v6 and CD44v10 may participate in the development of HAM/TSP, a chronic inflammatory disease of the spinal cord[64]. Alternatively, CD44v7/v10-deleted cells may infiltrate the spinal cord, but are more susceptible to apoptosis. Constitutive CD44v7-expression prevents T cells from going into apoptosis, as was shown by *in vitro* anti-CD3 stimulation of *in vivo* pre-activated mesenteric lymph node cells. CD44v7 deletion resulted in opposite results[60]. Also *in vivo*, reduced disease development in TNBS-induced colitis in CD44v7$^{-/-}$ mice was associated with high numbers of apoptotic cells in inflammatory lesions of the gut[8,24,25]. Taken together, absence of CD44v7 might protect from EAE by an impaired adhesion to spinal cord tissue. Additionally, induction of apoptosis of inflammatory cells or induction of regulatory T cells could also reduce inflammation in CD44v7$^{-/-}$ mice.

Concluding remarks

Here we show that CD44v3 and CD44v10 were expressed in inflammatory lesions in the CNS of MS patients. In the development of mouse EAE, the number of CD44v10-expressing brain infiltrating cells correlated with disease activity. Furthermore, we provide functional evidence that CD44v7 and CD44v10 deficiency reduced the clinical severity of mouse EAE. We have demonstrated a reduced number of inflammatory cells in the spinal cord tissue of CD44v7$^{-/-}$ and CD44v10$^{-/-}$ mice compared to wild type mice. CD44v10$^{-/-}$ mice produced elevated levels of IL-10 upon *in vitro* restimulation of lymph node cells with MOG_{35-55}. In addition, a reduced proliferation of CD44v7$^{-/-}$ lymph node cells was found in the remission phase of EAE, upon *in vitro* autoantigen restimulation. The contribution of CD44v7 and CD44v10 in the development of EAE is likely dependent on cell migration, adhesion, apoptosis and T cell regulation, functions already attributed to these variant isoforms of CD44. These data indicate that modulating CD44v isoform expression and function might be used as a directed therapeutical approach for MS.

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CHAPTER

6

General discussion

Discussion

Autoantigen-specific T and B cells are a part of the physiologic immune repertoire in humans[1,2], marmoset and rhesus monkeys[3,4], but in general do not cause autoimmune disease. Even abnormally high frequencies of autoantigen-specific T cells do not induce disease, as in naïve SJL/J mice in which $PLP_{139-151}$-specific T cells are not deleted during negative selection in the thymus[5,6]. So, an additional trigger is necessary to activate autoantigen-specific cellular effector functions. Several studies have demonstrated that the conditions in which autoantigen reactive cells are activated are crucial for the following events. In MS, autoreactive T and B cells might be activated in the CNS or in the periphery, as at both locations autoantigens can be presented by APC. In part I of this chapter it is discussed how PG and other TLR ligands may contribute to pathogenic immune responses in the CNS and in the periphery during MS and EAE.

The interactions between cell-cell and cell-ECM components are of crucial importance in the immunopathogenesis of MS and EAE. CD97 and CD44v isoforms have several cellular and ECM-related ligands and direct a wide variety of biological processes, e.g. leukocyte activation, costimulation, adhesion, transmigration and apoptosis. Both molecules participate in the immunopathogenesis of other chronic inflammatory diseases, as colitis and RA. We have examined the functional relevance of CD97 and CD44v isoforms in the immunopathogenesis of MS and EAE, which is discussed in part II.

Part I, Does PG contribute to autoantigen-specific attacks against the CNS?

PG-containing cell subsets in MS and in EAE-affected non-human primate brain tissue

The first indication that PG might be involved in the immunopathogenesis of MS arose from the fact that significantly higher numbers of PG containing cells were found in *post-mortem* brain tissue of MS patients than in control brain tissue[7]. PG was mostly present within MΦ, but also in DC. In chapter 2 we demonstrated that PG is also present within granulocytes in MS brain tissue. The cells that contained PG were localized in the edge of active lesions, around blood vessels, and in the normal appearing white matter. PG-containing cells in MS brain expressed antigen presenting (HLA-DR) and costimulatory molecules (CD40, CD80, CD86). Furthermore, PG-containing cells expressed proinflammatory (TNF-α, IL-1β, IL-6, IL-12, IFN-γ), anti-inflammatory (IL-4) and regulatory (IL-10) cytokines. These data indicate that PG-containing APC in MS brain are in a fully activated state.

In chapter 2 it is shown that numbers of PG-containing cells are also significantly elevated in the brain of marmoset and rhesus monkeys that developed

EAE, compared to non-EAE controls. The cells that contained PG in EAE-affected monkey brain tissue were mostly neutrophils, some DC and although not determined here, most likely also MΦ and microglia. Granulocytes are one of the first cells that are present at inflammatory sites. They have a limited life span of approximately 4-22 h. By producing chemokines and cytokines, granulocytes are important in the attraction and activation of other leukocytes. PG might induce the production of inflammatory mediators by granulocytes. In agreement with this, *in vitro* studies demonstrated that PG induced production of MIP-2 and TNF-α by mouse granulocytes[8].

Most of the cells that contained PG in EAE-affected monkey brain tissue expressed CD11b (84-100%). CD11b expression is mainly restricted to myeloid cells, including monocytes, DC, MΦ, microglia and granulocytes. CD11b (also known as αM integrin) complexes with CD18 (β2 integrin) to form the Mac-1 heterodimer (also known as CR3) and binds to ICAM-1, C3b, fibrinogen and coagulation factor X. Mac-1 is involved in adherence of monocytes and granulocytes to stimulated endothelium and also in the phagocytosis of complement coated particles. EAE studies demonstrated that CD11b is functionally involved in the pathogenesis of EAE, since administration of anti-CD11b Mab delayed the onset and reduced the disease load of EAE when treatment was initiated at the first appearance of clinical signs[9].

Recent *in vitro* data demonstrated that *S. aureus* PG induced expression of Mac-1 on human monocytes (Oude Nijhuis *et al.*, 2005, *submitted*). Under shear stress, PG-induced Mac-1 expression enhanced the adhesion of monocytes to L-cells and ICAM-1 coated beads that could be inhibited by β2-integrin blocking antibodies. Furthermore, PG-stimulated monocytes migrated better towards the chemoattractant C5a. Other cell types are likely to respond to TLR triggering in a similar way. In accordance, the TLR4 agonist LPS induced Mac-1 expression on murine granulocytes, whereas expression was absent in TLR4$^{-/-}$ mice[10]. These granulocytes also adhered and transmigrated across endothelial cells in a CD11b-dependent manner upon stimulation by LPS. Thus, PG and LPS induce CD11b expression (Figure 1), which is essential for the adhesion and transmigration of monocytes and granulocytes, and this likely applies to other myeloid cells as well. In addition, PG-induced CD11b expression may stimulate myelin phagocytosis by microglia and MΦ, a functional characteristic of CD11b[11,12].

Persistence of PG in the brain in relation to expression of lytic enzymes

Lysozyme and NAMLAA/PGRP-L are the best characterized enzymes which in joint action abrogate the stimulatory capacity of PG[13]. Strikingly, lysozyme was only expressed occasionally by cells near blood vessels in MS and EAE-affected brain tissue (chapter 2), which is in agreement with previous studies[14,15]. These findings suggest that once cells have migrated into the brain, lysozyme production is turned off. In marmoset and MS brain tissue also NAMLAA was also expressed

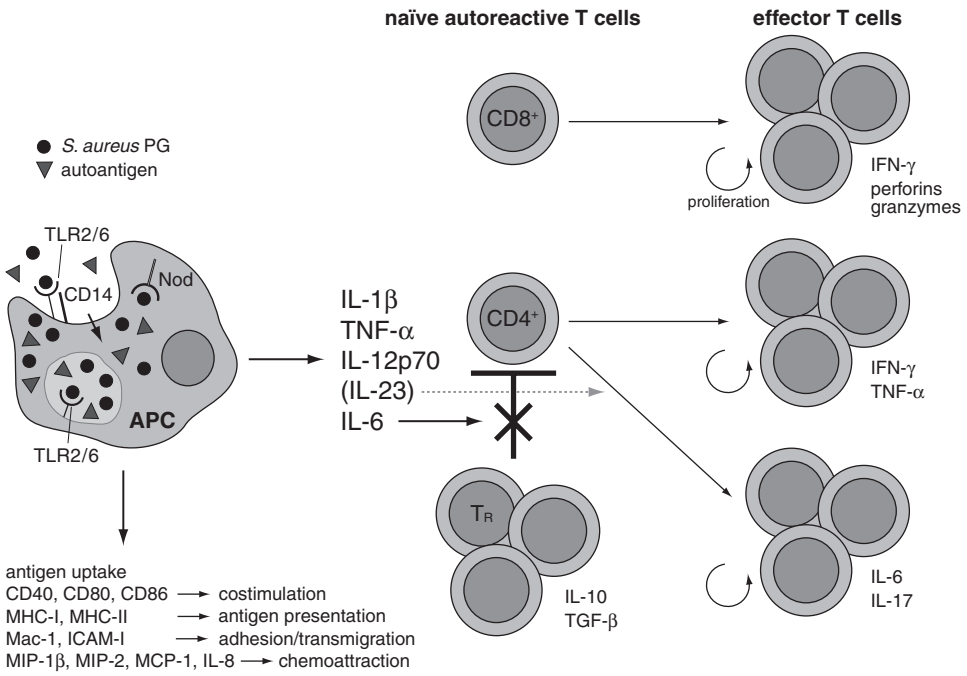

Figure 1. Model for the activation of autoreactive T cells by APC primed with *S. aureus* PG.
PG stimulates the immune system by binding to innate receptors (TLR2/Nod) on and within APC. *S. aureus*-derived PG is a potent activator of APC, by increasing antigen uptake, costimulatory and adhesion molecule expression, and antigen presentation. Furthermore, PG stimulates the production of many proinflammatory cytokines and chemokines. These proinflammatory factors can block immunosuppression by regulatory T cells (T_R)[44]. PG drives (auto)antigen-specific $CD4^+$ Th1 polarization and expansion. Experimental evidence for this is provided in chapter 3. In addition, $CD4^+$ Th1 cells may reciprocally activate APC, provide help for B cell responses (not depicted here), and help for the activation and expansion of autoreactive $CD8^+$ T cells. PG may also induce activation of IL-6/IL-17 producing $CD4^+$ T cells by stimulating IL-23 production. These events can occur both in the periphery, within secondary lymphoid organs, and at sites of inflammation, i.e. the CNS in MS and EAE. The term APC in this figure represents several cell types, dependent on the location, e.g. DC, MΦ and microglia.

in a restricted manner. In contrast, EAE-affected brain tissue from rhesus monkeys contained high numbers of NALMAA-expressing cells. This finding is not surprising, because rhesus monkeys develop an acute disease course, accompanied by large necrotic lesions with many neutrophils. Double labeling demonstrated that $NAMLAA^+$ cells in the rhesus monkey brain lesions were predominantly neutrophils. The restricted expression of lysozyme and NAMLAA (except for NAMLAA that is highly expressed in rhesus monkey EAE) in brain tissue suggests that PG is not at risk to be degraded by these enzymes in the CNS. This does not exclude the possibility that the PG within granulocytes, DC and MΦ is partially degraded,

since enzyme digestion might already have taken place in the periphery. The restricted expression of lyzosyme and NAMLAA in the CNS may contribute to PG accumulation and persistence inside the CNS, as described previously[16].

Only 25 to 48% of NAMLAA$^+$ cells coexpressed CD11b, whereas 86% of PG-containing cells expressed CD11b in rhesus monkey EAE brain tissue. This difference is even more pronounced in marmoset EAE brain tissue. In this case an average of 95% of PG-containing cells expressed CD11b, whereas of all NAMLAA$^+$ cells no more than 2% expressed CD11b. Comparable patterns were found in spleen tissue. In conclusion, these data strongly imply that PG and NAMLAA are mostly present in different cells. These data can be interpreted to mean that PG is partially or fully degraded in NAMLAA-expressing cells, at least to the degree that the remaining PG fragments are unrecognizable by Mab 2E9. Alternatively, NAMLAA expression might be switched on and off. That expression of NAMLAA can be induced is implied by the findings that peripheral blood monocytes and spleen MΦ do not express NAMLAA[17], but under inflammatory conditions, as in MS brain (chapter 2), a few to moderate numbers of foamy MΦ expressed NAMLAA. Future research should assess how the expression of NAMLAA is regulated in granulocytes, MΦ and DC and whether the presence of PG modulates NAMLAA expression.

Microbial stimulation of autoimmune inflammatory responses within the CNS

Can CNS cells respond to PG?

PG accumulates during local inflammation in the brain tissue of patients with MS and EAE-affected marmoset and rhesus monkeys (chapter 2). PG stimulates the innate immune system by engaging TLR2/6 or Nod receptors (Figure 1). Cultured human glial cells, i.e. microglia, astrocytes and oligodendrocytes all expressed TLR2 mRNA[18]. By using quantitative TaqMan® analysis it was shown that Nod2 mRNA was expressed at low levels in mouse brain tissue[19]. In contrast, expression of Nod 2 and Nod1 was not observed in human brain, as determined by Northern blot analysis[20,21]. This discrepancy may be related to the sensitivity of the techniques used to detect Nod2 mRNA in the different studies. It would be informative to determine which cells express Nod2 in brain tissue. Thus, in theory, glial cells can respond to PG, because they express the receptors that recognize PG. *In vitro* studies have indeed shown that PG is recognized by microglia and astrocytes. Primary mouse microglia[22,23] and astrocytes[24] produced several inflammatory cytokines (IL-1β, TNF-α) and chemokines (MIP-1β, MIP-2, MCP-1) after incubation with *S. aureus* PG (Figure 1). The inflammatory response of microglia and astrocytes was partially mediated by TLR2, since PG-induced responses were also present, although reduced, in TLR2$^{-/-}$ cells. *S. aureus* PG also induced expression of MHC-I and MHC-II and costimulatory molecules (CD80, CD86, ICAM-1) on murine microglia[22,25]. This means that PG-activated microglia

can potentially present antigens to CNS infiltrating T cells. This hypothesis was tested *in vitro* by using microglia from SJL/J mice[25]. Microglia were activated by *S. aureus* PG and subsequently incubated with $PLP_{139-151}$. It appeared that PG-stimulated peptide-pulsed microglia could induce proliferation of a costimulation-dependent $PLP_{139-151}$-specific $CD4^+$ T cell line. Microglia could also enhance the inflammatory potential of PG, because microglia have the capacity to digest *S. aureus* and release inflammatory muramyl peptides *in vitro*[26]. Taken together, microglia and astrocytes are fully equipped to recognize and respond to PG.

By which mechanisms might PG in the CNS contribute to the development of autoimmune disease?

A recent study has provided important insights on this issue[27]. In C57BL/6 mice, EAE does not develop unless PTX is injected systemically after immunization with MOG_{35-55} in CFA[28]. Immunization with MOG_{35-55} in CFA induced activation and clonal expansion of potentially pathogenic autoantigen-specific Th1 cells, since these cells could efficiently transfer disease after *in vitro* reactivation with MOG_{35-55}. The action of PTX is diverse and includes induction of differentiation and clonal expansion of Th1 cells, migration and adhesion to the BBB, and impairment of the BBB[29-32]. Nevertheless, migration of MOG_{35-55}-specific Th1 cells into the CNS is not enough to induce disease, because BBB damage induced by an aseptic cryoinjury did not lead to autoimmune disease. Strikingly, intracerebral injection of CpG-DNA, a TLR9 ligand, induced severe clinical symptoms in mice that were immunized with MOG_{35-55} in CFA, but not in $OVA_{peptide}$-CFA immunized control mice. Intracerebral injection of CpG induced mRNA expression of IP-10, MCP-3, MIP-1α, MCP-1 and CD40. Brain tissue injected with CpG-DNA contained an 11-fold increased frequency of MOG_{35-55}-specific T cells compared to $OVA_{peptide}$-specific T cells. Furthermore, *in vitro* studies with brain derived APC demonstrated that the amount of IFN-γ produced by individual MOG_{35-55}-specific $CD4^+$ T cells was 3-fold increased upon incubation with MOG-pulsed CpG-activated APC, as compared to non-CPG DNA-treated APC. Collectively, these data show that the immunostimulatory state of APC inside the target organ determines whether or not autoantigen-specific T cells are retained and reactivated.

In the model described above, a single cerebral injection of CpG-DNA induced an upregulation of CD40 mRNA for at least 5 days in CFA-MOG_{35-55} immunized mice, whereas expression was not detected on day 5 after injection in CFA-$OVA_{peptide}$ immunized mice[27]. This indicates that the effect of CpG-DNA on CNS resident APC is only transient and probably resolves as soon as the causative agent (CpG-DNA) is cleared from the CNS. It is therefore noteworthy that PG can persist in cells and accumulates in the brain after systemic administration[16]. Interestingly, brain pathology and mild clinical EAE can also be initiated by CFA-PTX immunization without CNS autoantigen, in transgenic mice with an astrocyte-targeted expression of IL-12p70[33]. CFA-PTX immunization induces a polyclonal T cell population,

reactive against the different antigens inside the immunization mix. Initial CNS infiltration in mice with an astrocyte-targeted expression of IL-12p70 was most probably independent on the recognition of self-antigen. However, autoreactive T cells may have co-migrated as bystander cells and could be activated in the CNS when autoantigens are released during inflammation. Upon release of new myelin epitopes bystander activation may also influence the process of epitope spreading, which is known to be associated with disease progression[34]. Thus even in the absence of peripheral autoantigens, a peripheral infection may result in CNS damage, when CNS microglia or astrocytes are already in an inflammatory state. This inflammatory state may be created by intracellular PG, which can act as a local adjuvant by producing chemokines, inflammatory cytokines and expressing MHC and costimulatory molecules by resident APC. To test this hypothesis, sPG from *S. aureus* can be injected intracerebrally into C57BL/6 wild type, $NOD1^{-/-}$, $NOD2^{-/-}$ and $TLR2^{-/-}$ mice. Upon systemic activation of T and B cells, by immunization with CFA-MOG_{35-55} (without PTX), it is expected that wild type and $NOD1^{-/-}$ mice will develop clinical EAE signs and neuropathology, since CNS resident APC will be activated by PG via ligation to TLR2 or Nod2 receptors. Accordingly, it is predicted that TLR2- and Nod2-deficient mice do not develop autoimmune disease. When PG is persistently present inside CNS APC, myelin-specific T cells may be activated in the CNS, even without prior activation in the periphery. To test this, a similar experimental approach may be used in which mice are immunized with CFA-$OVA_{peptide}$ instead of CFA-MOG_{35-55}.

Microbial stimulation of autoimmune inflammatory responses in the periphery

PG-containing APC in secondary lymphoid organs, functional implications

Also in the periphery, PG may contribute to development or perpetuation of autoimmune disease, by stimulating APC (Figure 1). Previous studies have revealed that PG in sterile human spleen tissue was biologically active. Upon stimulation of whole blood cells, spleen-derived PG induced production of IL-1β, IL-6, IL-8, IL-12, IL-10, TNF-α and MMP-9[35]. Interestingly, spleen-derived PG was 10-100 times more potent in the induction of proinflammatory cytokines compared to PG isolated from human feces[36]. As in human spleen tissue, we demonstrated in chapter 2 that intracellular PG is also present in marmoset spleen. In marmosets, the number of PG-containing spleen cells was similar in CFA-MOG immunized monkeys, compared to control monkeys, which were not immunized or immunized with OVA in CFA. In CFA immunized monkeys, PG in spleen and brain may have originated from the adjuvant depots, which contained *M. tuberculosis*. Furthermore, PG may have been transported by APC from mucosal tissues[37-40] that are continuously exposed to large quantities of bacteria. Notably, in rhesus monkeys with an acute EAE course, clusters of PG-containing APC were present in the marginal zone of the spleen (chapter 2, Figure 4a, b). PG-clusters were

also present in draining lymph nodes and spleens from mice immunized with MOG_{35-55} admixed in IFA with sPG from *S. aureus* and were identified as DC (chapter 3, Figure 4). PG-containing DC clusters were already found at 4 hours after immunization, and were still present at day 37 (unpublished data). Most likely, DC transported sPG and autoantigens from the injection site to the draining lymph nodes and spleens where naïve autoantigen-specific T cells were activated and expanded in an inflammatory environment. Additional *in vitro* studies using bone marrow-derived DC demonstrated that PG stimulated antigen uptake, DC maturation and antigen-specific Th1 cell development and expansion (Figure 1). In accordance, immunization of autoantigen in sPG-IFA stimulated the polarization and expansion of MOG_{35-55}-specific Th1 cells. Thus, in the periphery, inflammatory PG stimulated autoantigen-specific immune responses by modulating APC and subsequent T cell effector functions.

PG promotes the production of proinflammatory cytokines

Also other TLR ligands have been shown to contribute to autoimmune disease development by stimulating APC function in the periphery. By using a similar approach as used in chapter 3, EAE can be induced in SJL/J mice by immunization with autoantigen admixed in CpG-DNA and IFA[41]. Disease induction by administration of autoantigen in microbial adjuvant was related to the ability of CpG-DNA to induce IL-12, since EAE did not develop in IL-$12^{-/-}$ mice. IL-12 and IL-23 share a common p40 subunit, but these cytokines have different activities. IL-12 promotes the differentiation of naïve T cells into IFN-γ producing Th1 cells. IL-23 does not promote Th1 cell development, but is required for the expansion of a IL-6, IL-17 and TNF-α producing highly encephalitogenic T cell population[42,43]. It is not known whether PG stimulates IL-23 production by APC. Nevertheless, sPG from *S. aureus* is a potent inducer of IL-12p70 and IL-6 (chapter 2 and 3). PG-induced IL-6 may block immunosuppression by regulatory T cells (Figure 1), as observed with other TLR agonists[44]. *S. aureus* PG induced high levels of IL-12p70 following stimulation of mouse bone marrow-derived DC or marmoset and rhesus monkey PBMC. Subsequent culture of sPG-stimulated OVA-pulsed murine DC with naïve T cells transgenic for the TCR recognizing $OVA_{peptide}$ promoted the development and expansion of IFN-γ producing Th1 cells. It would be interesting to determine whether PG promotes IL-23 production by APC and subsequent development of IL-17 producing T cells (Figure 1).

We and others have demonstrated that immunization of EAE-susceptible mice with myelin antigens in IFA did not induce EAE. Myelin-specific T cells were generated in the draining lymph nodes and expanded upon *in vitro* restimulation, proving that these cells were not deleted and were responsive to the antigen of immunization. However, IFA-autoantigen primed T cells did not have the capacity to adoptively transfer disease except when microbial or inflammatory (LPS, CpG, IL-12)[41,45,46] stimuli were applied during *in vitro* reactivation. Even when mice

were tolerized to myelin antigens prior to EAE induction by systemic injection of autoantigen, unresponsive autoantigen-specific lymph node cells could be converted into pathogenic effector T cells, when stimulated *in vitro* in the presence of IL-12, CpG-DNA or agonistic anti-CD40 Mab[47]. Obviously, these model systems create an optimal situation for autoantigen-specific Th1 cell polarization and expansion, because all APC are exposed to the autoantigen and inflammatory stimulus simultaneously. It is questionable whether this process can also take place *in vivo*. In that respect, it is interesting to note that additional systemic administration of CpG at the time of autoantigen immunization in IFA did not lead to EAE development[41]. It is unknown whether PG administered separately from MOG/IFA is able to overcome tolerance to self-antigens *in vivo*, when tolerance is promoted by immunization with autoantigens in IFA. It can be argued that CpG is less efficient than PG, because CpG may be rapidly cleared from the circulation, whereas PG can persist inside cells. Therefore, it would be interesting to determine whether EAE can develop upon systemic administration of PG at the time of IFA-autoantigen immunization. In such a scenario, PG-containing cells in spleen or brain tissue may exert dangerous proinflammatory bystander affects on autoantigen-specific APC-T/B cell interactions.

Figure 1 provides a hypothetical scheme by which PG may exert its effect, focused on autoreactive T cell activation, either in the periphery or in the CNS. In addition, PG likely stimulates the development of activated autoreactive B cells as well.

Structural characteristics of inflammatory PG in relation to its degrading enzymes

Inflammatory potential of insoluble versus soluble PG

Several factors can determine differences in immunostimulatory capacity of PG, which are all related to the structure of PG. The composition of PG is highly variable between different bacterial strains and even varies within a single strain. As an example, Figure 2 depicts the structural diversity of PG in *S. aureus*. In our studies we have used two different PG forms purified from *S. aureus*, insoluble PG (iPG) or soluble PG (sPG) (chapter 2 and 3). The sPG was predominantly composed of low molecular weight multimers, whereas in iPG multimers of high molecular weight predominated (Figure 2). Both *in vitro* and *in vivo* data demonstrated that sPG was much more effective than iPG. sPG, but not iPG, was able to induce IL-8 production in TLR2 transfected HEK293 cells (unpublished observation). Both iPG and sPG could induce EAE in mice when admixed in IFA with MOG_{35-55}[48](chapter 3). However, the dosage required for EAE induction was 8-10 times lower for sPG compared to iPG. sPG induced IL-6, IL-12p70 and TNF-α by bone marrow-derived DC, whereas a similar dose of iPG induced TNF-α only. Thus, at similar concentrations, *S. aureus* iPG is less inflammatory than sPG.

As recently described, both Nod1 and Nod2 recognize specific motifs of PG,

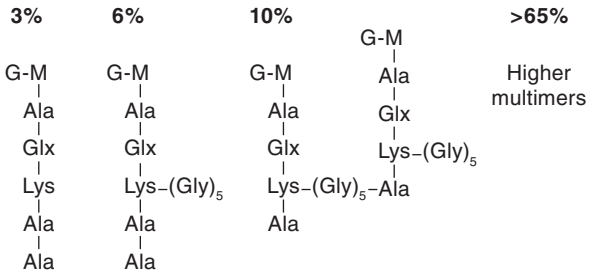

Figure 2. Structural diversity of *S. aureus* PG.
Stem peptides crosslink the glycan chains and differ in composition between bacterium strains. In *S. aureus* the stem peptides are composed of L-alanine-D-isoglutamine-L-lysine-D-alanine-(D-alanine). Stem peptides can be attached directly to each other or indirectly via interpeptide bridges. The interpeptide bridges in *S. aureus* PG are formed by pentaglycines $(Gly)_5$. There is a great variety in crosslinking and composition of stem peptides. *S. aureus* PG is predominantly composed of high molecular weight multimers (65%), and to a lesser extent of low molecular weight forms. Percentages indicate the relative abundance in overall cell wall composition. (G-M) GlucNAc-MurNAc. Adapted from de Jonge *et al*., 1992[205].

as depicted in Table 1 (and Figure 3c Introduction). Nod1 does not recognize *S. aureus* PG, since *S. aureus* PG does not contain the DAP at position 3 of the stem peptide which is required for Nod1 recognition. In contrast, Nod2 does not recognize MurNAc-Tri_{DAP}, but instead recognizes GM-DP and longer stem peptides with an L-lysine *(*in *S. aureus)* or L-ornithine at position 3[49]. In the compact structure of insoluble *S. aureus* PG, the Nod recognition sites are most likely hidden from detection by Nod2 proteins, whereas these motifs are likely accessible for detection in sPG. This idea is supported by studies in which PG from *Eubacterium aerofaciens* was partially digested by lysozyme or bacterial-derived mutanolysin, which both cleave PG identically[50]. The biological activity of the arthritogenic *E. aerofaciens* (ATCC 25986) was significantly increased after mutanolysin treatment[51]. Muramidase treatment does not abolish recognition for either Nod1 or Nod2, since after cleavage both GM-Tri_{DAP} and GM-DP can be formed (Figure 3b/c Introduction). In contrast, digestion of PG by NAMLAA results in loss of PG detection by Nod2, since PG recognition by Nod2 is dependent on the presence of MurNAc. Accordingly, combined treatment of *E.* aerofaciens (ATCC 25986) PG by lysozyme and NAMLAA abrogated its inflammatory effects both *in vivo* and *in vitro*[13]. *Staphylococci* are resistant to lysozyme degradation. In *S. aureus* bacteria, this resistance depends on the *O*-acetyl moieties at the C6 hydroxyl group of *N*-acetylmuramyl residues of PG[52]. This perhaps explains why iPG from *S. aureus* is not as effective as sPG, since partial degradation by lysozyme does not occur.

Taken together, partial digestion of PG by muramidases can result in the release of highly immunogenic PG motifs. In the periphery, phagocytes contain lysozyme, as a part of their machinery to attack invaded microbes. However, lysozyme is

only sporadically expressed in MS and EAE-affected monkey brain (chapter 2), despite the presence of many activated phagocytes in infiltrates. This does not necessarily mean that the PG inside APC of the CNS is not partially digested, since APC most likely have transported PG from the periphery, where they are able to produce lysozyme. It would be interesting to determine the effects of lysozyme and NAMLAA expression during EAE, induced by immunization with autoantigen in PG-adjuvant. This could be accomplished by generating mice that constitutively express lysozyme and/or NAMLAA under the MHC-II promoter, specifically targeting enzyme expression in APC, including microglia. The other way around, EAE induction in mice with a single or combined deletion for lysozyme and NAMLAA would provide valuable insights into the role of these enzymes in EAE. Both lysozyme- and NAMLAA-deficient mice are viable and available on the EAE-susceptible C57BL/6 background[53,54].

Inflammatory potential of PG depends on the composition of stem peptides
Not all bacteria are susceptible to lysozyme degradation, as described above. Besides structural modification by *O*-acetylation, also other PG modifications increase the resistance to lysozyme degradation, including the degree of crosslinkage between stem peptides[56]. Other bacterial cell wall components, such as polysaccharides, LTA and TA can also reduce muralytic degradation[56,57]. Resistance of bacterial cell walls to lysozyme degradation is important for the retention of PG in liver, spleen, lymph nodes and synovial tissues[51,58]. There appears to be a positive correlation between the capacity of bacterial cell walls to induce chronic arthritis and the resistance of cell walls to lysozyme degradation, as summarized by Šimelyte *et al.*[55]. However, there are exceptions, e.g. *Streptococcus faecium* cell wall is sensitive to lysozyme degradation and induces arthritis in rats[55,59]. On the other hand, *E. alactolyticum* cell walls are resistant to lysozyme degradation, but could not induce arthritis[57]. Thus, lysozyme-resistance alone is not decisive for the arthritogenicity of bacterial cell walls.

Several studies have indicated that the amino acid at position 3 of the stem peptide plays an important role in the stimulatory capacity of PG in experimental arthritis, as shown in Table 1. Cell walls from the arthritogenic *E. aerofaciens* (ATCC 25986) and the nonarthritogenic *E. aerofaciens* (ATCC 35085) differ in amino acid 3 of the stem peptide. The nonarthritogenic cell walls have L-ornithine at position 3 of the stem peptide, whereas the arthritogenic cells walls have L-lysine. Arthritogenic cell walls were more resistant to lysozyme degradation. Upon digestion with lysozyme, arthritogenic PG induced elevated levels of TNF-α and MCP-1 upon stimulation of peritoneal MΦ[60]. No differences were found when undigested PG was used. Moreover, after i.p. injection, arthritogenic PG accumulated in significantly higher quantities in spleen and liver compared to nonarthritogenic PG, and was also detected in synovial tissue, whereas nonarthritigenic PG was not. It should be noted that L-lysine at position 3 of the stem peptide is not crucial for the induction

Table 1. Nod recognition sites in PG from Gram-positive and Gram-negative bacteria.

PG molecular motif	Nod1	Nod2	arthritis induction[55]	(predicted)/proven adjuvant function in EAE
Gram-positive	-*	+		
GM-DP	-	+	n.d.	(-)
GM-Tri$_{ornithine}$	-	+	-	(-)
GM-Tri$_{lysine}$	-	+	+	+ (chapter 3)
Gram-negative	+	+		
GM-DP	-	+	n.d.	(-)
GM-Tri$_{DAP}$ / mDAP**	+	-	- §	(-)

Composition of PG (see also Figure 3 Introduction): (GM) GlcNAc-MurNAc, (GM-DP) GM-L-Ala-D-Glu, (mDAP) meso-diaminopimelic acid, (GM-Tri$_{ornithine/lysine/DAP}$) GM-L-Ala-D-Glu-ornithine//lysine/mDAP.
(*) Exceptions to the general rule: several Gram-positive bacteria contain GM-Tri$_{DAP}$, and can therefore be recognized by Nod1; e.g. *Eubacterium alactolyticum*, *Lactobacillus plantarum, Listeria monocytogenes*, *Bacillus cereus* and *Bacillus anthracis*.
(**) For Nod1 binding mDAP needs to be present as the terminal amino acid[49].
(§) arthritogenic bacteria with mDAP at position 3 of the stem peptide, e.g. *L. plantarum*.
(n.d.) not determined.

of arthritis since *L. plantarum* bacteria with mDAP at position 3 can induce arthritis as well (Table 1)[55]. But, all other bacteria with ornithine or mDAP at position 3 of the stem peptide did not induce arthritis and among the nonarthritogenic bacteria none contained L-lysine at potision 3 of the stem peptide. Within the structure of PG, Nod2 both recognizes GM-Tri$_{lysine}$ and GM-Tri$_{ornithine}$, while the latter does not appear to induce arthritis (Table 1). It remains to be determined whether Nod2 responds differently to these two distinct structures. Taken together, L-lysine at position 3 of the stem peptide appears important for the inflammatory capacity of PG, while lysozyme resistance appears important for retention of PG at inflammatory sites. For the induction of EAE, it is expected that bacteria that contain L-lysine at position 3 of the stem peptide of PG (e.g. *S. aureus*) will stimulate autoimmune disease development, whereas PG with other amino acids at this position will be less efficient (mDAP) or incapable (ornithine) to such effects (Table 1).

Where does PG present in lymphoid organs and CNS come from?

PG can be derived from all mucosa, and both from bacterial infections and from the normal physiological flora of skin and mucosa, notably the gut. PG is released throughout bacterial infection, both during bacterial replication through cell wall biosynthesis, and by uptake/infection and degradation by phagocytes. Exacerbations of MS are often associated with bacterial infections[61-63]. Moreover, bacterial products, including PG, are continuously exposed to the immune system at all mucosal sites, with the highest concentration of bacteria being present in the gut. The highest number of intestinal bacteria occurs in the lumen of the distal ileum and colon. In the gut lumen, obligate anaerobic bacteria (10^8-10^{12} per gram of luminal content) outnumber aerobic bacteria (10^6-10^8)[64,65]. Of the obligate

anaerobes, the dominating Gram-positive species include *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Lactobaccilus* and *Peptostreptococcus*[64]. Cell walls from particular substrains of these commensals could efficiently induce arthritis by i.p. injection into rats[55] and induced inflammatory cytokine and chemokine responses by *in vitro* stimulation of mononuclear cells[51]. PG polysaccharides can be absorbed in the gastrointestinal tract, as demonstrated by oral feeding of these complexes to rats. Orally fed radiolabeled PG polysaccharides were found in liver, spleen, mesenteric lymph nodes, cardiac blood and joints, demonstrating that PG was absorbed across the epithelial barrier of the mucosa and redistributed into different tissues[66]. Intestinal absorption of PG was increased when high concentrations of PG polysaccharides were injected into the gut lumen or when intestinal bacteria were experimentally overgrown in a surgically created jejunal blind loop[67]. Both in humans[68-70] and in experimental models[71], bacterial overgrowth in the small bowel was associated with joint inflammation, indicating that PG translocation may stimulate the development of joint inflammation in susceptible hosts.

Is there a role for the intestinal flora in MS?

In healthy individuals, the mucosal immune response to antigens of the indigenous flora is tightly regulated, in order to prevent chronic inflammation in the intestine. It is generally assumed that a loss of tolerance to the commensal flora is involved in the development and perpetuation of IBD. In accordance, it was shown that immune tolerance is generated against antigens of the enteric flora, since intestinal lymphocytes of mice do not proliferate upon stimulation with antigens of their own intestinal flora, but do proliferate when antigens of intestinal flora of other mice are encountered[72]. In patients with IBD, a defective epithelial barrier may cause a loss of tolerance to the normal enteric flora. The additional development of RA in 5-23% of patients with IBD highlights that the bacterial flora may play an important role in autoimmune disease development[64].

Although limited, there are also indications that the intestinal flora plays a role in MS and EAE. Spontaneous EAE in MBP-specific T cell receptor transgenic mice only developed in a non-sterile environment[73]. Oral administration of different *Lactobacillus* strains, prior to the induction of EAE, either enhanced or reduced EAE[74], and was positively related to the adjuvant inducing capacity of the different strains[75]. Conversely, EAE was ameliorated by treatment with the oral antibiotic vancomycin, which specifically targets Gram-positive, but not Gram-negative bacteria[76]. Increased gut permeability to lactulose / mannitol was observed in 5 out of 20 MS patients[77]. Evidently, larger patient numbers need to be screened to assess whether an increased intestinal permeability is associated with MS. Patients with CD and successive development of MS-like disease have been described[78,79], but this link is not strong in epidemiological terms. One study describes the co-occurrence of CD and MS in 4 out of 832 patients with CD[80]. Of the total group of IBD patients, 3% suffered from neurological complications.

Hypointense T2-weighted focal white matter lesions were present in 50-70% of IBD subjects compared to 16% in healthy age-matched controls[81]. Additionally, a case study presented a patient with CD in which inflammatory brain lesions were accompanied by either clinical signs of ADEM or a first attack of MS[82].

NOD2/CARD15 has been identified as susceptibility gene for CD[83,84]. Nod2 recognizes MDP or longer GM-Tri_{Lys}-containing structures in PG and is expressed on monocytes, granulocytes, DC and MΦ. In CD patients, three common single nucleotide polymorphisms of the *NOD2* gene have been identified, i.e. *IBD8* (Arg702Trp), *IBD12* (Gly908Arg) and *IBD13* (1007fsins)[85]. These mutations are carried by 30-50% of CD patients and 15-20% of healthy controls. The risk for CD is only 2-3 times elevated in heterozygous carriers; however, the relative risk is increased to 20-40 in homozygous carriers. Genetic analysis of 631 MS patients showed no significant difference in allele frequency of *IBD8*, *IBD12* and *IBD13* as compared with controls[86]. These data suggest that the *NOD2* gene does not contribute to MS susceptibility. However, there are 29 additional rare *NOD2* mutations described for CD. With respect to their basal expression levels of NF-κB and PG-induced NF-κB activation, 13 of these additional *NOD2* variants were shown to differ from wild type *NOD2*[87]. It would be interesting to determine whether these *NOD2* variants are also present and associated with MS susceptibility.

What is the functional role of Nod2 in the intestine? Normal intestinal epithelial cells express low levels of Nod2, whereas expression is greatly upregulated by epithelial cells in CD patients[88]. Nod2 expression has also been found in Paneth cells[89]. Paneth cells are located in deep crypts of the intestinal epithelium. Paneth cells can efficiently kill bacterial cells by the secretion of defensins upon stimulation by MDP[90]. Recently, animal models have demonstrated that Nod2 is important for bacterial clearance by Paneth cells. $Nod2^{-/-}$ mice displayed increased susceptibility to gastrointestinal infection. Microarray analysis revealed that the expression of a subgroup of cryptidins, the equivalent of human α-defensins, was significantly reduced in the terminal ileum of infected $Nod2^{-/-}$ mice[91]. In accordance, expression of α-defensins was reduced in CD patients, specifically in patients with *NOD2* mutations[92,93]. A defect in bacterial sensing by Nod2 by intestinal Paneth cells may result in increased numbers of intestinal bacteria and thus in greater quantities of PG in the circulation and tissues.

Proinflammatory PG as a cofactor in MS an EAE

We have shown previously that immunocompetent APC within the CNS of MS patients contain PG, a major cell wall component of Gram-positive bacteria. In the present study we assessed whether proinflammatory PG can act as a cofactor in MS and EAE development. Figure 3 illustrates how PG might be distributed and how PG might contribute to MS and EAE, as described below.

Where does PG come from under physiological conditions? PG (circles) can be derived from bacteria at all mucosal sites ①. The flora of the intestine represents

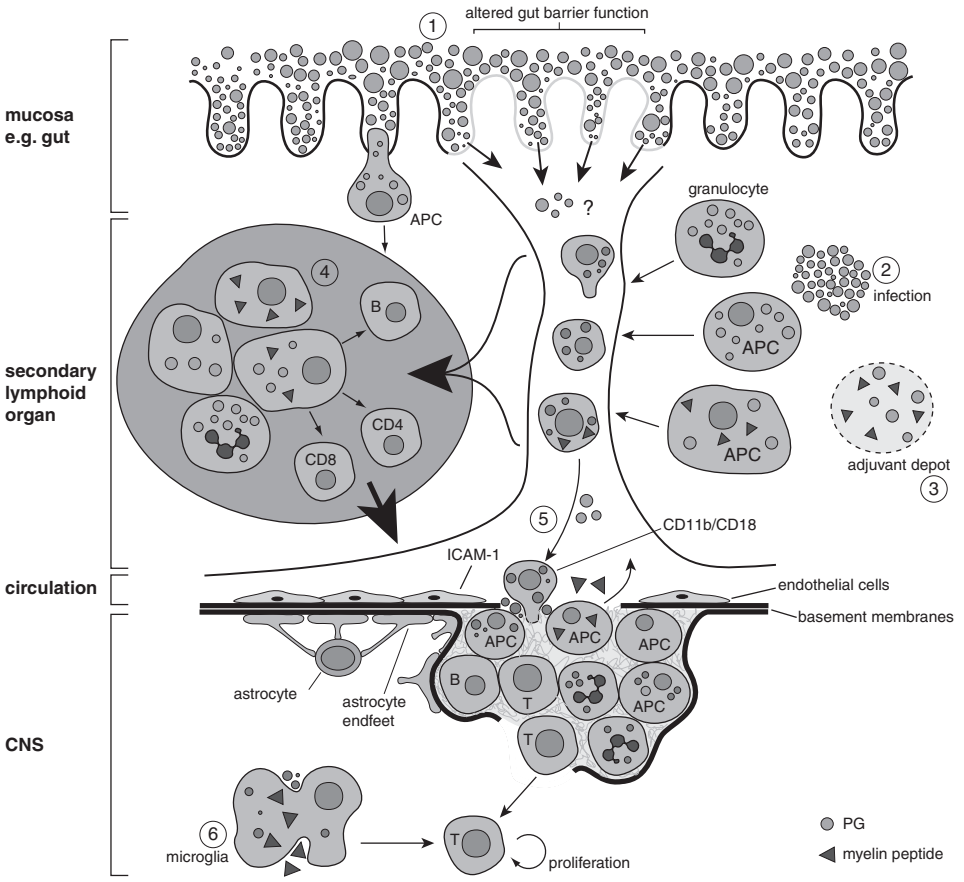

Figure 3. Proinflammatory PG as a cofactor in MS an EAE.
(for explanation see text)

the largest load of bacteria among mucosal surfaces. In the intestine, bacteria can be translocated across the epithelial barrier by M cells and specialized APC. In this way, bacteria are sampled and transported into the circulation and peripheral lymphoid organs as a part of a physiologically occurring process. However, when the mucosal integrity is disturbed, e.g. by infection or by chemical substances, higher numbers of bacteria and perhaps also other bacterium strains can cross the gut barrier, resulting in higher levels of PG in the circulation and tissues. PG can also be derived from bacterial infections at non-mucosal sites ②, by the release of PG fragments during bacterial replication or by uptake and processing of bacteria by APC. In MS, bacterial infections are associated with clinical relapses. Thus,

PG can be derived from different anatomical locations (all mucosa) and levels are increased during bacterial infection or when the permeability of mucosal sites is increased.

In our mouse EAE model, we have artificially administered PG from the pathogenic bacterium *S. aureus* by s.c. immunization in IFA ③. In EAE, *S. aureus* PG could replace whole *M. tuberculosis* in CFA. Addition of PG to an emulsion of IFA and myelin peptides (triangles) was associated with autoreactive T cell priming and development of EAE. *In vitro* studies revealed that PG stimulates antigen uptake and maturation of DC. Most likely DC transported PG and myelin peptides from the adjuvant depot into the draining lymph nodes ④. Accordingly, PG-containing DC were found in the draining lymph nodes already at 4 h after immunization. It is apparent that immunization with myelin peptides in IFA-PG provides an optimal condition for PG to stimulate myelin peptide-specific immune responses, since phagocytes at the immunization site can engulf the PG and myelin peptides simultaneously. However, also in non-artificial conditions, PG and myelin peptides may be in close proximity, as both substances are confined within APC of peripheral lymphoid organs.

PG-stimulated APC can prime naïve T and B cells in secondary lymphoid organs, and in the presence of (auto)antigens induce development of (auto)antigen-specific effector lymphocytes (Figure 1). These effector lymphocytes can enter the circulation and adhere to activated endothelial cells in the CNS. Encounter with their targets (myelin peptides) in the CNS parenchyma, presented by DC or MΦ/microglia initiates an inflammatory cascade that includes the attraction of other leukocytes. Among these leukocytes, PG-containing phagocytes may also enter the CNS by utilizing CD11b/CD18-mediated adhesion and transmigration across the BBB endothelium ⑤. Thus, during CNS inflammation, PG-containing cells accumulate in the CNS, and may promote local inflammation and disease perpetuation. PG may also be present within resident microglia ⑥, by uptake of PG-fragments which originated from the blood stream, by uptake of apoptotic cells that contained PG, or by uptake of bacteria that have infected the CNS (although there is little evidence for local replication). We propose that PG persists in the CNS and that this persistence may be related to the restricted expression of PG-degrading enzymes in the CNS. Taken together, our data show that *S. aureus* PG is a sufficient proinflammatory stimulus to break T cell tolerance. PG likely mediates this by stimulating DC-T cell interactions in lymph nodes, and potentially also acts in the CNS.

Future research

When we consider that PG is important in autoimmune disease development and perpetuation, the following research questions and experiments can be envisaged to provide additional insight in how and where PG may contribute to the immunopathogenesis in MS and EAE.

Is the quantity and nature (chemical composition) of PG important in MS and EAE?

PG-containing cells are present in peripheral blood leukocytes of a number of healthy subjects and patients (incl. MS and RA), but no clear differences were detected in the occurrence of PG between the different groups[94]. The proportion of individuals with circulating PG-containing leukocytes decreases with age. However, there may be differences between MS patients and healthy controls with respect to 1) the quantity and nature (chemical composition) of PG in the circulating leukocytes and 2) the cellular subsets (monocytes, MΦ, DC and granulocytes) that can contain PG. Moreover, it is not clear whether relapse-associated infections in MS patients are accompanied with elevated numbers of PG-containing cells in the peripheral blood. These questions can be addressed by using immunocytochemistry and mass spectrometry. EAE can be used to functionally address the question whether PG is able to induce relapses. EAE should be induced by adoptive transfer, to exclude effects of previous adjuvant depots. After recovery from EAE, animals may be injected with PG to evaluate whether such injections can induce relapses.

In MS, aside from bacterial infections, other factors may contribute to increased PG concentrations within secondary lymphoid organs, the circulation and inflammatory sites including the activity of PG-degrading enzymes (see below) and permeability of the gut. Does the permeability of the intestine contribute to disease development and does bacterial overgrowth in the intestine influence disease development? These questions can be addressed in EAE, where the gut permeability can be increased by chemical disruption. Bacterial overgrowth can be established by creating an intestinal blind loop.

In accordance with previous findings in MS[7], chapter 2 shows that significantly higher numbers of cells contain PG (quantity) in marmoset and rhesus monkeys that had developed EAE, compared to controls. We also demonstrated that low numbers of PG-containing cells are present inside the CNS of humans and non-human primates that did not develop CNS disease. This raises the question whether intracellular PG in healthy brain tissue is structurally different (nature) from PG in MS brain tissue, which can be assessed by *in situ* NMR, or laser dissection followed by mass spectrometry. Assuming that the chemical structure of PG is important for its proinflammatory effects, it will be of interest to determine whether PG with GM-Tri$_{lysine}$, GM-Tri$_{DAP}$ or GM-Tri$_{ornithine}$ exerts different effects on APC function. Additionally, these distinct substances can be tested for their capacity as adjuvant in EAE induction (Table 1).

Is the interaction of PG with its degrading enzymes and receptors important in MS and EAE?

In chapter 2, we demonstrate that expression of lysozyme and NAMLAA is restricted in EAE-affected marmoset and MS brain tissue. This finding was surprising, because several cell types that normally express these enzymes in

the periphery were present in the infiltrates. This raises the question which factors determine the expression of these PG-degrading enzymes. In view of that, it would be interesting to determine whether PG modulates the expression of NAMLAA and lysozyme, and whether the expression of NAMLAA and lysozyme differs between PG-activated APC subsets (incl. microglia). PG-degrading enzymes are known to affect the immunostimulatory potential of PG[13]. Can the inflammatory potential of PG (sPG and iPG) be increased or decreased by modulating the NAMALAA and lysozyme expression and does NAMLAA or lysozyme expression influence EAE, induced by autoantigens in IFA-PG? These questions can be addressed by using NAMLAA- and lysozyme-deficient mice.

It has been shown previously that PG stimulates the innate immune system by binding to TLR2/6 and Nod1/2 receptors (for details see Introduction). However, it remains unclear whether or not a combination of these receptors is required for the inflammatory action of *S. aureus* PG in EAE. Both *in vitro* studies (using bone marrow-derived DC) and *in vivo* studies (using EAE) in mice with single deletions for TLR2, Nod1 and Nod2 will provide answers to this question. In this respect, it would be of interest to determine whether PG-containing cells in secondary lymphoid organs and CNS tissue express TLR2/6 and Nod1/2 receptors.

Is the location of PG in relation to myelin antigens important for MS and EAE?
We and others have demonstrated that myelin antigens are present within APC in secondary lymphoid organs[95-97]. Thus, in principle, PG-containing APC may exert proinflammatory effects on APC that contain myelin antigens, by bystander activation. Alternatively, and less likely, PG and myelin antigens are present within the same APC. To address these issues, the following hypothesis can be tested: 1) myelin antigens and PG need to be in close proximity of each other to induce autoimmune disease (see page 162: *PG promotes the production of proinflammatory cytokines*), 2) PG is present within microglial cells during EAE, 3) PG can exert its adjuvant effect solely in the CNS (see page 160: *By which mechanisms might PG in the CNS contribute to the development of autoimmune disease?* and 4) PG is present in APC that contain myelin within lymph nodes of MS patients, non-human primates with EAE and not in controls.

Part II, Functional contributions of CD97 and CD44v isoforms in MS and EAE

Implications for involvement of CD97 in MS and EAE

CD97 has been associated with several chronic inflammatory conditions, as RA[98,99]. Until recently, CD97 has not been studied in relation to CNS inflammation. The best-described cellular ligand for CD97 is CD55, also known as decay accelerating factor (DAF). The complement system is thought to contribute to MS and EAE development and as a component of this system, CD55 may participate in the inflammatory process as well. Hypothesizing that CD97-CD55 interactions are involved in the inflammatory process in MS, we determined the expression of CD97 and its ligand CD55 in MS brain lesions, and measured sCD97 in serum and CSF of MS patients. Additionally, we assessed whether CD97EGF1 interactions are functionally involved in the development of EAE, by administration of blocking CD97EGF1 Mab.

In chapter 4, we demonstrate abundant expression of CD97 in MS brain lesions. In confirmation of previous findings we found that CD97 was not expressed by glial cells under normal conditions[100], but was expressed in all stages of MS brain lesions. In MS brain lesions, CD97 was expressed by different cellular subsets e.g. MΦ or microglial cells and T cells. CD55 was highly expressed by endothelial cells in pre-active and active lesions, while in normal white matter only modest expression by endothelial cells was found. In pre-active lesions, CD55 was only occasionally expressed by T cells and MΦ or microglial cells, while in active lesions, many MΦ or microglial cells expressed both CD55 and CD97. Furthermore sCD97 was found by ELISA in serum, but not in CSF, of a significant number of MS patients (37%) compared to healthy controls (8%).

CD97 and/or EMR2 expression in MS brain lesions?

CD97 can be expressed as three different isoforms, containing three, four or five EGF domains (Figure 3 Introduction). Here we studied the expression of CD97 by using an antibody recognizing EGF domain 1 (Mab CLB-CD97/1) that is present in all three isoforms. EMR2 is one of the other five EGF-TM7 family members that have been identified in humans. The EGF domains of EMR2 and CD97 differ by only 6 amino acids and Mab CLB-CD97/1 does not distinguish between EMR2 and CD97. We also determined whether EMR2 was expressed in MS brain tissue by staining with Mab 2A1 that specifically detects the stalk region of EMR2, but not of CD97. We concluded that CLB-CD97/1 staining was specific for CD97, since EMR2 expression was absent in MS lesions (data not shown). Immunoreactivity with 2A1 was observed in adenoid tissue, demonstrating that lack of expression in MS lesions was not caused by failing of the staining procedure. To ascertain that CD97 was expressed in MS brain, we also confirmed expression of CD97 by Mab MEM-180 and CLB-CD97/3 that specifically detect the stalk region of CD97 (data not shown).

Table 2. The composition of CD97 EGF domains determines its ligand binding capacity.

ligand / hCD97 isoform	CD55	CS-B	VLA-5 αvβ3 integrin
EGF 1,2,5	+++	-	+
EGF 1,2,3,5	++	-	+
EGF 1,2,3,4,5	+	+++	+

(hCD97) human CD97, (EGF) epidermal growth factor domain, (CS-B) chondroitin sulfate B. Binding is indicated as none (-) to strong (+++).

Variation in CD97EGF domains influences ligand binding

The known ligands of CD97 include CD55[101-104], CS-B[105,106], α5β1 (VLA-5) and αvβ3 (CD51/CD61) integrins[107]. Integrins mediate a diversity of biological responses e.g. angiogenesis, proliferation, survival and adhesion. The VLA-5 and αvβ3 integrins were recently identified as CD97-ligands important for CD97-mediated angiogenesis[107]. The EGF domain composition in CD97 controls ligand binding (Table 2). At least 3 EGF domains are needed to establish CD55 binding, such that all CD97 isoforms can bind CD55. However, the short isoform CD97(EGF1,2,5) binds CD55 with higher affinity than the larger isoforms (EGF1,2,3,5) and CD97EGF(1-5)[102].

In contrast, binding of the glycosaminoglycan CS-B is solely dependent on EGF domain 4 (in the mouse EGF domain 3) that is only present in the larger CD97 isoforms. The EGF domains 4 and 5 are identical for CD97 and EMR2, thus EMR2 also binds CS-B[105]. CS-B is present in the ECM and on cell surfaces, attached to proteoglycans (Figure 4). Cell-surface associated glycosaminoglycan are heterogeneous in strucure with differences in chain length, degree of sulfation and epimerization[108]. These factors co-determine binding to their receptors, as for CD97. CD97-binding of CS-B is so far detected on primary B cells and within ECM of arthritogenic synovial tissue[106,109]. EMR2-binding of CS-B furthermore occurs on fibroblasts, monocyte-derived MΦ and within ECM of different tissues, but not in normal brain tissue[105].

Three EGF domains are required for CD97-integrin interaction[107]. All human CD97 molecules contain an Arg-Gly-Asp (RGD) sequence at 110 amino acids from the carboxy-terminal site of EGF5[98] that is required for VLA-5 and αvβ3 integrin binding[107]. Taken together it can be speculated that the expression of different isoforms takes place under specific regulation, leading to a different outcome in cellular adhesion or activation in short CD97 isoforms than in larger ones. Therefore, it would be of interest to determine whether different splice variants of CD97 are expressed in MS brain lesions, and if these splice variants can be correlated with a specific lesion stage or cell type. This can be done by using an immunohistochemical double labeling procedure with Mab MEM-180 directed against the common stalk region of CD97, and Mab 1B5 directed against the fourth EGF domain of CD97. An alternative approach includes the analysis of mRNA expression levels of CD97 isoforms in MS and control brain tissues.

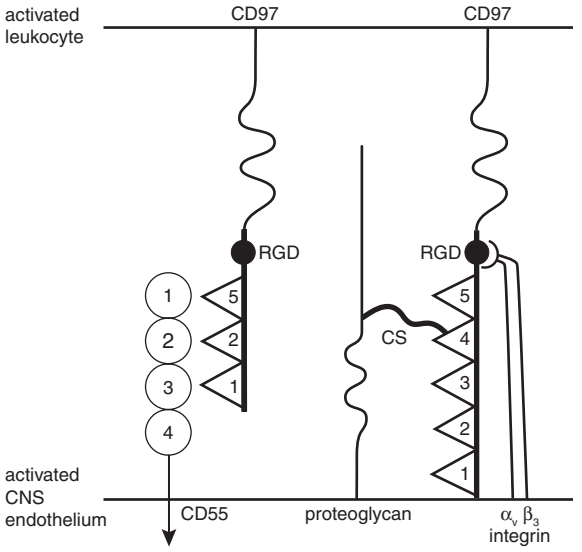

Figure 4. CD97-mediated leukocyte adhesion to activated CNS endothelium in MS.
In MS brain tissue activated endothelial cells express increased levels of CD55, αvβ3 integrin and possibly CS. These cell surface molecules and cell-associated (by glycoproteins) ECM molecules may facilitate the adhesion of activated leukocytes by binding their respective receptors, as explained in the text.

CD97 ligand expression in MS brain lesions

In MS brain lesions, perivascular T cells and MΦ or microglial cells expressed CD97 (chapter 4). Both CD97-ligands CD55 and αvβ3 integrins were expressed on the luminal side of endothelial cells in MS brain lesions and their expression was dramatically increased compared to normal appearing white matter of controls (chapter 4)[110,111]. Luminal surface expression implies the potential of αvβ3 integrins and CD55 molecules to bind CD97-expressing leukocytes, which may facilitate extravasation of these cells into the CNS parenchyma. It would be of interest to determine whether other integrins with specificity for RGD sequences[112,113] can also bind CD97. CD97-CD55/integrin interactions may contribute to adhesive events at the BBB, together with other adhesive interactions which are known to be involved in MS and EAE, such as LFA-1/ICAM-1 and VLA-4/VCAM-1[114].

Is there also a possibility for CD97-CS-B interaction in the CNS? CS proteoglycans are the most abundant ECM components in the adult CNS and are produced by neurons, astrocytes and other cells[115]. In controls, CS-B was expressed homogeneously in the white matter[116]. In contrast, scattered granular heterogeneous aggregates of CS-B were present in the ECM of the normal appearing white matter and in peri-lesional areas in MS brain. Additionally,

elevated expression of CS-B was detected at edges of active lesions, associated with astrogliosis. It has been implied previously that ECM accumulation in the CNS inhibits tissue repair, e.g. axonal regeneration and remyelination[117]. Conversely, chondroitinase ABC treatment has been shown to promote tissue regeneration and functional recovery after spinal cord injury[118]. Increased CS-B in MS white matter may serve as binding regions for CD97-expressing cells, and may be involved in the retention of infiltrating cells. It will be of interest to determine whether CD97-CD55, CD97-CS and CD97-integrin interactions are involved in BBB adhesion and transmigration, and retention in the CNS during MS and EAE. Figure 4 illustrates the potential CD97-ligand interaction between activated leukocytes and activated CNS endothelial cells in MS and EAE. By using CD97-specific multivalent probes, MS brain tissue can be analyzed for CD97-specific ligands, as has been reported recently for RA synovial tissue[109]. Whether CD97-ligand interactions are functionally involved in MS can be modeled in EAE, by using Mab against different EGF domains of CD97, as described below.

Blocking CD97 interactions in mouse EAE

We assessed whether blocking CD97-CD55 interactions affects mouse EAE. SJL/J mice were immunized with $PLP_{139-151}$ in CFA and additionally injected with heat-killed *B. pertussis* bacteria. Mice were treated with 0.25 - 0.5 or 1 mg blocking Mab hamster anti-mouse CD97EGF1 (clone 1B2) or hamster immunoglobulin as a control from two days before immunization and every other day thereafter, until day 12 after immunization. It appeared that EAE onset and severity were not reduced by the anti-CD97EGF1 (1B2) treatment (Figure 5). Treatment of collagen-induced arthritis in mice with the same antibody, however, significantly reduced disease onset and severity, ankle joint swelling and bone destruction (E.N. Kop, personal communication). In this case, the arthritis was induced by immunization with collagen II in CFA. Mice received a booster injection with collagen on day 21 and were subsequently treated with 0.25 or 0.5 mg Mab anti-CD97EGF1 (1B2) for 3 times a week until the day of sacrifice. The early phase of collagen-induced arthritis was characterized by synovial tissue infiltration by T cells, which was followed by infiltration of neutrophils and MΦ. Clinical signs developed between day 21 and 28 and subsided around day 42. Anti-CD97EGF1 treatment significantly reduced the synovial tissue infiltration and analysis of the cellular subsets revealed a trend in reduction of neutrophils at day 49 after immunization. However, as the authors point out, this does not exclude the possibility that CD97-CD55 blockade inhibits infiltration of other cell subsets at earlier time points.

The reason for the different results between EAE and experimental arthritis with respect to CD97-CD55 interactions in disease development is not clear at this point, but may simply relate to differences in treatment duration. Alternatively, differences may relate to anatomical differences (joint in experimental arthritis vs. CNS in EAE), or to the possible dependency on neutrophils in disease

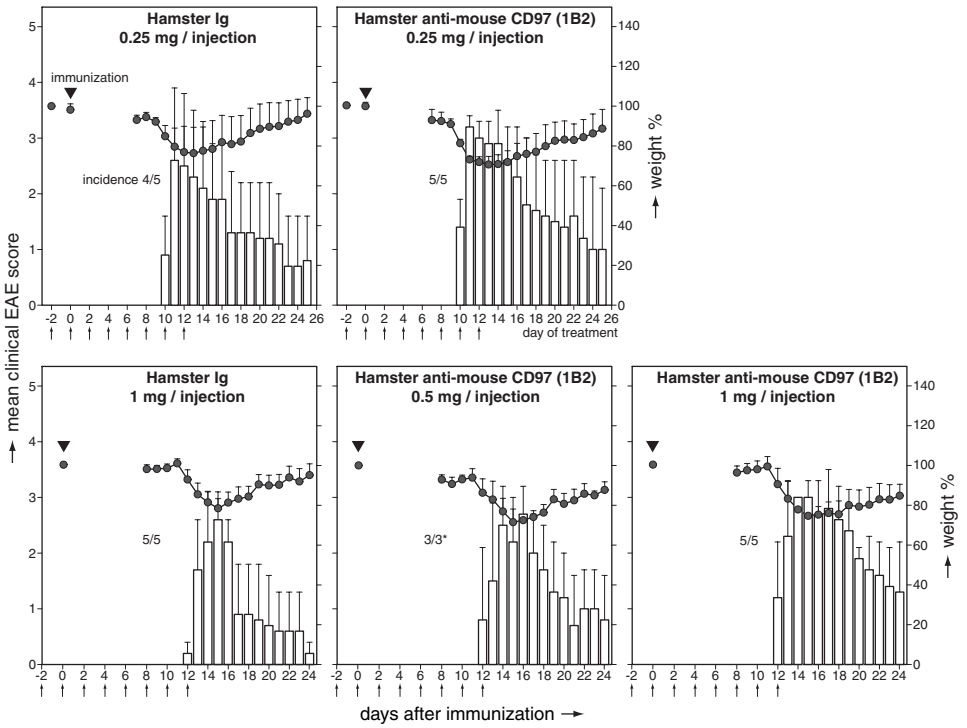

Figure 5. Early treatment with anti-CD97EGF1 (1B2) does not affect EAE development.
SJL/J mice were immunized with $PLP_{139-151}$ in CFA. Treatment (arrows) with CD97EGF1 Mab or hamster immunoglobulin control antibody was initiated two days before immunization (arrowheads) and applied every other day thereafter, until day 12 after immunization. In two separate experiments mice received 0.25, 0.5 or 1 mg CD97EGF1 Mab. Mice were weighed and scored for clinical signs of EAE daily. Bars represent the mean clinical scores, lines the mean weight. (*) two mice died before the onset of EAE.

development. Blocking of CD97-CD55 interactions alters neutrophil function, as demonstrated in two different *in vivo* models. Mab anti-CD97EGF1 (1B2) inhibited neutrophil migration in experimental colitis and reduced the neutrophil-dependent IL-8 induced hematopoietic stem cell mobilization[119](M.J. Kwakkenbos, personal communication). Taken together, these data indicate that CD97-CD55 interactions are specifically important for neutrophil adhesion or function, and may affect other cell types as well. Abundant numbers of neutrophils are present in rhesus monkeys[120,121], rats[122] and mice[123] with hyperacute EAE, representing models for acute forms of MS or ADEM. In this respect, it would be of interest to determine whether blocking CD97-CD55 interactions in these models can reduce EAE.

Apart from Mab anti-CD97EGF1, also Mab anti-CD97EGF3 (clone 1C5), which targets CS-binding EGF domains, inhibited neutrophil migration[119] and experimental

arthritis development (E.N. Kop, personal communication). It is of note that 1C5 binds CD97, but not EMR2, since mice lack the *EMR2* gene. This also applies for all other anti-mouse CD97 Mab. Combined CD97-ligand interaction with either CD55/CS or VLA-5/CS have been shown to act synergistically on cell function[106,107]. This is interesting, because combined targeting of EGF domain 1 and EGF domain 3 (in the mouse) of CD97 by Mab treatments would likely result in enhanced treatment efficacy. The functional role of CD97 in EAE can be elucidated using mice with a targeted deletion for the total CD97 gene. Other species than mice should be used to determine the functional effect of CD97-integrin interactions, since mouse CD97 does not contain the RGD motif that is present in human CD97[103,124].

Role for CD55 in protection from complement-mediated damage in MS brain tissue

Complement activation is thought to contribute significantly to inflammation of the CNS in MS and EAE[125,126]. The anaphylatoxins C3a and C5a are involved in the recruitment of inflammatory cells into the CNS[127,128]. Deposition of C3b increases myelin phagocytosis and interaction with its receptor on MΦ or microglial cells can induce release of TNF-α and nitric oxide[129]. Complement can also exert direct cytotoxic effects by deposition of the membrane attack complex on myelin producing cells in the CNS, the oligodendrocytes[130]. Membrane bound or soluble regulatory proteins inhibit formation of C3 and/or C5 convertases and normally protect tissues against the complement system. These proteins include CD46, CD55, CD59, complement receptor type-1 (CR-1), C4b binding protein, and factors H and I. CD55 protects cells from complement-mediated damage by accelerating the decay of C3/C5 convertases and is therefore also known as decay accelerating factor (DAF)[131]. CD55 plays the major role in protecting endothelial cells from complement-mediated damage[132]. *In vitro* expression of CD55, but not of CD46 and CD59, by endothelial cells can be increased by the proinflammatory cytokines TNF-α and IFN-γ, and also by the membrane attack complex. This increase in CD55 expression reduced complement deposition significantly[132]. In MS brain lesions different cytokines and complement components are present which might also be capable of upregulating CD55. Indeed, we observed upregulated CD55 expression by endothelial cells in MS brain lesions compared to expression in normal white matter (chapter 4). In MS brain, CD55 may also protect neurons from complement-mediated damage by expressing CD55. In corroboration, it was recently demonstrated that neurons in EAE-affected grey matter tissue of marmoset monkeys also expressed CD55[133]. Neurons that expressed CD55 were located in close vicinity to inflammatory lesions, whereas CD55 was not expressed by neurons in non-affected areas. These findings indicate that CD55 is induced on neurons under inflammatory conditions. Additional *in vitro* studies showed that ectopic expression of CD55 was critical in protecting human neuroblastoma cells from complement-mediated damage. In conclusion, during inflammatory attacks

on the CNS, as in MS, brain endothelial cells and neurons are likely protected from complement-mediated damage by increased expression of CD55.

CD55 is involved in cellular activation and signal transduction

Besides preventing cells from complement-mediated damage, CD55 is also involved in cell activation and signal transduction of different cell types, even though the CD55 molecule does not traverse the plasma membrane[134]. Incubation of monocytes with an antibody against CD55, which blocks complement protection, has been shown to result in increased glucose consumption and phagocytic uptake of latex beads as well[135]. Crosslinking of CD55 on human monocytes and granulocytes nduced cytoplasmic Ca^{2+} influx and oxidative burst[136]. Also T cells respond to CD55-mediated signaling. CD55 expressed at low levels by resting human T cells was upregulated after activation by mitogens. Crosslinking of CD55 by antibodies, together with costimulation by phorbol esters, induced T cell proliferation. This anti-CD55 induced proliferation was blocked by phospholipase C, which removes the GPI-linked CD55 from the cell[137].

These data imply that CD55 acts as a receptor involved in signal transduction. The involvement of CD55 in signal transduction was demonstrated by transfection of murine thymoma cells with CD55. Transfection resulted in the transduction of early activation events, leading to tyrosine phosphorylation and association with Scr-related kinases p56lck and p59fyn[138]. More recently, it has been shown that crosslinking of CD55 on Jurkatt T cells was insufficient to induce Ca^{2+} influx and phosphorylation of PLC-γ. However, TCR-ζ, p56lck and ZAP-70 tyrosine residues were phosphorylated, leading to IL-2 secretion[139]. Taking these data into consideration, it is tempting to speculate that binding of CD97 to CD55 can lead to cell activation and signal transduction of the CD55 expressing cell. Therefore, it is possible that binding of CD97 to CD55 leads to reverse signaling of the CD55 expressing cell. As CD55 is expressed by infiltrating leukocytes (T cells and MΦ) and by resident activated microglia cells in MS brain lesions, CD97-CD55 ligation on MΦ or microglial cells may stimulate the phagocytosis of myelin and the production of oxygen radicals, while T cells might be able to proliferate when appropriate costimulatory signals are available.

Functional aspects of CD44v3, v7 and v10 in MS and EAE

Under defined conditions, such as leukocyte activation, CD44v isoforms are specifically upregulated[140-142]. Particular CD44v isoforms have been implicated to play a role in the development of autoimmune disease in man. Experimental models for IBD, RA and alopecia areata (a skin-associated autoimmune disease) have confirmed that a selected number of CD44v isoforms contribute significantly to disease development, by using antibody treatment or genetic deletion of specific isoforms. We therefore hypothesized that CD44v isoforms are crucial for the development of MS and EAE. Previously, we showed that $PLP_{139-151}$-induced EAE could be ameliorated by early treatment (day 0, 2, 4 and 6 post immunization) of SJL/J mice with a mixture of

antibodies against CD44v6, v7 and v10 isoforms. Mice treated with control antibody showed expression of CD44s and CD44v10 by infiltrated mononuclear cells in the CNS.

As previously demonstrated, CD44v4-v6 were not expressed in normal human white matter, whereas astrocytes expressed CD44s and endothelial cells expressed both CD44s and CD44v10[143]. Expression of CD44s was upregulated in inflammatory brain lesions of MS patients[144] and EAE-affected mice[145,146], however it was not known whether these molecules also contained the variant regions. We therefore explored whether CD44v3-v10 (excl. v7) isoforms are expressed in MS brain lesions (chapter 5). The CD44v4, v5, v6 and v9 isoforms were not expressed in MS and in control brain tissue. Expression of CD44v7 could not be assessed by immunohistochemistry (for details, see chapter 5), but it is known that murine astrocytes and activated leukocytes express CD44v7 upon activation[147]. We demonstrated that both CD44v3 and v10 were expressed in MS brain lesions. The expression patterns of CD44v3 differed from CD44v10. Endothelial cells expressed CD44v10, but not CD44v3 in MS and control brain. Perivascular cells in pre-active lesions expressed both CD44v3 and v10, but more cells expressed CD44v10 than CD44v3. Moreover, in activated (HLA-II$^+$) areas, large numbers of astrocyte-like cells expressed CD44v10, but not CD44v3. In active lesions, MΦ-like cells with a foamy appearance expressed both CD44v3 and CD44v10. Taken together, increased expression of CD44v3, (v7) and v10 in MS brain lesions suggests that these isoforms functionally contribute to MS. By using mice with a single genetic deletion for CD44v7 or CD44v10, we analyzed whether these isoforms contribute to the development of EAE, induced by active immunization or adoptive transfer.

CD44 ligands in MS brain tissue

During MS and EAE, leukocytes adhere to the vascular endothelium in the CNS, extravasate and form perivascular infiltrates (Figure 2, Introduction). Adhesion and transmigration of cells through the BBB involves a multi-step process, consisting of capture, rolling, firm adhesion and subsequent diapedesis. This process depends on the expression of several adhesion molecules, chemokines and interactions with ECM components. CD44 and its variant isoforms play an important role in leukocyte adhesion and transmigration by binding different components of the ECM (HA, CS, HS, collagen, fibronectin and laminin), chemokines (MIP-1β, RANTES), OPN and gelatinases (MMP-2/9). All these CD44-ligands are present in the brain during MS and EAE and may regulate CD44-mediated leukocyte entry during inflammation.

CD44-ECM interactions in the CNS

GAG-modified CD44 molecules can bind collagen, fibronectin and laminin. In contrast to other tissues, these ECM components are limited to vascular and perivascular areas of the brain, since connective tissue is lacking[148]. CD44-

expressing infiltrating leukocytes may utilize these components to facilitate their CNS transendothelial migration. HA is a linear polysaccharide that is non-covalently linked to many other ECM components, such as proteoglycans of the lectican family (e.g aggrecan and versican) and CS. HA is not bound to a core protein, in contrast to CS and HS. The ECM of the brain is mainly composed of HA, which is probably produced by astrocytes[149]. HA is not present in the perivascular basement membrane, but expression of HA is inducible on microvascular endothelial cells by stimulation with LPS or proinflammatory cytokines (TNF-α, IL-1β)[150]. Activated cells that express an active form of CD44 are recruited to sites of inflammation by binding to endothelium-bound HA (Figure 6)[151]. CD44-HA interactions are required for initial capture and rolling of leukocytes on endothelial cells. The binding of CD44 to HA results in upregulation of VCAM-1 and ICAM-1 on endothelial cells[152]. It was recently demonstrated that the subsequent step of transmigration, i.e. firm adhesion, is crucially dependent on the association of CD44 with VLA-4 that form a bimolecular complex within the cytoplasm (Figure 6). Both anti-CD44 and anti-VLA-4 Mab can reduce CNS infiltration and EAE development[153,154]. Mab anti-CD44 specifically prevents leukocyte migration into the CNS and does not affect lymph node migration, whereas Mab anti-VLA-4 prevents cells from trafficking into the CNS and lymph nodes[153,154]. Interestingly, a placebo-controlled double-blind clinical trial demonstrated that treatment of relapsing-remitting MS patients with the α4-integrin antagonist Natalizumab reduced the number of inflammatory brain lesions and clinical relapses[155].

Binding of CD44 to HA can be regulated by CD44v isoforms. In rat carcinoma cells, CD44v isoforms form homotypic molecular aggregates in the plasma membrane. This aggregation of CD44v proteins significantly increased the binding capacity to HA[156]. In activated human T cells, combined expression of CD44v isoforms is required for adhesion to HA, and can be induced by cross-linking with Mab[157]. Conversely, depending on the cell type used, splice variants can also inhibit HA-mediated binding[158]. Thus, depending on the cell type, ligation of CD44 to HA appears to depend on the presence and combination of spliced variants. By an *in vitro* tissue-binding assay (Stamper-Woodruff), using human cerebellar tissue, it was demonstrated that CD44-HA interactions contributed to peripheral blood lymphocyte adherence to white matter regions. This interaction was abrogated by anti-CD44 Mab and hyaluronidase treatment[159]. By using specific CD44v-transfected Namalwa cells, it was subsequently demonstrated that CD44v isoforms did not contribute to CD44-HA-mediated binding to white matter regions[160]. However, as discussed above, this does not exclude the possibility that CD44v isoforms may contribute to HA-mediated binding by other cells to brain tissue. In this particular model, the brain tissue was not in an inflammatory state. In inflamed brain tissue, HA may be present in immunostimulatory low molecular weight forms. Endothelial cells likely also express HA, which may change the binding capacity for variant isoform regions in CD44. Alternatively, CD44v-mediated binding may occur

independent of HA, as discussed below. It will be of interest to determine whether particular isoform combinations adhere to normal and MS brain tissue. This can be studies by using multivalent CD44v isoform probes, in a comparable approach used for CD97.

CD44 as a docking site for inflammatory mediators in MS and EAE brain tissue

Several CD44 binding components such as gelatinases, MIP-1β, RANTES and OPN are produced by inflammatory cells in MS and EAE lesions. In normal human brain different cell types express MMP-9, e.g. endothelial cells, perivascular mononuclear cells, astrocytes and microglia. There is a widespread dominant expression of MMP-9 by MΦ and reactive astrocytes in demyelinating MS lesions[161,162]. In EAE, increased MMP-9 mRNA expression levels in brain tissue coincided with the peak of the clinical disease course[163,164]. Intracerebral injection or induction of MMP-2 and MMP-9 resulted in leukocyte recruitment, ECM degradation and impairment of BBB function[165-167]. MIP-1β and RANTES were produced by perivascular and parenchymal foamy MΦ in MS brain lesions[168]. Additionally, in MS brain tissue RANTES was also expressed by endothelial cells, astrocytes and perivascular T cells[169,170]. In a relapsing-remitting EAE model, relapses were associated with elevated MIP-1β and RANTES expression levels in the spinal cord[171]. OPN transcripts are exclusively upregulated in MS patients and not in controls, as demonstrated by cDNA microarray analysis of brain tissue samples from MS patients (plaques) and controls[172]. Both MS and rat EAE CNS lesions contained OPN-expressing astrocytes and MΦ[146]. In EAE, OPN levels were significantly increased at the onset of disease and expression of CD44 was detected on similar cell types. Taken together, these findings suggest that in MS and EAE brain lesions, CD44 molecules can collect and locally concentrate matrix-degrading enzymes, chemokines and cytokines, which may contribute to the development and chronicity of the disease.

Are CD44v3 and v10 involved in CNS infiltration in MS and EAE?

Peripheral blood lymphocytes of MS patients express elevated levels of CD44s compared to healthy controls. It was previously demonstrated by RT-PCR that CD44v6-v10 isoforms are expressed on PBMC of MS patients[173]. This study focused on the expression of CD44v6+v10 in patients with human T-lymphotropic virus type 1 (HLTV-1)-associated myelopathy/tropical spastic paraparesis (HAM-TSP), a chronic disease of the spinal cord. Expression of CD44v6+v10 isoforms was increased in PBMC of HAM-TSP patients compared to controls. The number of MS patients was, however, too small to conclude whether or not there are significant differences in CD44v6+v10 expression compared to healthy controls. It has been shown that specific splice variants of CD44 are upregulated in other autoimmune diseases, including CD44v3 and CD44v7 on activated peripheral blood leukocytes of patients with IBD, RA and Sjögren's syndrome[174]. It would be

interesting to determine whether the combination of CD44v isoforms expressed by peripheral blood leukocytes from MS patients differs from healthy controls and whether their expression is related to disease activity. Furthermore, it is of interest to assess whether leukocytes from MS patients exhibit an increased CD44v isoform specific adherence and transmigration through endothelial cells, by using blocking CD44v Mab.

During MS and EAE leukocytes may utilize CD44v isoforms for adhesion and transmigration into the CNS. In chapter 5 we demonstrate that CD44v3 and v10-expressing cells were present in perivascular infiltrates in MS brain. Increased numbers of brain infiltrated $CD4^+$ T cells expressed CD44v10 in EAE mice, compared to control immunized mice. The number of $CD4^+CD44v10^+$ cells was positively correlated with the disease course. CD44v3 plays an important role in leukocyte extravasation. This was demonstrated by the fact that anti-CD44v3 Mab impaired leukocyte egress in mouse DTH responses, where CD44v3 was expressed by endothelial cells and infiltrated leukocytes[175]. However, in MS brain CD44v3 was not expressed by the endothelium. Nevertheless, CD44v3 may facilitate transmigration of brain infiltrating leukocytes by attaching to HS and CS (Figure 6). In brain tissue, HS-proteoglycans are expressed by endothelial cells and within basement membranes. By using an *in vitro* adhesion and transmigration assay, it was demonstrated that the transmigration of monocytes across an endothelial cell monolayer is partly dependent on HS structures on both monocytes and endothelial cells[176]. It remains to be determined whether CD44v3 also contributes to this process. The maturation of monocytes into mature DC is accompanied by an increased CD44v3 expression. That CD44v3 participates in adhesion is demonstrated by the fact that adhesion of mature DC to immobilized HA was inhibited by anti-CD44v3 Mab. Thus CD44v3 may facilitate diapedesis by enhanced binding of leukocytes to HA and HS on brain endothelium (Figure 6).

Like CD44v3, CD44v10 is mainly involved in cell migration, adhesion and extravasation[177,178]. Blocking of CD44v10 by Mab in mouse DTH and alopecia areata reduced the cellular infiltration at the site of inflammation[175,179]. In white matter of MS patients and controls we found that CD44v10 was expressed by endothelial cells (chapter 5)(Figure 6), which is in accordance with previous findings[143]. CD44v10-expressing cells can attach in a HA-independent manner to CD44s-expressing activated microvascular endothelial cells. This binding was significantly reduced by pre-incubation of lymphocytes with Mab against CD44v10. Moreover, in a cellular aggregation assay, CD44v10-expressing lymphocytes formed homotypic clusters that were not formed in the presence of anti-CD44v10 Mab[178]. More recently, it was demonstrated that homotypic CD44v10/CD44v10 and heterotypic CD44v10/CD44s interactions are controlled by CS-side chains (Figure 6) which bind to a Bx7B motif within the CD44v10 region[180]. Additionally, CD44-CS interactions have been shown to mediate leukocyte rolling under shear stress[181]. However, it is unclear whether CD44v10 participated in this process. Altogether,

these data provide evidence that CD44v10 and CD44s on activated MS brain endothelial cells may serve as ligands for CD44v10-expressing blood leukocytes. In activated (HLA-II$^+$) areas in MS and control brain tissue astrocyte-like cells expressed CD44v10. In accordance, CD44v6/v7 and v10 isoform expression can be induced *in vitro* on murine astrocytes by stimulation with phorbolesters or TNF-α/IFN-γ[147]. Co-incubation of activated astrocytes with CD44v-expressing T cells resulted in HA-dependent adhesion. In this way, CD44v-HA-CD44v interactions between astrocytes and T cells may control the retention of lymphocytes within the CNS.

CD44v3+v10 may also stimulate inflammation in MS in a different way. By their capacity to obtain HS (binding CD44v3) and CS (binding CD44v3+v10) side chains[182,183], CD44v3 and CD44v10 can collect and locally concentrate proinflammatory OPN[184,185], growth factors (i.e. heparin-binding growth factor)[186], MIP-1β[187] and RANTES[188], thereby attracting cells and creating a proinflammatory environment. Moreover, blocking CD44v3 or CD44v10 expression resulted in reduced production of proinflammatory cytokines by monocytes[175,189]. These data indicate that expression of CD44v3 and CD44v10 in MS brain may promote chronic

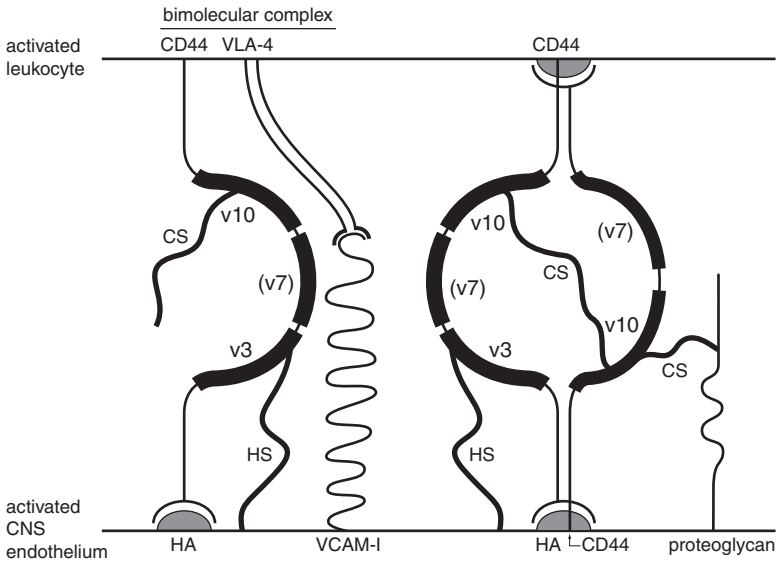

Figure 6. CD44v-mediated leukocyte adhesion to activated CNS endothelium in MS.
In MS brain tissue activated endothelial cells express increased levels of HA, HS and possibly CS. Furthermore, endothelial cells in brain tissue constitutively express CD44v10. These cell surface and cell-associated (by glycoproteins) ECM molecules may facilitate the adhesion of activated leukocytes by binding their respective receptors, as explained in the text.

inflammation by stimulating cell adhesion and transmigration and by increasing the levels of proinflammatory mediators. Figure 6 illustrates the potential CD44v-ligand interactions between activated leukocytes and activated CNS endothelial cells in MS and EAE.

Possible contributions of CD44v7 in autoimmune disease

Using two different mouse EAE models (active immunization and adoptive transfer), and two different mouse strains (SJL/J and C57BL/6) we demonstrated in chapter 5 that EAE was reduced by genetic deletion of CD44v7. At present it is not clear by which mechanism CD44v7 exerts its effects in EAE, but previous studies suggest that CD44v7 isoforms modulate 1) cellular activation, 2) Th1 cell development and 3) apoptosis, extrapolated from the following observations.

1) CD44v7 is expressed after CD40 ligation on lamina propria mononuclear cells[190]. Transgenic mice that constitutively express CD44v4-v7 on thymocytes and peripheral T cells exhibit accelerated responsiveness to stimulation with T cell mitogens and T-dependent antigens[191]. Conversely, blocking of CD44v7 expression by Mab or genetic deletion downmodulates CD40L expression and lymphocyte proliferation[192,193]. These studies furthermore demonstrated that CD44v7 affects lymphocyte priming, whereas antigen-specific recall responses where not or only slightly affected. In agreement, we did not observe effects of CD44v7-deletion on autoantigen-specific T cell proliferation around the day of EAE onset, by *in vitro* restimulation.

2) Blocking CD44v7 by Mab treatment or genetic deletion reduces the production of proinflammatory cytokines (TNF-α, IL-12, IFN-γ)[174,190,192,194,195]. In a Th1-dependent DTH model, anti-CD44v7 Mab treatment reduced the number of IFN-γ producing cells and increased the number of IL-10 producing cells at the inflammatory site[194]. In TNBS-induced colitis, treatment with anti-CD44v7 did not prevent mild infiltration of the intestine[195]. However, inflammation in treated mice was only transient and was followed by complete recovery with increased production of IL-10 and decreased production of IL-12 at the inflammatory site as well as in the periphery. The IL-10 contributed significantly to the recovery from inflammation, as could be deduced from the observation that co-administration of neutralizing anti-IL-10 antibodies completely abolished the therapeutic effect of Mab anti-CD44v7[196]. Moreover, chronic enterocolitis in IL-10$^{-/-}$ mice was diminished in mice with an additional deletion of CD44v7[190]. Also in CD patients, increased levels of IL-10 were observed after *in vitro* treatment of peripheral blood leukocytes with CD44v7 Mab[174]. It is intriguing that CD44v7 is one of the isoforms binding OPN. OPN is a Th1 cytokine/chemokine and has been described to play an important role in MS and EAE[146,172,197]. OPN-deleted mice developed EAE, but the disease severity was reduced in the progressive phase with an increased occurance of remissions compared with wild type mice. The reduced EAE in OPN-deleted mice was associated with increased IL-10 and reduced IFN-γ and IL-12

levels. Furthermore, DNA vaccination against OPN, protected mice from EAE development[198]. CD44v7-cell binding to OPN can promote spreading, motility and chemotactic behavior of cells[199].

3) It has been suggested that upregulation of CD44v7 in response to CD40 ligation protects leukocytes from apoptosis. In experimental colitis, the reduction in the initial inflammatory response in CD44v7$^{-/-}$ mice correlates with increased numbers of apoptotic cells in the inflamed mucosa[190,200,201]. In patients with CD, CD44v7 expression is constitutively upregulated on lamina propria mononuclear cells at inflammatory sites[174]. *In vitro* blockade of CD44v7 with Mab or recombinant fusion protein induces apoptosis of lamina propria leukocytes of CD patients, which was not observed for cells obtained from normal mucosal tissue[201]. Constitutive CD44v7 expression prevents T cells from going into apoptosis, as was shown by *in vitro* anti-CD3 stimulation of *in vivo* pre-activated mesenteric lymph node cells, whereas CD44v7 deletion resulted in opposite effects[193]. Increased susceptibility to apoptosis in CD44v7$^{-/-}$ mice was associated by an upregulation of anti-apoptotic genes.

Does genetic deletion of CD44v7 promote apoptosis in EAE?

Reduced EAE burden in CD44v7$^{-/-}$ SJL/J mice is associated with a reduced number and size of infiltrates in spinal cord tissue compared with wild type mice (chapter 5). This finding may imply an impaired migration into the spinal cord or an increase in apoptosis of infiltrated cells. Moreover, autoantigen-specific lymph node cell proliferation appeared to be reduced in CD44v7$^{-/-}$ mice at late time points after EAE induction in both SJL/J and C57BL/6, compared to wild type mice. This may mean that at late time points after EAE induction the frequency of autoantigen-specific lymphocytes is reduced in CD44v7$^{-/-}$ mice or that autoantigen-specific CD44v7$^{-/-}$ cells divide less or are more susceptible to apoptosis after late restimulation. In a pilot experiment we aimed to detect differences in MOG_{35-55}-specific *in vitro* proliferation versus cell death between wild type and CD44v7-deficient mice. Draining lymph node cells were isolated and labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester) at day 12 after immunization with MOG_{35-55}. Four days after *in vitro* restimulation with MOG_{35-55}, cells were harvested and stained with annexin-V and propidium iodide to determine the number of apoptotic cells. Percentages of proliferating cells (diluted CFSE) and apoptotic cells (annexin-V positive) were similar between wild type and CD44v7$^{-/-}$ cells (unpublished data), arguing that CD44v7 deletion does not promote apoptosis.

These data contradict with the finding that CD44v7$^{-/-}$ cells are more susceptible to apoptosis than wild type cells in a TNBS-induced experimental colitis model[193]. In this model it was shown earlier that increased apoptotic activity was present in inflamed lesions of CD44v7$^{-/-}$ mice, as demonstrated by TUNEL assay[190]. Mice were treated with TNBS and mesenteric lymph node cells were subsequently stimulated with anti-CD3 Mab or PHA. Possible explanations for the discrepancy

in data may include differences in the mouse strains, cells, and stimulations used. Similar findings were reported about OX40 (by using AND TCR transgenic mice), whereas early cellular division rates and apoptotic cell numbers, at day 4 after antigen stimulation, were not affected by stimulation or deletion of OX40[202,203]. However, a dramatic increase in cell death of $OX40^{-/-}$ T cells occurred between day 6 and day 12 after *in vitro* antigen stimulation[203]. OX40 stimulation promoted the accumulation of dividing cells through more cellular division cycles and decreased apoptosis of antigen-specific T cells *in vivo* and *in vitro*[202,203]. It is still unclear whether or not CD44v7 deficiency influences antigen-specific survival versus apoptosis in EAE development. By using $PLP_{139-151}$ TCR transgenic mice[204] backcrossed with $CD44v7^{-/-}$ mice, the hypothesis that CD44v7 deficiency decreases the life span of antigen-activated $CD4^+$ T cells by promoting the induction of apoptosis could be further tested.

Concluding remarks

Taken together, the studies described in this thesis suggest that PG, CD97 and CD44v isoforms are involved in MS and EAE. We postulate that during demyelinating disease, phagocytes carry PG into the CNS. We have demonstrated that PG accumulates in the CNS of MS patients and of non-human primates with EAE inside different cell subsets including granulocytes, MΦ and DC. PG may persist in the CNS, because the CNS lacks lysozyme and NAMLAA, enzymes that can degrade PG. In mice, *S. aureus* PG facilitated an autoantigen-specific inflammatory immune reponse in the CNS, that resulted in EAE development. *S. aureus* PG stimulated antigen uptake, antigen presentation, costimulation and proinflammatory cytokine production by DC *in vitro*. Furthermore, PG induced Th1 cell polarization and expansion via DC. Therefore, in MS as well as in EAE, PG may stimulate autoimmune-mediated reactions in the periphery and in the CNS.

CD97 and CD44v isoforms contribute to a variety of cellular processes, e.g. cell activation, adhesion and apoptosis. We have demonstrated that CD97 and its ligand CD55, and CD44v3 and CD44v10 were expressed by different cell types in MS brain lesions. In functional experiments in mice, it appeared that EAE was not reduced by blockade of the CD97-CD55 interaction early after disease induction. Further research is required to determine whether CD97 parcipitates in MS and EAE by interacting with its other ligands (CS-B, VLA-5 and $\alpha v\beta 3$ integrins). Genetic deletion of CD44v7 or CD44v10 in mice did reduce EAE. Additional studies are required to elucidate by which mechanisms CD44v7 and CD44v10 contribute to MS and EAE. Based on studies in experimental colitis and DTH responses, CD44v7 and CD44v10 may participate in EAE and MS by stimulating cellular activation, adhesion, Th1 cell development and/or apoptosis.

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Abbreviations

ADEM	acute disseminated encephalomyelitis
APC	antigen presenting cell(s)
BBB	blood-brain barrier
CARD	caspase recruitment domain
CD	Crohn's disease
$CD44^{-/-}$	CD44 knock-out
CD44s	CD44 standard
CD44v	CD44variant
CFA	complete Freund's adjuvant
CFSE	carboxyfluorescein diacetate succinimidyl ester
CNS	central nervous system
CpG	cytosin-guanosin dinucleotide
CR-1	complement receptor type-1
CS	chondroitin sulfate
CSF	cerebrospinal fluid
DAF	decay accelerating factor
DC	dendritic cell(s)
DTH	delayed-type hypersensitivity
DTPA	diethylene-triamine-pentaacetic acid
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
EGF	epidermal growth factor
EMR	EGF-like module containing mucin-like receptor protein
ETL	EGF-TM7-latrophilin-related protein
FIRE	F4/80-like-receptor
GAG	glycosaminoglycan
GlucNAc	*N*-acetylglucosamine
GM	GlucNAc-MurNAc
GM-DP	GM-L-ala-D-Glu
$GM\text{-}Tri_{DAP/lysine}$	GM-L-ala-D-Glu-mDAP/lysine
GPI	glycosylphosphatidylinositol
HA	hyaluronic acid
HAM-TSP	HTLV-1-associated myelopathy/tropical spastic paraparesis
HC	healthy controls
HLA	human leukocyte antigens
HLTV-1	human T-lymphotropic virus type 1
HS	heparan sulfate
IBD	inflammatory bowel disease
IFA	incomplete Freund's adjuvant

iPG	insoluble peptidoglycan
IRF-3	interferon regulatory factor 3
LFB	luxol fast blue
LPS	lipopolysaccharide(s)
LRR	leucine-rich repeat
LTA	lipoteichoic acid(s)
Mab	monoclonal antibody
MAG	myelin associated glycoprotein
MΦ	macrophage(s)
MBP	myelin basic protein
mDAP	meso-diaminopimelic acid
MDP	muramyl dipeptide
MHC	major histocompability complex
MMP	matrix metalloproteinase(s)
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
MurNAc	*N*-acetylmuramic acid
NAMLAA	*N*-acetylmuramyl-L-alanine amidase = PGRP-L
NBS	nucleotide-binding site
NO	nitric oxide
Nod	nucleotide-binding oligomerization domain
OIND	other inflammatory neurological diseases
OND	other neurological diseases
OPN	osteopontin
PAMP	pathogen associated molecular pattern(s)
PAS	periodic acid-Schiff
PBMC	peripheral blood mononuclear cells
PG	peptidoglycan
PGRP-L	peptidoglycan recognition protein long form = NAMLAA
PHA	phytohemagglutinin
PLP	proteolipid protein
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptors
PTX	*Bordetella pertussis* toxin
RA	rheumatoid arthritis
RGD	Arg-Gly-Asp
sCD97	soluble CD97α
sPG	soluble peptidoglycan
TA	teichoic acid(s)
TCM	cell-culture medium
Th	T helper

TIR	Toll/IL-1 receptor
TLR	Toll-like receptor(s)
TM7	seven-span transmembrane domain
TNBS	trinitrobenzene sulfonic acid

chemokine nomenclature

IL-8	CXCL8
IP-10	CXCL10
MCP-1	CCL2
MCP-3	CCL7
MIP-1β	CCL4
MIP-2	CXCL2
RANTES	CCL5

Summary

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). We are interested in the mechanisms involved in the immunopathogenesis of MS, especially in microbial signals able to break T cell tolerance, and in the function of molecules on the activated cells in costimulation, migration, adhesion and apoptosis.

Peptidoglycan (PG) is a major cell wall component of Gram-positive bacteria, that activates the innate immune system by binding to TLR2/6, Nod1 (Card4) and Nod2 (Card 15) receptors. The enzymes lysozyme and NAMLAA are produced by phagocytic cells and can degrade PG. However, after bacterial phagocytosis, PG can persist intracellularly when the digestion is incomplete. During chronic inflammation, PG-containing antigen presenting cells (APC) accumulate at inflammatory sites, as previously demonstrated in patients with colitis, arthritis and MS. At these inflammatory sites or in peripheral lymphoid tissues PG may serve as a costimulatory factor in autoimmune disease by overriding tolerance against self-antigens.

To obtain more insight into the functional relevance of PG in MS, we compared the presence of PG and its degrading enzymes in the CNS of two distinct non-human primate EAE models **(chapter 2)**. As in brain tissue of MS patients we here demonstrate that EAE-affected brain tissue from marmoset and rhesus monkeys contained elevated numbers of cells with PG, compared to control immunized animals. Interestingly, chronic EAE in marmoset monkeys was accompanied by a modest number of PG-containing cells in the brain, whereas brain tissue from rhesus monkeys that had developed acute EAE contained abundant numbers of PG-containing cells. Lysozyme was only sporadically expressed in EAE-affected brain tissue from both monkey species. In contrast, NAMLAA was expressed on many perivascular cells in EAE-affected brain tissue from rhesus monkeys, but only by few perivascular cells in marmoset monkeys. Double-labeling revealed that NAMLAA was mostly expressed by neutrophils. Thus, in EAE-affected brain tissue, PG was present within significantly higher numbers of APC compared to controls. Conversely, lysozyme was mainly absent in normal and inflamed brain tissue, and NAMLAA was only abundantly expressed during acute CNS inflammation in rhesus monkeys. These data suggest that PG persists inside APC in the CNS and may contribute to the inflammation by stimulating TLR/Nod receptors.

To further elucidate the functional contribution of PG to autoimmune disease we investigated the hypothesis that PG acts as a costimulatory factor for disease development in MS, by using mouse EAE **(chapter 3)**. EAE is normally induced by s.c. immunization of autoantigens admixed in a strong adjuvant with attenuated *Mycobacterium tuberculosis* bacteria. Inflammatory *Staphylococcus aureus* PG could replace whole *M. tuberculosis* in EAE induction. We demonstrate that

S. aureus PG was transported to the spleen and draining lymph nodes after immunization with *S. aureus* PG-containing adjuvant. In the draining lymph nodes *S. aureus* PG was localized within dendritic cell (DC) clusters. Clusters of PG-containing cells were also present in the spleen of rhesus monkeys that developed EAE **(chapter 2)**. Compared to control mice that were immunized with autoantigen in incomplete Freund's adjuvant (IFA), PG increased autoantigen-specific T cell proliferation and Th1 cell development, when the autoantigen-IFA mix was supplemented with PG. By using bone marrow-derived DC, *S. aureus* PG was shown to stimulate antigen uptake by DC, DC maturation, and subsequent differentiation and proliferation of Th1 cells. Taken together, proinflammatory PG may stimulate autoimmune-mediated processes in MS, either in the CNS or in the periphery, by accelerating (auto)antigen uptake, initiating DC maturation, and polarizing and expanding (auto)antigen-specific T cells.

CD97 and CD44 are membrane-expressed molecules required for cell-cell and cell-matrix interactions. These molecules contribute to disease development in experimental models for other chronic inflammatory diseases, e.g. arthritis and colitis and may determine interactions between leukocytes, endothelium and the extracellular matrix (ECM) of the CNS during MS and EAE. Here we assessed whether CD97 and CD44v isoforms are involved in the immunopathogenesis of MS and EAE.

CD97 is a member of the 7-span transmembrane receptor family that is expressed on leukocytes early after activation. CD97 is involved in migration and adhesion, by binding to CD55 (decay accelerating factor), $\alpha 5/\beta 1$ and $\alpha v\beta 3$ integrins and ECM components. The CD97-ligand CD55 is expressed on several cell types, protecting them from complement-mediated damage. Here we examined the expression of CD97 and CD55 by immunohistochemistry in MS brain tissue. **Chapter 4** shows that cells in normal white matter did not express CD97, whereas many MΦ or microglial cells and T cells in MS brain lesions expressed CD97. Endothelial cells in normal white matter only modestly expressed CD55. In contrast, in MS brain lesions, endothelial cells expressed high levels of CD55 and different leukocyte subsets also expressed CD55. In active lesions, expression of CD55 was predominantly detected on MΦ or microglial cells. In these lesions, a substantial proportion of cells also expressed CD97. The soluble form of CD97 was found in serum, but not in CSF of a significant number of MS patients.

These findings suggest that local CD97-CD55 interactions may contribute to the immunopathogenesis of MS. CD55 expressed on brain endothelial cells may serve to protect the vessels from complement-mediated damage. Additionally, CD97-expressing cells may utilize CD55 on activated brain endothelial cells to facilitate adhesion and transmigration into the CNS. To determine whether CD97-CD55 interactions are important in disease development, we blocked the interaction

between CD97 and CD55 by CD97 Mab treatment in the priming phase of mouse EAE. This treatment regimen did not affect the onset and severity of disease. However, these data do not exclude the possibility that CD97-CD55 interactions are involved in EAE development, since the treatment duration might have been suboptimal. Alternatively, CD97 may participate in MS and EAE by binding to integrins or ECM components. By using mice with a genetic deletion for CD97, we can elucidate whether CD97 functionally contributes to EAE development.

CD44 and its variant isoforms (CD44v1-v10) are required for many different biological processes such as lymphocyte activation, costimulation, adhesion/ extravasation into inflammatory sites and apoptosis. The CD44 molecule also serves as a docking site for mediators of inflammation by binding cytokines, growth factors and chemoattractants. One gene encodes different CD44v isoforms, which emerge as a result of complex RNA splicing. We determined whether CD44v isoforms are involved in the immunopathogenesis of MS and EAE **(chapter 5)**.

It appeared that only CD44v10 was expressed by normal white matter endothelium, whereas both CD44v3 and v10 were expressed by cells in perivascular infiltrates and by microglia or MΦ in active lesions. Due to technical difficulties, we could not assess expression of CD44v7. All other isoforms were not expressed in MS and control brain. Also in mouse EAE, brain-infiltrating cells expressed CD44v10 and were already detected before clinical signs. The number of leukocytes that expressed CD44v10 paralleled the extent of clinical EAE symptoms. CD44v10-expressing $CD4^+$ T cells persisted in EAE brain until late time points after immunization, whereas these cells were absent in control-immunized mice. Single deletion for either CD44v7 or CD44v10 resulted in reduced EAE, which was accompanied by reduced infiltration of the spinal cord. Adoptive transfer experiments demonstrated that CD44v7 exerted its effect on the donor lymphocyte compartment as well as on the recipients APC. No clear differences were detected in autoantigen-specific T cell proliferation and cytokine production around the day of onset. In the remission phase of EAE, CD44v7-deleted lymph node cells showed a reduced proliferation compared to wild type cells. At present, it is unclear by which mechanism CD44v7 and CD44v10 reduced EAE. Currently we are exploring the possibility that deficiency of CD44v7 or v10 promote clearance of inflammation by the induction of apoptosis. Additionally, CD44v10 may also affect cell migration, as indicated in animal models for Th1-mediated DTH responses. It remains to be determined whether CD44v3 can functionally contribute to EAE.

Taken together these data show that a single bacterial component, PG, can create an inflammatory environment. During an autoimmune attack, PG-containing cells most likely enter the inflammatory site and contribute to the pathological process. Intracellular PG may activate cells by engaging TLR or Nod receptors. PG promotes antigen uptake, DC maturation and proinflammatory cytokine production.

In the presence of autoantigens, PG induces autoantigen-specific Th1 cell priming and expansion. This is relevant in MS, since elevated numbers of PG-containing APC are found in the CNS, where autoantigens are released during inflammatory attacks. Moreover, autoantigens and PG are present within APC of peripheral lymphoid organs, where similar events may take place.

CD97 and CD44v isoforms may facilitate cellular activation, costimulation, adhesion, extravasation and survival. By their interactions with other cellular ligands and different ligands in ECM these molecules likely contribute to tissue damage and chronic inflammation in MS. We here show that CD97 and CD44v molecules are expressed in MS brain lesions. EAE models demonstrated that by targeting CD44v7 or CD44v10 the clinical symptoms of the disease can be reduced. Total prevention of CD97 expression, by genetic deletion or long-lasting antibody treatment against ligand-binding CD97EGF domains may give similar effects. Eventually, altering the immune response by the modulation of PG, CD97 and CD44v isoforms in EAE will give more insights in the possibilities for therapeutic interventions in MS.

Samenvatting voor niet-ingewijden

Multipele sclerose (MS) wordt beschouwd als een chronische ziekte van het centrale zenuwstelsel (CZS: hersenen en ruggenmerg). Bij MS patiënten wordt gedacht dat het afweersysteem ontspoord is. Het afweersysteem is normaliter gericht tegen lichaamsvreemde, bedreigende componenten, zoals virussen en bacteriën. Bij MS patiënten worden door het afweersysteem ook lichaamseigen componenten in het CZS aangevallen, zoals de beschermende schede rondom de uitlopers van zenuwcellen (de zogeheten myelineschede). Schade aan de myelineschede en zenuwcellen verstoort de zenuwfunctie. Het klinische beeld wordt bepaald door de plaats van myeline afbraak. MS wordt gekarakteriseerd door ontstekingshaarden (infiltraten) in het CZS. Deze infiltraten worden gevormd door cellen van het afweersysteem, de leukocyten. Er bestaan verschillende typen leukocyten, zoals antigeen presenterende cellen (APC), T en B cellen. APC kunnen bacteriën of virussen, maar ook andere componenten opnemen. De APC herkent bepaalde structuren van bacteriën en virussen en worden hierdoor geactiveerd.

In dit proefschrift hebben we onderzocht of een bacterieel celwand component, het peptidoglycaan, kan bijdragen aan MS. Verder hebben we onderzocht of de cel oppervlakte moleculen CD97 en CD44 betrokken zijn bij MS.

Peptidoglycaan (PG) is een belangrijk bestanddeel van de celwand van bacteriën en werkt ontstekingsbevorderend via activering van specifieke receptoren voor PG. Deze receptoren zijn aanwezig op en in APC. APC kunnen PG vervoeren van de slijmvliezen of van plaatsen van infectie naar de lymfoïde organen. Daarnaast worden PG-bevattende APC ook in hoge aantallen gevonden op plaatsen van chronische ontsteking, zoals in de gewrichten van patiënten met reuma. Eerder onderzoek heeft aangetoond dat ook in hersenweefsel van MS patiënten meer PG-bevattende APC aanwezig zijn in vergelijking met controle personen.

Wij denken dat PG bijdraagt aan de chronische ontsteking bij patiënten met MS door activering van APC. Om deze hypothese te toetsen hebben we gebruik gemaakt van diermodellen voor MS. In die diermodellen wordt het op MS gelijkende ziektebeeld experimentele autoimmuun encefalomyelitis (EAE) genoemd. Lysozym en amidase zijn twee enzymen die geproduceerd worden door APC, en die PG kunnen afbreken. Hierdoor verliest PG zijn ontstekingsbevorderende werking. **In hoofdstuk 2** hebben we de aanwezigheid van PG-bevattende cellen en PG-afbrekende enzymen bepaald in hersenweefsel van twee verschillende aapmodellen voor MS. Voor deze studie hebben we ingevroren weefsels gebruikt uit voorgaande experimenten. EAE in marmoset apen verloopt chronisch, terwijl resus apen een acuut ziekteverloop vertonen. In beide modellen werden bij apen met EAE meer PG-bevattende cellen in de hersenen gedetecteerd in vergelijking met controle dieren zonder EAE. Hersenweefsel van resus apen die een acuut

ziektebeeld vertonen bevatte veel meer cellen met PG in vergelijking met marmoset apen waar het ziektebeeld chronisch verloopt. Verder zagen we dat amidase alleen aanwezig was in infiltraten in het hersenweefsel van resus apen met EAE, en niet in marmoset apen. Lysozym daarentegen, was afwezig was in alle dieren (resus en marmoset), met of zonder EAE. Deze gegevens impliceren dat tijdens EAE cellen naar de hersenen migreren die PG bevatten, waarbij het aantal PG-bevattende cellen afhangt van het verloop van de ziekte. De afwezigheid van PG-afbrekende enzymen in de hersenen suggereert dat PG op deze plek niet afgebroken kan worden en mogelijk bijdraagt aan de chronische ontsteking.

In hoofdstuk 3 hebben we aangetoond dat PG daadwerkelijk kan bijdragen aan EAE ontwikkeling in muizen. EAE treedt niet spontaan op, maar wordt geïnduceerd door een injectie van myeline antigenen in een emulsie van olie met ontstekingbevorderende componenten (gewoonlijk dode tuberculose bacteriën). Het huidige onderzoek toont aan dat PG, als een enkel component van de bacteriële celwand, de gehele tuberkel bacterie kan vervangen voor de inductie van EAE. Verder zagen we dat het ingespoten PG al na 4 uur aanwezig was in de lymfoïde organen in een gespecialiseerd type APC, de dendritische cel. APC kunnen andere cellen activeren, zoals T en B cellen die specifiek gericht zijn tegen de antigenen (deeltjes) die de APC presenteren. Dit gebeurt onder normale omstandigheden in lymfoïde organen, zoals de lymfeklieren en de milt. In een kweeksysteem hebben we dit nagebootst. We hebben aangetoond dat PG de dendritische cel kan stimuleren tot een verhoogde opname van antigenen en productie van ontstekingsbevorderende eiwitten. PG kon via dendritische cellen ook T cellen laten delen en uitrijpen tot ontstekingsbevorderende T helper 1 cellen. Deze gegevens suggereren dat PG in lymfoïde organen of zelfs in het CZS kan bijdragen aan MS, door het geven van ontstekingssignalen aan APC.

Activatie en migratie van cellen vanuit de lymfoïde organen naar plaatsen van ontsteking (bij MS patiënten het CZS) is een zorgvuldig gereguleerd proces. Dit proces wordt onder andere gecoördineerd door moleculen die aanwezig zijn op het oppervlak van zowel de migrerende cellen als de cellen van de bloedvaatjes op plaatsen waar een ontstekingsreactie is. De moleculen van de migrerende cellen en de receptor moleculen op andere cellen grijpen in elkaar als een sleutel en slot. Door deze signalen kunnen de cellen de bloedbaan uit migreren. *In dit proefschrift hebben we tevens onderzocht of de cel oppervlakte moleculen CD97 en CD44 bijdragen aan het ziekteproces in MS, zoals hieronder beschreven.*

CD97 en CD44 zijn moleculen die voorkomen op het oppervlak van verschillende celtypen. Eerder onderzoek heeft aangetoond dat deze moleculen een belangrijke rol spelen bij reuma en chronische darmontsteking (colitis). Deze moleculen dragen mogelijk bij aan het ziekteproces in MS door coördinatie van verschillende cellulaire processen, zoals activatie, deling en migratie naar plaatsen van ontsteking.

CD97 is betrokken bij de activatie, migratie en adhesie van cellen. CD55 was de enige ligand voor CD97 dat in de literatuur bekend was tijdens het onderzoek voor dit proefschrift. Inmiddels is bekend dat CD97 zich ook kan binden aan integrinen en componenten van de extracellulaire matrix (tussenstof). CD55 beschermt cellen tegen complement factoren. Het complement systeem bestaat uit moleculen die helpen bij het bestrijden van infecties. Doordat lichaamseigen cellen moleculen zoals CD55 op hun oppervlakte dragen worden zij niet aangevallen door het complement. Het onderzoek beschreven in **hoofdstuk 4** toont aan dat CD97 en CD55 aanwezig zijn op plaatsen van ontsteking in het CZS van MS patiënten. CD97 kwam niet en CD55 kwam nauwelijks tot expressie op cellen in normaal hersenweefsel. Daarentegen werd in ontstekingsgebieden in MS hersenweefsel een verhoogde expressie van CD55 gevonden op endotheelcellen (cellen die de wand van bloedvaten aan de binnenzijde bekleden). Direct naast deze endotheelcellen kwam CD97 tot expressie op infiltrerende APC, T en B cellen. Daarom veronderstellen wij dat CD97 op leukocyten bijdraagt aan de migratie van cellen uit de bloedbaan naar het CZS, door interactie met CD55 op endotheelcellen. Om te bepalen of CD97 interacties met CD55 van belang zijn voor MS, hebben we EAE in muizen behandeld met een blokkerende antistof die de interactie tussen CD97-CD55 voorkomt. Het gebruikte behandelingsschema resulteerde niet in een verminderde ziektelast. Dit kan betekenen dat 1) de CD97-CD55 interactie niet van belang is in muizen met EAE, of 2) de behandeling van te korte duur was om de ziektelast te verminderen. In vervolgonderzoek kunnen we gebruik maken van muizen die het gen voor CD97 ontberen, om zodoende een beter inzicht te krijgen in de functionele rol van CD97 in MS.

CD44 en de variant isovormen van CD44 (CD44v1-v10) spelen een belangrijke rol bij allerlei biologische processen, zoals celactivatie, -costimulatie, -migratie, -adhesie en geprogrammeerde celdood. Geprogrammeerde celdood is van belang bij het beëindigen van een ontstekingsreactie, b.v. als het infectiegevaar geweken is. Daarnaast kan CD44 als een magneet fungeren voor ontstekingsmediatoren. Zodoende kan CD44 ervoor zorgen dat er lokaal een verhoogde concentratie ontstaat van deze ontstekingsmediatoren. **In hoofdstuk 5** hebben we aangetoond dat bepaalde CD44v isovormen specifiek tot expressie komen in hersenweefsel van MS patiënten en muizen met EAE. In muizen met EAE hebben we op verschillende tijdstippen na ziekte inductie de expressie van CD44v10 bestudeerd in de hersenen. CD44v10 kwam tot expressie op verschillende CZS infiltrerende cellen (APC, T en B cellen). Met name $CD44v10^+$ T helper cellen konden zelfs nog op late tijdstippen na EAE inductie gedetecteerd worden, terwijl deze cellen afwezig waren in controlemuizen. Daarnaast laten we zien dat muizen, die het gen voor CD44v7 of CD44v10 missen, een verminderde ziektelast vertoonden na EAE inductie. Deze gegevens tonen aan dat CD44v isovormen een belangrijke bijdrage kunnen leveren aan EAE. Deze isovormen zijn mogelijk ook betrokken bij het ziekteproces

in MS. Verder onderzoek moet aantonen op welke manier CD44v moleculen een rol spelen in de ziekte (b.v. bij adhesie of geprogrammeerde celdood).

Kort samengevat heeft dit proefschrift de volgende inzichten opgeleverd. PG kan bijdragen aan MS door activatie van APC. PG-bevattende cellen kunnen mee migreren naar het CZS tijdens MS of EAE en dragen daar mogelijk bij aan een ontstekings-bevorderend milieu. Niet alleen in het CZS, maar ook in lymfoïde organen zou PG een bijdrage kunnen leveren aan MS en EAE. Hier kan PG bijdragen door stimulatie van T en B cellen die gericht zijn tegen componenten van het CZS (zoals myeline), via APC. CD97 en CD44v isovormen kunnen ervoor zorgen dat celactivatie, -migratie, -adhesie en -overleving gestimuleerd worden tijdens MS en EAE. We laten zien dat CD97 en CD44v isovormen tot expressie komen in ontstekingshaarden in het CZS van MS patiënten. EAE in muizen kan worden verminderd door afwezigheid van CD44v7 of CD44v10. Soortgelijke effecten worden verwacht bij afwezigheid van het CD97 molecuul. Dit proefschrift verschaft een verbeterd inzicht in de pathogenese van MS en draagt mogelijk bij in een rationele doelgerichte ontwikkeling van nieuwe therapeutische benaderingen.

Dankwoord

En dan is het is zover, het boek is af! Mijn naam staat dan wel als enige op de voorkant, maar dit boek is met hulp van velen tot stand gekomen, die ik hierbij dan ook eerst allemaal in het algemeen wil bedanken,

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Om in volgorde van het proefschrift te beginnen, wil ik eerst Bert 't Hart bedanken voor de mogelijkheid tot het ontstaan van chapter 2 in dit boekje! De laatste maanden hebben we dit tot een mooi artikel gemaakt (al zeg ik het zelf), ook onder andere met de hulp van Sandra, Ella en Jeffrey, ook daarvoor mijn dank. Het hoofdstuk waarbij we gewerkt hebben aan peptidoglycaan in muizen EAE (chapter 3) is mede tot stand gekomen dankzij samenwerking met Edward Nieuwenhuis en Bart Lambrecht. Edward, bedankt voor je aanstekelijke enthousiaste kijk op de wetenschap en alle input bij het bedenken van proeven en schrijven van het artikel. Ook wil ik Bart bedanken voor de wetenschappelijke input en de mogelijkheid om proeven uit te voeren met mooie modelsystemen. Ook met name Hendrik Jan wil ik daarbij bedanken voor alle inzet!

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Chapter 2: Figure 2

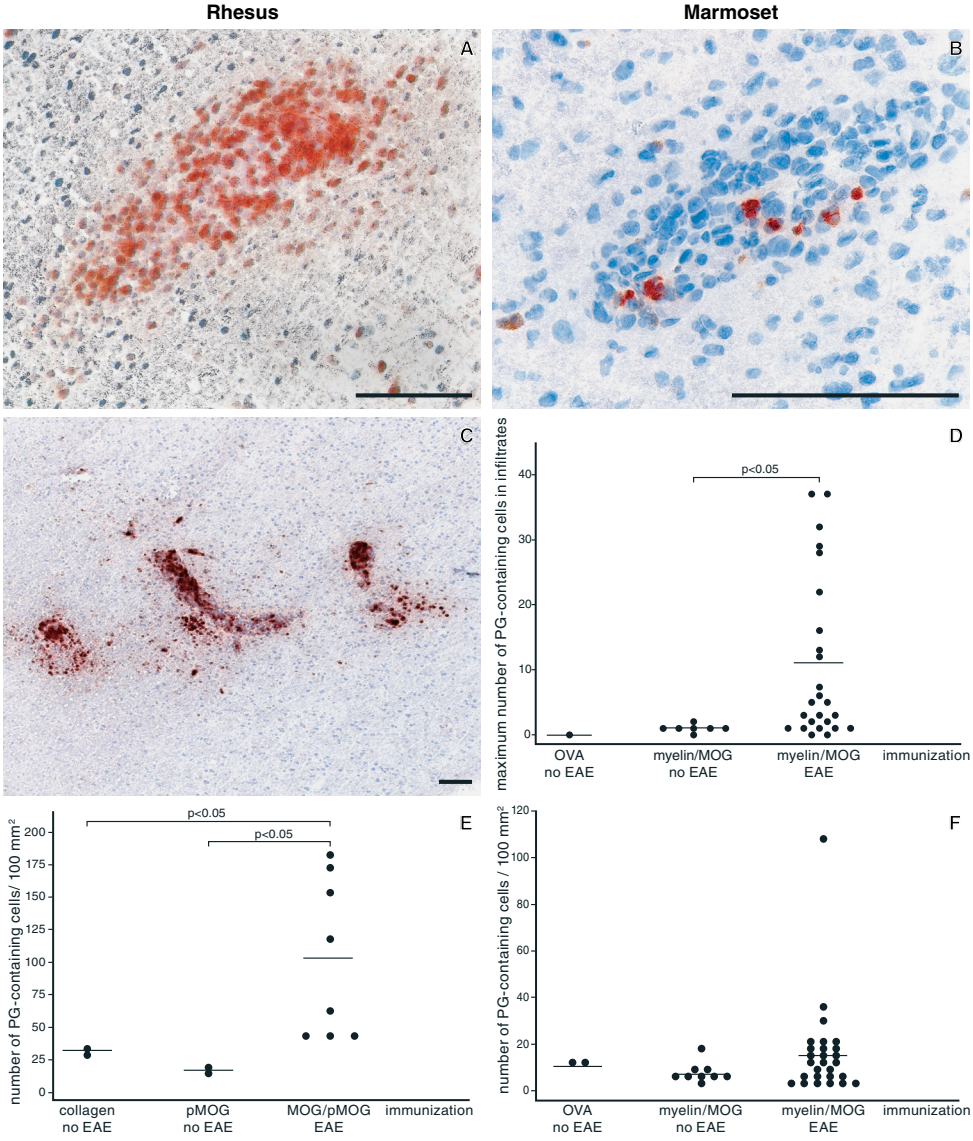

Figure 2. PG-containing cells are present in rhesus and marmoset brain.
Left column: rhesus brain tissue and right column marmoset brain tissue. Many cells in infiltrates of (A) rhesus EAE brain tissue (animal 9) and a modest cell number in infiltrates of marmoset EAE brain tissue (animal 10) contain PG (B, D). Significant higher numbers of cells in infiltrates of marmoset EAE brain tissue (D) contain PG when compared to control brain tissue. Many PG-containing infiltrates are present in rhesus EAE brain tissue (C). Significantly elevated numbers of PG-containing cells are present in the parenchyma of rhesus (E), but not in marmoset EAE brain tissue (F), compared to control brain tissue. Bar = 100 μm.

Chapter 2: Figure 3

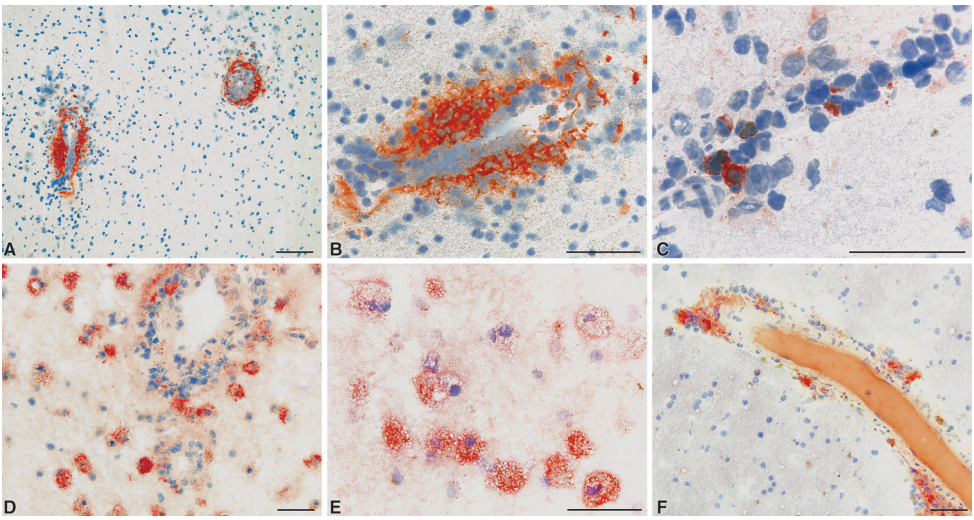

Figure 3. Restricted NAMLAA expression in MS and monkey EAE brain.
In rhesus EAE brain tissue (animal 9) many perivascular infiltrates are present with a moderate to high number of NAMLAA-containing cells (A, B). In marmoset EAE brain tissue (animal 10) some NAMLAA-containing cells are localized near blood vessels (C). Foamy MΦ in active MS brain lesions (sample 97-160) express NAMLAA (D). Expression of NAMLAA is restricted to a certain subpopulation of foamy MΦ (E). Occasionally also perivascular cells express NAMLAA in MS brain tissue (sample 00-082) (F). Bar = 100 μm.

Chapter 2: Figure 4

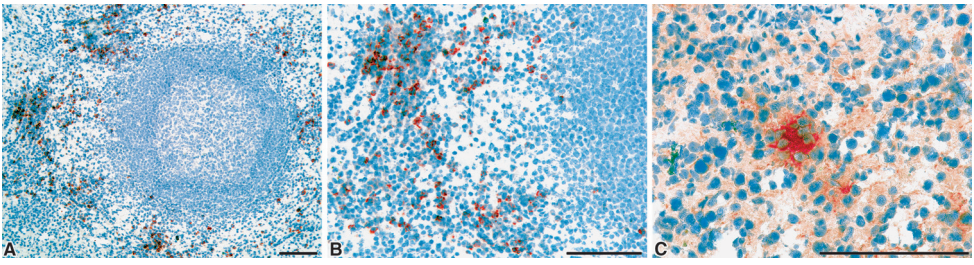

Figure 4. High numbers of PG-containing cells in rhesus EAE spleen.
High numbers of PG-containing cells are present in the marginal zone of rhesus monkeys that developed EAE (animal 10)(A, B). In control immunized rhesus monkeys (animal 5) NAMLAA-expressing cells are present in clusters within the red pulp (C). Bar = 100 μm.

Chapter 3: Figure 2

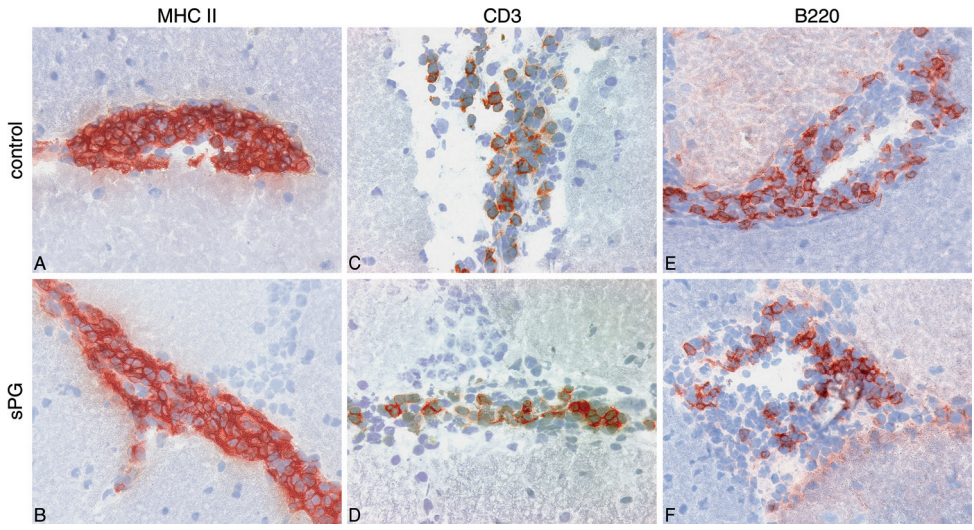

Figure 2. Immunization with PG-containing adjuvant induces classical EAE histopathology. Brain tissues were isolated between 4 to 7 weeks after immunization. Frozen brain sections were stained with hematoxylin in combination with MHC-II (A, B), CD3 (C, D) and B220 (E, F).

Chapter 3: Figure 4

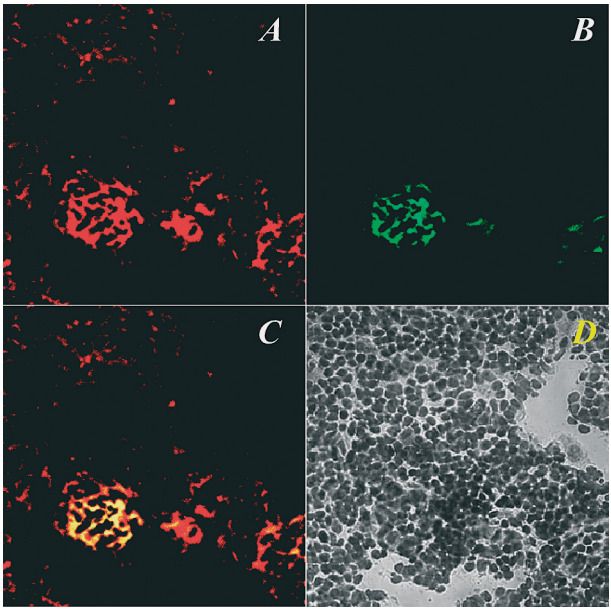

Figure 4. PG is redistributed from the site of immunization to DC in draining lymph nodes. Draining lymph nodes were isolated at 4 h after s.c. immunization with MOG_{35-55} in IFA-sPG. Frozen sections were stained by immunofluorescence for DC (CD11c in red, A) and for PG (green, B). Overlay in C demonstrates clusters of DC containing PG. D is a lightmicroscopic image of the same area.

Chapter 4: Figure 1

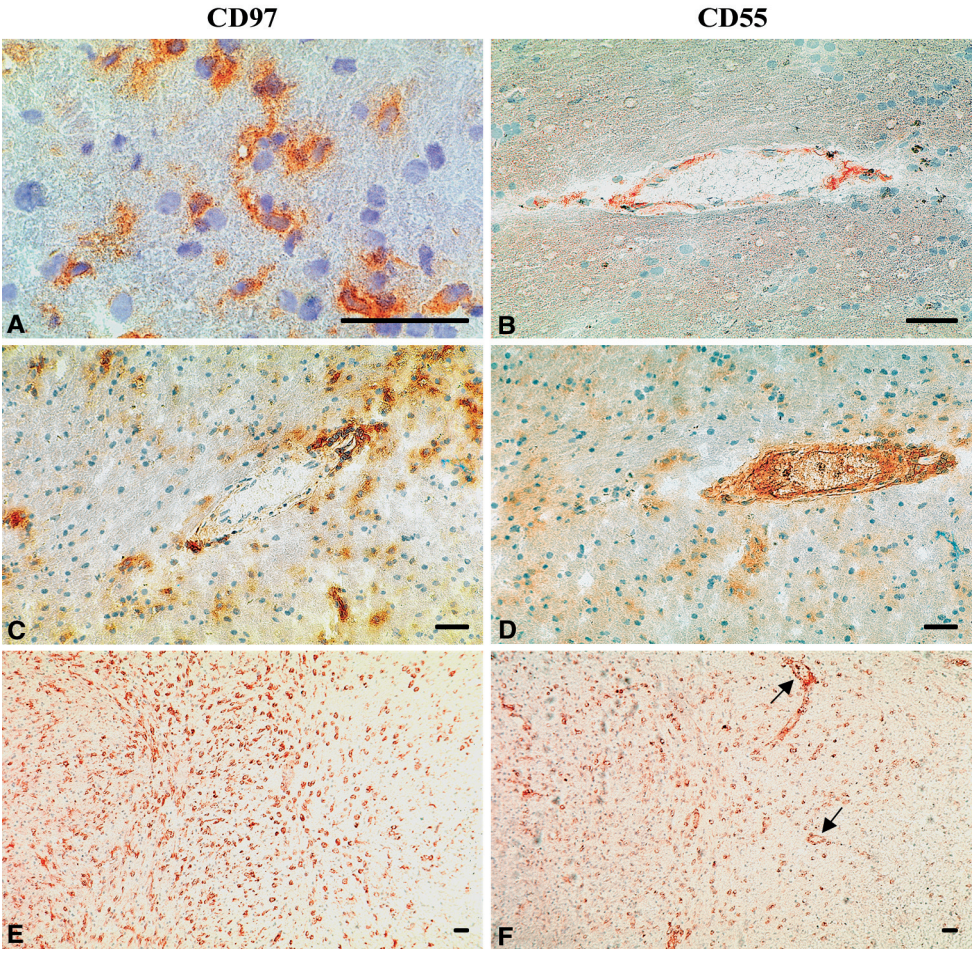

Figure 1. Expression of CD97 and CD55 in human MS brain.
Left column, CD97 expression; and right column, CD55 expression. In pre-active lesions, parenchymal cells as well as infiltrating cells expressed CD97 (A and C, respectively). In normal white matter, CD55 was expressed at low levels by endothelial cells (B), while in pre-active lesions, CD55 was expressed at high levels by endothelial cells and by some infiltrating cells (D). In active lesions, in which phagocytic cells contain myelin debris, numerous cells expressed CD97 (E) and also a moderate number of cells expressed CD55 (F). Bar = 45μm.

Chapter 4: Figure 2

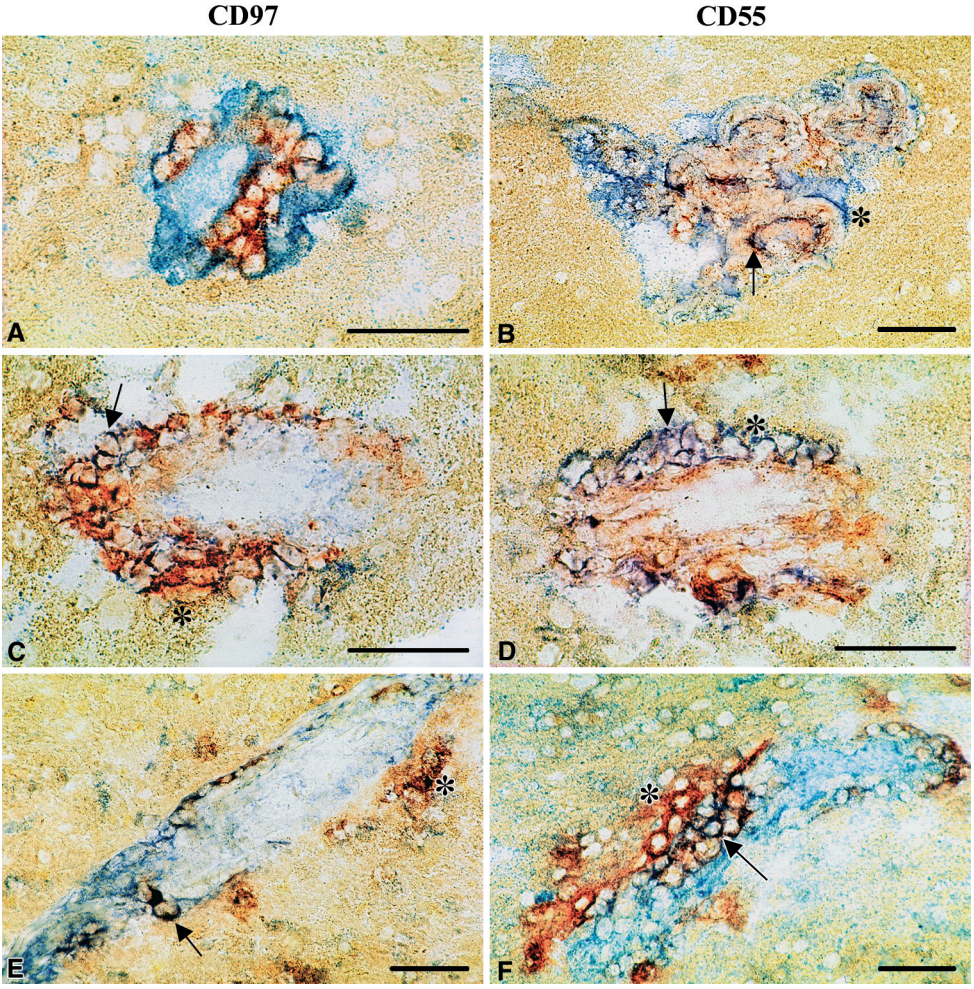

Figure 2. Identification of CD97- and CD55-expressing cell types in pre-active MS brain lesions. Left column, CD97 expression; and right column, CD55 expression. CD97 (red) is not expressed by endothelial cells (laminin in blue)(A). In contrast, double labeling showed co-expression (arrow) for CD55 (red) and laminin (blue; asterisk)(B). Many infiltrating $CD3^+$ T cells (blue) expressed CD97 (arrow)(C), and some expressed CD55 (arrow)(D). A moderate number of HLA-DR positive (red; asterisk) infiltrating MΦ and resident microglial cells co-expressed CD97 (arrow)(E), while only a few co-expressed CD55 (arrow)(F). Bar = 45μm. Asterisks indicate single expression (red or blue). Arrows indicate double expression (purple).

Chapter 5: Figure 1

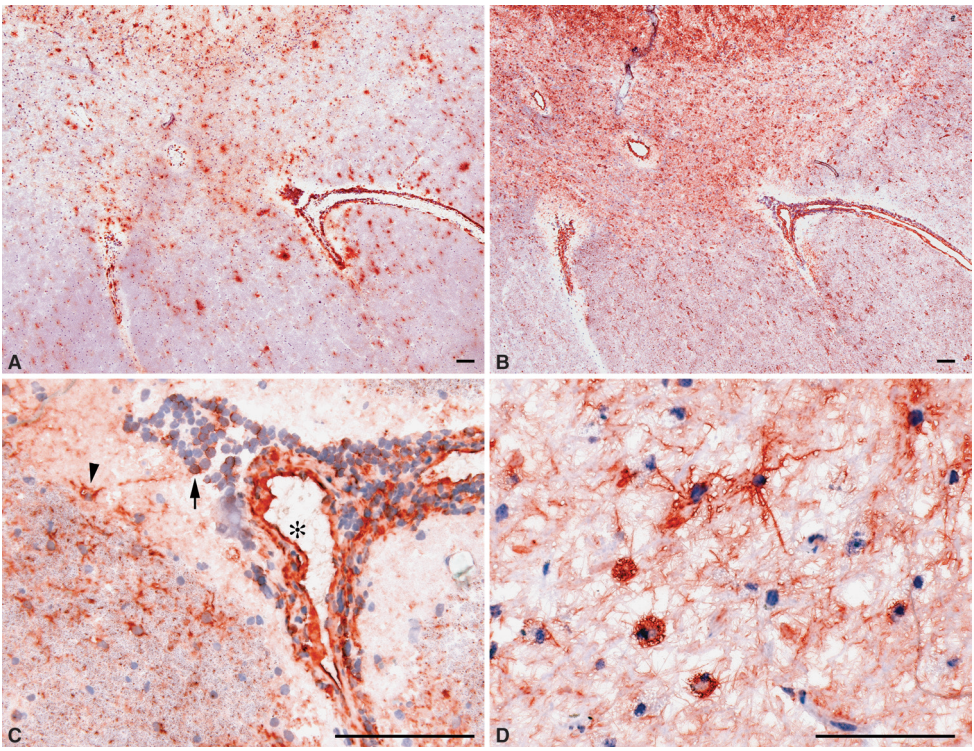

Figure 1. CD44v10 is expressed in MS brain tissue
Pre-active lesions are characterized by HLA-DP/DQ/DR-expressing activated MΦ/microglia (A), which are located in perivascular infiltrates or in clusters in the brain parenchyma (A). An abundant cell number expressed CD44v10 in the parenchyma within pre-active lesions (B). These parenchymal v10-expressing cells had a fibrillary glial morphology (arrowhead) (C). Besides glial cells also few perivascular cells (arrow) and endothelial cells (asteriks) expressed CD44v10 (C). In active lesions a moderate to an abundant number of foamy MΦ expressed CD44v10 (D). Bar = 100 μm.