Immune modulation by mannosylated peptides

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Een rolstoel glijdt geweid voorbij en ik streel over mijn knie
Snel draai 'k mijn kijker nu langszij ontroerd door wat ik zie
In waardigheid verkoren zit daar haar levenslange rit
Het lamme nekje hoog opgeschoren, dat meisje met het kunstgebit
Multiple Sclerose heeft haar verkozen, de mensen wijken beschaamd
Haar beentjes steken gelijk doornrozen, als een ziekte die niet betaamt

Theo van Gogh

Contents

1

Introduction

I Multiple Sclerosis

1.1 Epidemiology, clinical features and etiology

Multiple Sclerosis (MS) is an inflammatory, degenerative disease of the central nervous system (CNS), characterized by the formation of sclerotic plaques in the CNS. The first clinical signs usually appear during young adulthood and symptoms may include fatigue, a wide variety of motor deficits, paralysis and frequently also sensory problems such as visual impairment. MS develops heterogeneously and different subtypes have been defined based on the clinical course of the disease. Most MS patients suffer from relapsing-remitting MS, characterized by discrete episodes of clinical symptoms, followed by periods of improvement. Many of these patients eventually develop a disease course of slow, but continuous deterioration, which is defined as secondary progressive MS. A minority of patients show a progressive form of MS without remissions from the onset, called primary progressive MS (1-3). Epidemiological studies have revealed that individuals with a North European heritage have a high risk to develop MS (approximately 0.1%) and that female patients outnumber men by a ratio of 2:1 (4-6).

Our knowledge of MS etiology is currently limited, although strong evidence for a genetic contribution has been provided by twin studies. Monozygotic twins display a 150-300 fold increased risk to develop MS, compared to unrelated individuals and for dizygotic twins this risk is still 20-40 fold higher (7). HLA class II gene expression has been linked to MS susceptibility and recently several non-HLA candidate susceptibility genes have been proposed (8,9).

Migration studies indicate that next to a genetic predisposition, the exposure to certain environmental factors may contribute to MS development (10). Although causation has not been proven, an association between infectious agents, such as Epstein-Barr virus, human herpes virus 6 or C. Pneumoniae and the development of MS has been suggested. Molecular mimicry between pathogenic antigens and autoantigens resulting in aberrant immune responses is one of the favorite hypotheses to explain the association between MS and such concomitant infection(s) (11,12).

Summarizing, the factors that contribute to MS susceptibility are complex and remain poorly understood, although it is believed that MS arises in genetically predisposed individuals as a consequence of environmental factors.

1.2 Neuropathology and myelin destruction

Charcot was the first to correlate the symptoms observed in MS patients to the formation of lesions in CNS tissue (13). Myelin is composed of oligodendrocyte membranes that are tightly wrapped around axons to form a protective and isolating layer. Short sections of unmyelinated axon, the nodes of Ranvier, enable rapid conduction of electric pulses along myelinated axons (Figure 1). The high lipid content of myelin is responsible for the white appearance of myelinated areas, which are therefore referred to as CNS white matter. The myelin content of CNS gray matter, as present in the cerebral cortex and in the spinal cord medulla, is much lower. Proteins comprise approximately one third of the myelin dry weight and within this fraction proteolipid protein (PLP, 50%) and myelin basic protein (MBP, 30%) are the major components. Although the exact function of most myelin proteins is unknown, a role in stabilization of membrane layers and compaction of myelin has been indicated (14).

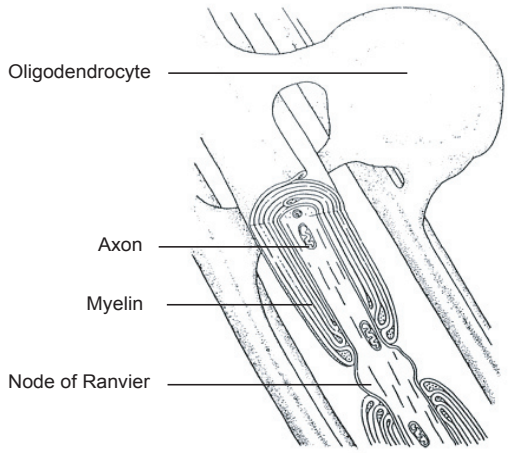

Figure 1. *Myelin is composed of oligodendrocyte membranes that are tightly wrapped around axons. Short sections of unmyelinated axon form the nodes of Ranvier. Adapted from Bunge, 1968 (15).*

Lesion formation is most abundant in CNS white matter, but the presence of lesions and demyelination in CNS gray matter has also been described (16). The formation of new lesions is detectable in acute MS patients, but also during the chronic phases of the disease. The heterogeneous appearance of such lesions has been classified by Lucchinetti et al (17). Type 1 and type 2 lesions reflect the inflammatory processes involved in MS and both types are characterized by accumulation of T lymphocytes and demyelination mediated

by infiltrating macrophages. Moreover, autoantibodies are present in type 2 lesions. The ongoing demyelination in active lesions of MS patients is reflected by macrophages that contain intracellular myelin proteins. Type 3 and type 4 MS lesions are characterized by severe primary loss of oligodendrocytes, due to apoptosis or another (currently unknown) mechanism, in the absence of inflammation (18). The clinical symptoms in MS are the result of irreversible damage of CNS tissue that is caused by the pathological processes occurring in the lesions (19). For example, the loss of axons impedes correct transmission of electric pulses (20) and tissue repair is inhibited due to the accumulation of astrocytes that contributes to the formation of scar tissue (21).

1.3 Neuroimmunology

The accumulation of inflammatory cells in MS lesions suggests that the immune system is involved in disease. However, it is still a topic of debate whether autoimmune responses initiate MS development or that these responses should be considered as a secondary event that is initiated by preceding tissue damage (22,23). It is unclear to what extent peripheral tolerance to myelin antigens exists in humans, because only part of the myelin components is presented in the thymus and therefore several myelin antigens may be recognized by the immune system as non-self (24,25).

Accumulating evidence indicates that both myelin-specific $CD4^+$ and $CD8^+$ T cells play a role in MS (26) and that the frequency of such T cells is similar in healthy individuals and MS patients (27). Importantly, the T cells in MS patients show a memory phenotype with a decreased threshold for activation, while T cells in healthy persons display a naive phenotype (28,29). In healthy persons, myelin antigens are present in the periphery and thus available to autoreactive T cells. Therefore the inactivity of these T cells cannot simply be explained by immunological ignorance (24). Recent studies show that active suppressor mechanisms prevent the derailment of autoreactive T cells under healthy conditions and suggest that this regulation might be less functional in MS patients (30-32). Besides myelin components, stress-related proteins are considered as potential autoantigens in MS, although the relevance to disease pathology is unclear (33).

Myelin-specific autoantibodies can be detected in CNS tissue, in the cerebrospinal fluid and in the circulation of MS patients. Moreover, B cells can be detected in MS lesions. Autoantibodies are also present in healthy controls, although at much lower levels. Abnormal synthesis of immunoglobulin is applied as a read-out in the diagnosis of MS patients, although the pathological significance of myelin-specific autoantibodies is still unclear (34-36).

Resident glial cells in the CNS contribute to the cascade of immunological events in lesion formation. The migration of T cells through the blood brain barrier (BBB), consisting of tightly arranged endothelial cells and astrocytes, is a crucial event in the formation of plaques. Activated microglia can act as antigen presenting cells (APC) and they can stimulate infiltrating T cells by presenting myelin antigens (37,38). Moreover, activated glial cells can produce noxious factors, such as nitric oxide (NO), which induces myelin damage and the killing of oligodendrocytes (39). After activation, T cells in CNS tissue produce a variety of proinflammatory factors, such as lymphotoxin-α and TNF-α, which results in the accumulation of other inflammatory cells. Full-blown inflammation in the CNS involves the infiltration of monocytes that contribute to myelin destruction (40,41).

1.4 Current treatment

At present, MS cannot be cured and treatment of patients is focused on resolving exacerbations and slowing down disease progression. The immune modulators Glatiramer acetate (Copaxone ©) and IFN-β form the basis for therapy nowadays and are applied with variable success (42). IFN-β is a pleiotropic molecule that is particularly effective in relapsing-remitting MS patients. Although the underlying mechanism is elusive, growing evidence suggests that IFN-β alters the balance between proinflammatory and anti-inflammatory cytokine production (43). Glatiramer acetate is a synthetic polypeptide composed of the most prevalent amino acids in MBP and it is believed to modulate autoreactive T cells and inhibit monocyte activity in the CNS. Moreover, it has recently been published that glatiramer acetate stimulates the development of active immune regulation in MS patients (44).

The identification of compounds that provide a synergistic benefit when administered in combination may improve the efficacy of MS therapy. Statins were initially applied in atherosclerosis patients to reduce cholesterol levels, but they were additionally shown to modulation the immune system (45). The effect of statins on autoimmune responses occurring in EAE and MS has gained considerable attention since and recently a combination therapy of statins and Glatiramer was successfully tested in EAE (46).

A different strategy for MS treatment is the inhibition of trafficking of inflammatory cells through the BBB and therefore a humanized monoclonal antibody against α4-integrin has been designed (Nataluzimab ©). Clinical trials in MS patients were promising, but severe side effects in a few patients forced a reevaluation of the safety profile of this drug (47).

All conventional therapies of MS patients entail considerable side effects due to a lack of specificity. Antigen-specific immunotherapy may offer the perspective of a highly specific

reduction of pathological autoimmunity, without disturbing normal immune function. Several approaches to restore immunological tolerance, such as administration of myelin components or altered peptide ligands, have been evaluated with success in preclinical studies. Some of these treatment strategies were also applied in human clinical trials and resulted in variable outcome. This indicates that antigen-specific immunotherapy might be a promising approach for MS treatment, but that the complexity of the disease impedes the transition from preclinical studies towards application in humans (48,49).

Box 1 An overview of frequently used EAE models

Peptide-induced EAE models

Mouse strain	**Epitope**	**Disease pattern**
SJL/J (53)	$PLP_{139-151,}$ MOG_{92-106}	Relapsing-remitting
Biozzi ABH (54)	$PLP_{56-70,}$ MOG_{8-22}	Chronic-relapsing
C57BL/6 (55)	MOG_{35-55}	Chronic-progressive
PL/J and B10.PL (56)	MBP_{1-9}	Relapsing-remitting

Rat strain	**Epitope**	**Disease pattern**
Lewis (57)	$gpMBP_{73-86}$	Monophasic
DA (57)	$gpMBP_{63-81,}$ MBP_{79-99}	Monophasic
DA (58)	MOG_{1-125}	Relapsing-remitting

Non-human primates (59)	**Epitope**	**Disease pattern**
Marmoset	hMOG	Heterogeneous
Rhesus Macaque	hMBP , hMOG	Acute or chronic

Virus-induced EAE models (60,61)

Mouse strain	**Virus**	**Disease characteristics**
SJL/J, C57BL/6	Theiler's virus	Demyelination (cytotoxicity towards infected CNS cells)
Biozzi ABH, C57BL/6	Semliki-Forest virus	Demyelination (cross-reactivity between SFV- and myelin epitopes

II Experimental autoimmune encephalomyeltitis

2.1 Animal models used for MS research

In 1933 experimental autoimmune encephalomyelitis (EAE), an MS-like disease model, was described for the first time. Monkeys were repeatedly injected with fresh rabbit myelin, which elicited an inflammatory response in the CNS (50). Since then the EAE model has evolved into an important tool in the field of MS research. EAE can be induced in several animal strains by immunization with myelin components in the presence of adjuvant or by adoptive transfer of myelin-specific T cells (51,52). In Box 1 an overview of frequently used EAE models is presented.

In many aspects the EAE model resembles the features of MS. In the brain and spinal cord of EAE animals lymphocytes and antibodies can be detected, which resembles the type 1 and type 2 lesions that are described in MS patients. The accumulation of inflammatory cells in EAE and MS suggests that in both cases the BBB is compromised. In EAE, the inflammatory response in the CNS results in disability and in some models also in demyelination.

Like in MS, EAE susceptibility is closely linked to the expression of certain MHC class II molecules (62). Several studies in mice have demonstrated that females are more susceptible to develop EAE than their male counterparts, which is consistent with the fact that the majority MS patients is female (63,64). Castration of male mice abrogates the resistance to EAE induction and on the other hand it was shown that treatment with androgens results in protection against the disease (65). It has been suggested that gender differences in EAE susceptibility are the result of less potent immune regulation in females (66), which may also influence the effectiveness of experimental protocols for tolerance induction (67).

Despite the considerable resemblance between both diseases, one should be aware of the limitations of EAE as a model of MS (68). The large variety of EAE models has contributed to a better understanding of the mechanism(s) underlying autoimmunity in the CNS and several MS therapeutics currently applied in patients were first discovered in EAE (69). However, the translation to the clinic of therapeutics that were successful in EAE has also yielded some disappointing results. For example, neutralization of TNF-α resulted in exacerbation of MS symptoms, while it was protective in EAE (70). Such unexpected results may be explained by the complexity of the disease processes that contribute to MS, which can only be partially covered by individual EAE models (71,72).

2.2. *The immune cascade in EAE*

EAE is mediated by $CD4^+$ myelin-specific T cells that are primed in peripheral lymphoid organs after immunization with myelin components in Complete Freund's adjuvant (73). The injected myelin fragments can be taken up by immature dendritic cells (iDC) in the periphery and the presence of adjuvant ensures DC maturation. Mature DC (mDC) express the chemokine receptor CCR7, which enables their migration into draining lymph nodes (74). Here, mDC can interact with naive T cells, resulting in the induction of a powerful (autoreactive) immune response (75,76). The production of IL-12 by mDC promotes the development of T_h1 cells (77) and for a long time it was believed that these T cells were the key effector cells in EAE (78). However, studies in mice that were deficient for IL-12 or IFN-γ suggested otherwise, because these mice were still susceptible to EAE (79,80). Several recent publications showed that mDC can produce IL-23, which is crucial for the differentiation of T cells that produce IL-17 (81,82). Importantly, these so called T_h17 cells play a major role during autoimmune inflammatory responses, as occurring in EAE (83,84). Accordingly, it has been shown that inhibition of IL-23 signaling indeed prevented the development of EAE (85,86).

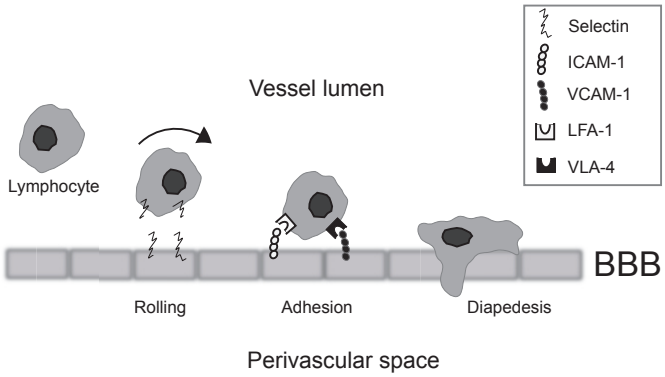

Figure 2. *Lymphocyte migration across the blood brain barrier. The activated blood brain barrier endothelium expresses selectins that mediate tethering and rolling of lymphoctes. After firm adhesion lymphocytes can transmigrate into the perivascular space. Based on Engelhardt, 2006 (94).*

Under healthy conditions the entrance of immune cells into the CNS is limited, but in MS and EAE the barrier function of the BBB is compromised (87). It is known, that adjuvant components increase the permeability of the BBB and in this way can promote EAE development (88,89). The expression of P- and E-selectin is elevated on activated endothelium and these

molecules can mediate the initial contact with traveling leukocytes (87). This interaction results in tethering and rolling of leukocytes along the endothelium (Figure 2). Although the reduced speed of leukocytes facilitates subsequent firm adhesion, the importance of selectin-mediated interactions for EAE development remains controversial (90). The firm adhesion of leukocytes to endothelial cells is mediated by the integrins LFA-1 and VLA-4 expressed on leukocytes, which bind to the endothelial adhesion molecules VCAM-1 and ICAM-1, respectively. These adhesion molecules not only function as docking sites for leukocytes, but also induce signaling inside the endothelial cell that promotes leukocyte extravasation (91). After diapedesis, inflammatory cells accumulate in the perivascular space and can further infiltrate the CNS parenchyma. The application of blocking antibodies against VLA-4 in EAE and in initial trials in MS patients significantly ameliorated the clinical disease course, demonstrating the importance of integrin-mediated interactions in the development of CNS inflammation (92,93).

Life-imaging studies have revealed that both naive and activated myelin-specific T cells are able to cross the BBB, but that only highly activated T cells induce CNS inflammation. The reactivation of myelin-specific autoreactive T cells in situ is required for the development of full-blown EAE (95,96). Both resident microglia in the parenchyma and DC that have infiltrated the CNS are able to reactivate T cells (97,98). The production of TNF-α and lymphotoxin by reactivated T cells further contributes to a proinflammatory milieu in CNS tissue. Other inflammatory cells, such as macrophages, are attracted by T cell-derived chemokines. The chemotactic factors RANTES, MCP-1, MIP-1α, TCA-3 and IP-10 have been shown to play a role in EAE development (99,100).

Several studies have implicated that immune reactivity to additional self-antigens, different from the disease-inducing epitope, may develop during EAE. This phenomenon of epitope spreading occurs as a result of continuous tissue damage and may contribute to disease progression (101-103).

III Immune regulation, Autoimmunity and Tolerance

The function of the immune system is the eradication of harmful agents, while simultaneously tolerance towards self-components should be preserved. During development of the immune repertoire, lymphocytes that originate from the bone marrow are selected in the thymus. A large variety of self-antigens is presented here and T cells with high affinity for self-epitopes become deleted, a process called negative selection (104,105) Nevertheless, considerable numbers of self-reactive lymphocytes escape this process of elimination and regulatory

mechanisms in the periphery have evolved to prevent derailment of these autoreactive lymphocytes.

Naturally occurring $CD4^+CD25^+$ regulatory T cells arise from the thymus and are characterized by the expression of FoxP3 (106). This regulatory T cell population controls the activity of autoreactive T cells via cell-cell contact and is therefore highly important in preventing autoimmunity (107-109). Indeed, it has been shown that injection of $CD4^+CD25^+$ regulatory T cells before EAE induction by active immunization prevents disease development and that these cells can also inhibit EAE when co-transferred together with highly encephalitogenic T cells into naive animals (110).

In addition, several other $CD4^+$ regulatory T cell subtypes have been identified (111,112). For example, the immune regulation of the gut is associated with Th3 cells that produce TGF-β and oral administration of autoantigens results in tolerance induction mediated by this regulatory T cell population (113). Levings et al. first described Tr1 cells that can mediate immune suppression via the production of regulatory cytokines, such as IL-10 (114). Importantly, EAE studies in IL-10 deficient mice have demonstrated the important role of this cytokine in the suppression of autoimmune responses (115,116).

T cell anergy is another mechanism that contributes to silencing of T cell activity, which may involve signaling via CTLA-4 expressed by the T cell (117). T cells that recognize their antigen in the absence of sufficient costimulation may become anergic, which means that T cell proliferation and effector functions are inhibited (118). Several publications report that the systemic administration of non-physiologically high amounts of self-antigen induces tolerance due to clonal deletion of autoreactive T cells or due to deviation of T cells towards a non-pathogenic phenotype (119,120).

The balance between tolerance and (auto)immunity is carefully controlled by dendritic cells (DC) that provide tolerizing or immunogenic stimuli to T cells, depending on their maturation status. Immature DC are highly ramified and avidly endocytose antigens from their environment. In this state, DC express no costimulatory molecules and only low numbers of MHC molecules and are therefore considered tolerogenic (121,122). Maturating DC upregulate several costimulatory molecules on their cell surface, including CD80, CD86 and CD40. These molecules can interact with CD28 and CD40L expressed by T cells, contributing to the development of effector T cells (123-125). Indeed, it has been demonstrated that such interactions between costimulatory molecules are important in the disease progression of MS as well as of EAE (126,127). The increasing knowledge on how DC can be modulated paves the way towards a the application of DC with a tolerogenic phenotype to prevent transplant rejection and to treat autoimmunity (128).

IV Pathogen recognition receptors

Dendritic cells are the key APC for the initiation and regulation of immune responses and based on the expression of CD11b and CD8α, different DC subtypes have been defined in vivo. It is not completely clear whether differences in surface expression of these molecules correlates with functional activity of the cells (129). One way to discriminate DC that are functionally different may be via the expression of pathogen recognition receptors, that are expressed to scan the environment for the presence of danger signals (130). The families of Toll-like receptors (TLR) and C type lectin receptors (CLR) are most intensively studied in this respect and represent an important link between innate and acquired immunity (131).

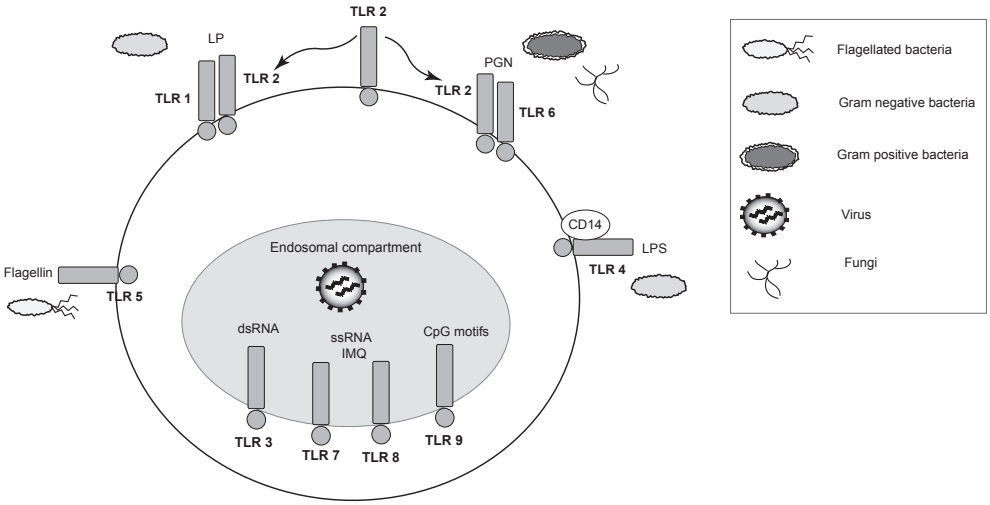

Figure 3. *TLR receptors and their ligands. The intracellular localization of TLR 3,7,8 and 9 enables the recognition of viral components. TLR2 can dimerize with TRL1 and TLR6 to recognize extracellular pathogens. The adaptor molecule CD14 is required for LPS binding to TLR4. PGN: peptidoglycan, LP: Lipoprotein, IMQ: Imiquimod*

TLR are non-phagocytic receptors that recognize molecular patterns present in microbial lipids, lipoprotein, lipopolysaccharides, nucleic acids or bacterial DNA (Figure 3). The TLR expression profile on DC is heterogeneous, suggesting that functionally different DC use a specialized set of TLR for pathogen recognition. TLR 1,2,4,5 and 6 are present on the cell surface and specialize in the recognition of products that are unique to bacteria and are not made by the host. On the other hand, TLR 3,7,8 and 9 recognize nucleic acids that are not unique to the microbial world. These TLR are localized intracellularly and are specialized in the detection of internalized viral components in endosomes and lysosomes. Normally, host-

derived nucleic acids are absent in these organelles, which prevents TLR activation by self-components. The current knowledge suggests that triggering of TLR results in maturation of DC and in the induction of proinflammatory T cell responses (132,133). Although APC have been most extensively studied with regard to their TLR profile, also other immune cells have been shown to express TLR. For example, it has recently been described that TLR expressed on T cells are involved in the tuning of TCR-mediated stimulation (134).

The CLR family can recognize carbohydrate structures and this receptor family has a broad repertoire of functions (135). The mannose receptor (MR) and DC-SIGN are involved in the migration of lymphocytes by facilitating their adhesion to endothelial cells (136,137). Studies in mannose receptor deficient mice have demonstrated that this receptor can also act as a scavenging receptor, mediating the clearance of glycosylated self-molecules (138,139). In addition, a role for the mannose receptor in the transport of antigens from the periphery towards the lymph node has been suggested (140).

Importantly, several CLR family members recognize sugar moieties in pathogenic cell walls, which can result in efficient uptake and processing of (microbial) antigens. During maturation, the endocytic capacities of DC are decreased and in line with this the expression of most CLR becomes downregulated (141). Surprisingly, it was demonstrated that DEC-205 expression is preserved on mDC, suggesting that the function of DEC-205 may extend beyond the endocytosis of antigens (142). In box 3 an overview of several CLR family members and their ligand specificity is presented.

The binding of microorganisms to CLR family members MR, DC-SIGN and Dectin-1 can result in their internalization. Although the ligand-specifity of DEC-205 is currently unknown, antibody-mediated targeting of antigens towards DEC-205 similarly induces endocytosis. The receptor-mediated route of uptake facilitates antigen processing and presentation, resulting in very efficient loading of antigens in both MHC class I and II molecules. The intracellular targeting motifs present in the cytoplasmic tail differ between CLR family members, resulting in several intracellular routes of endocytosed receptor-antigen complexes. For example, the MR releases its ligand in endosomes and recycles back to the cell surface. This ensures multiple rounds of antigen uptake and thus the internalization of large amounts of antigen (153). In contrast, DEC-205 and DC-SIGN are targeted directly towards lysosomes were they are degraded together with their cargo. Trimer or tetramer clustering of CLR on the cell surface can increase the affinity for ligands that contain multiple carbohydrate structures (154,155).

It is generally believed that CLR have developed during evolution as a way to effectively discriminate between self and non-self. However, pathogens have evolved as well to take

Box 3 The C-type lectin receptor family

CLR can recognize sugar moieties containing mannose, galactose or fucose. Each CLR contains one or multiple domains for the Ca^{2+}-dependent binding of carbohydrate structures (CRD). Moreover, the mannose receptor contains a cysteine-rich domain (CR) for the binding of sulphated carbohydrates and a fibronectin domain (FN) (143).

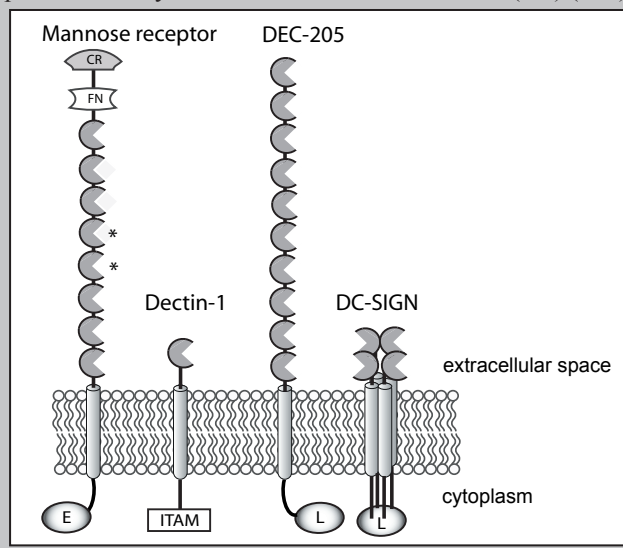

Figure 4. *Schematic representation of several CLR family members. CRD4 and CRD5 in the mannose receptor are highly important for the binding of glycosylated ligands(*). Multimerization of DC-SIGN enables the high affinity binding of ligands. E: endosomal targeting motif, L: lysosomal targeting motif, ITAM: immunoreceptor tyrosine-based activation motif.*

Mannose receptor family (Type 1 membrane proteins with multiple CRD)

Receptor	Expression	Ligand(s)
Mannose receptor (144, 145)	MΦ, DC, epithelial cells	End-standing mannose
DEC-205 (142, 146)	$CD8^+$ DC, Langerhans cells	ligand(s) unknown
Endo180 (147)	Chondrocytes	Mannose, fucose, collagen

Asialoglycoprotein receptor family (Type 2 membrane proteins with a single CRD)

Receptor	Expression	Ligand(s)
MGL (148)	APC	GalNac
DC-SIGN (149)	DC	High mannose
Langerin (150)	Langerhans cells	Mannose, fucose, GlcNac
Dectin (151, 152)	$CD11b^+$ DC, monocytes	β-glucan

advantage of CLR. For example, the HIV virus uses DC-SIGN as a backdoor to enter immune cells in order to enhance their infectious capacity and Mycobacterium tuberculosis targets DC-SIGN to circumvent immune responses that would otherwise result in their elimination from the host (156).

This information further contributed to the concept that CLR family members play a role in the regulation of immune responses and may therefore be a suitable target for immune modulating therapy. On one hand, CLR are considered potential candidates for efficient targeting of tumor antigens to DC, in order to induce immunity against otherwise non-immunogenic tumors. On the other hand, it is demonstrated that targeting of CLR under steady state conditions can result in suppression of potent immune responses, which may be used as a strategy to induce tolerance in autoimmunity or transplantation.

V Scope of the thesis

The treatment that is currently available for MS patients is rather palliative and non-specific. Selective inhibition of the autoimmune responses that contribute to disease would therefore provide a valuable contribution to treatment of MS. The development of vaccines consisting of synthetic peptides representing autoreactive T cell epitopes may be a successful strategy (157,158).

As mentioned previously, CLR expressed on APC have gained considerable attention as modulators of the immune system (159). In the case of erroneous immune responses such as autoimmunity, targeting of CLR may be a useful tool to skew the immune system towards a healthy state. It has been shown that targeting of CLR family member DEC-205 expressed by iDC induces unresponsiveness to both class I and class II restricted antigens (160,161). Moreover, targeting of a self-antigen towards DEC-205 indeed inhibited the development of EAE in mice (162). The immature status of the DC is crucial in this strategy of tolerance induction, because ligation of CD40 on DC results in a powerful immune response. In line with this, it has been shown that targeting of tumor antigens towards DEC-205 and the simultaneous administration of a TLR ligand results in functional immunity and eradication of the tumor (163).

Previous in vitro studies revealed that the use of mannosylated peptides elicits very efficient peptide uptake via the mannose receptor, resulting in increased antigen presentation by human DC (164-166). In addition, mannosylated altered peptide ligands could successfully be applied to inhibit antigen-specific T cell proliferation in vitro (47).

In this thesis, the capacities of mannosylated peptides to modulate the immune system in vivo were studied. The studies described in Chapter 2 demonstrate that immunization with mannosylated self-peptide prevented EAE development and induced antigen-specific tolerance against the disease. The biodistribution pattern of mannosylated peptide after in vivo administration is presented in Chapter 3. In Chapter 4, IL-10 deficient mice were used to study the role of this regulatory cytokine in tolerance induction by mannosylated self-peptide. Studies with TCR transgenic T cells, as described in Chapter 5 and 6, were performed to monitor the fate of antigen-specific T cells in response to mannosylated antigens. A first step towards treatment of ongoing autoimmunity during EAE using mannosylated self-peptide is described in Chapter 7. The findings from this thesis are summarized in Chapter 8 in the context of recent knowledge in the field of autoimmunity and immune modulation.

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2

Mannosylated $PLP_{139-151}$ induces peptide-specific tolerance to experimental autoimmune encephalomyelitis

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Abstract

SJL mice immunized with mannosylated (M-) $PLP139_{139-151}$ in complete adjuvant do not develop EAE and little CNS mononuclear cell infiltration; other mannosylated peptides were ineffective in this experimental setting. Despite apparently normal T cell responses M-$PLP_{139-151}$ immunized mice show impaired delayed-type-sensitivity to $PLP_{139-151}$ but a normal response to other peptides. After re-immunization with $PLP_{139-151}$ in complete adjuvant these mice are largely tolerant to EAE, show less T cell proliferation and decreased peptide-specific IgG2a. Our data suggest that M-$PLP_{139-151}$ induces peptide-specific tolerance to EAE via a mechanism of deletion or impaired migration of encephalitogenic T cells.

Introduction

C-type lectins play an important role in the recognition of carbohydrate structures on pathogens and endogenous ligands. Members of this family play an important role in immune regulation, immune evasion and tolerance (1-3). One of these members is the mannose receptor that selectively binds carbohydrate groups such as mannose and fucose on glycoproteins of yeast, bacteria, parasites and certain viruses and is expressed on subsets of antigen presenting cells (4, 5). Binding of mannosylated ligands leads to internalization and delivery to the late endosomal compartments where low pH releases the ligand from the receptor, after which the latter is recycled to the cell surface (6-8). Mannose receptor-mediated endocytosis is a very efficient process allowing multiple rounds of antigen binding and endosomal delivery and can result in up to a 10,000-fold increased efficiency of presentation of mannosylated antigen compared to non-mannosylated antigen (9,10). Another member of the family of C-type lectins is DEC-205, a multilectin receptor homologous to the macrophage mannose receptor that is selectively expressed by immature DC (11). Targeting of ovalbumin to this receptor was shown to induce T cell unresponsiveness unless a second activation signal was provided simultaneously (12,13). Interestingly, binding of the mycobacterial cell wall component ManLAM to the DC specific C-type lectin DC-SIGN inhibits DC maturation and induces IL-10 production (14). Consequently, a role of C-type lectins in the maintenance of tolerance for self-antigens has been suggested (2,3). To address this possibility we studied a mannosylated self-peptide with regard to its ability to induce disease and show for the first time that a mannosylated form of $PLP_{139-151}$ (M-$PLP_{139-151}$) induces a state of tolerance to EAE.

Materials and Methods

Synthesis of peptides

Mannosylation of $PLP_{139-151}$ was performed as described elsewhere (7, 10). Briefly, $PLP_{139-151}$ was synthesized using solid phase synthesis. For M-$PLP_{139-151}$ the peptide was N-terminally elongated with a lysine building block containing two tetra-acetyl protected mannose groups. The $PLP_{139-151}$ peptide was elongated with bis-acetyl lysine. Cleavage of the peptides and removal of the protecting groups was performed with TFA. Removal of the acetyl protecting groups on the mannose moieties was performed by treatment with Tesser's base. The H-2^s binding peptides $OVA_{371-386}$ (15) and MOG_{92-106}, and the non-H-2^s-binding peptide KXQSKRFFKFRAAKSL - where indicated in a mannosylated form, served as control

peptides. All peptides were analyzed with Maldi-Tof mass-spectrometry and showed the expected masses.

Induction of EAE

Female SJL mice were bred and kept in our own SPF housing facilities. At an age of 9 to 11 weeks, mice were immunized subcutaneous with mannosylated or non-mannosylated $PLP_{139-151}$ dissolved in PBS and emulsified with an equal volume of incomplete Freunds' adjuvant supplemented with 1 mg/ml *M. tuberculosis* H37Ra (Difco, Detroit, MI). In initial experiments the amounts of peptide were compared on a per weight basis (Figure 1 and Table 1); all subsequent studies were performed with amounts based upon amino acid content only.

Development of EAE was monitored daily from day 7 after immunization by assessment of bodyweight and a disability score. This score ranges from 0: no symptoms, 0.5: partial loss of tail tonus, 1: complete loss of tail tonus, 2: limb weakness, 2.5: partial paresis, 3: complete paralysis of hind limbs, 3.5: complete paralysis from diaphragm and hind limbs, incontinence, 4: moribund, to 5: death due to EAE. Where appropriate, results are expressed as a mean maximal score or a cumulative disability score (area under the curve) during the indicated period of monitoring.

All of these studies were performed with the approval of the Animal Ethical Committee and in compliance with Dutch governmental regulations on Animal Experimentation. This approval has been filed in the protocol numbers DEC 933 and DEC 1317.

Delayed type hypersensitivity (DTH)

Mice were immunized with $PLP_{139-151}$, M-$PLP_{139-151}$, $OVA_{371-386}$ or MOG_{92-106} in complete adjuvant as indicated in the legends. Twelve to 15 days after immunization these mice were challenged by injecting 13.5 nMol of the relevant non-mannosylated peptide in 10 µl saline into the dorsal side of the right ear, employing a Hamilton syringe fitted with a 30 gauge needle. As a control, 10 µl saline was injected into the left ear. Ear thickness was measured before and 24 hours after challenge with the use of a Mitutoyo micrometer. For each individual mouse, results are expressed as the percentage specific swelling corrected by subtracting the percentage non-specific swelling.

Histology

Brain tissue and spinal cord of each individual mouse was fixated in 10% formalin and embedded in paraffin. From each individual mouse three spinal cord sections (5 µm) and six sections of cerebellum separated by 100 µm were stained with hematoxyllin. The area of each section was measured by morphometry and the number of infiltrating mononuclear cells per section was counted. For each individual mouse the mean number of infiltrating cells per square mm tissue was determined.

Lymphocyte culture

Lymph node cells (LNC) were isolated from inguinal, axillary and brachial lymph nodes and suspended in RPMI 1640 containing 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, 2 mM L-glutamin and 5% FCS (Gibco BRL, Gaithersburg, MD). Cells (3 x 10^5 LNC) were cultured in triplicate in 200 µl in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) and stimulated with various concentrations of M-$PLP_{139-151}$ or $PLP_{139-151}$. Cells were incubated with 0.5 µCi of [^{3}H]-labeled thymidine (2 Ci/mmol; Radiochemical Center, Amersham, UK) as indicated and harvested onto glass fiber filters (Packard, Meriden, CT). Incorporated label was counted using a Wallac 1450 micro beta counter.
CD4+ T cells were isolated from draining lymph nodes obtained from SJL mice 14 days after immunization with 50 µg $PLP_{139-151}$ in complete adjuvant and enriched to more than 95% employing a negative selection kit according to the instructions of the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). These cells (10^5/well) were stimulated with different concentrations of M-$PLP_{139-151}$ or $PLP_{139-151}$ in the presence of 10^4 bone marrow derived DC. The latter were obtained by culturing SJL bone marrow cells during 10 days in the presence of recombinant mouse GM-CSF (1.4 ng/ml; R&D Systems, Minneapolis, MN) and IL-4 (1000 U/ml, a kind gift of Dr. Satwant Narula, Schering-Plough); 1 µg/ml LPS (*E. coli* O127; Sigma, St. Louis, MO) was added during the last 2 days of culture.

ELISA

$PLP_{139-151}$-specific antibodies (IgG1, IgG2a and IgG2b) were measured by capture ELISA as described previously (16). IFN-γ, IL-10 and IL-4 concentrations in culture supernatants were determined by ELISA as described by Luca et al. (17).

Statistical analysis

Statistical analyses were performed with the Mann-Whitney U-test using SPSS 8.0 for Windows**.** Where indicated, ANOVA was used combined with a post-hoc LSD-test for multiple groups.

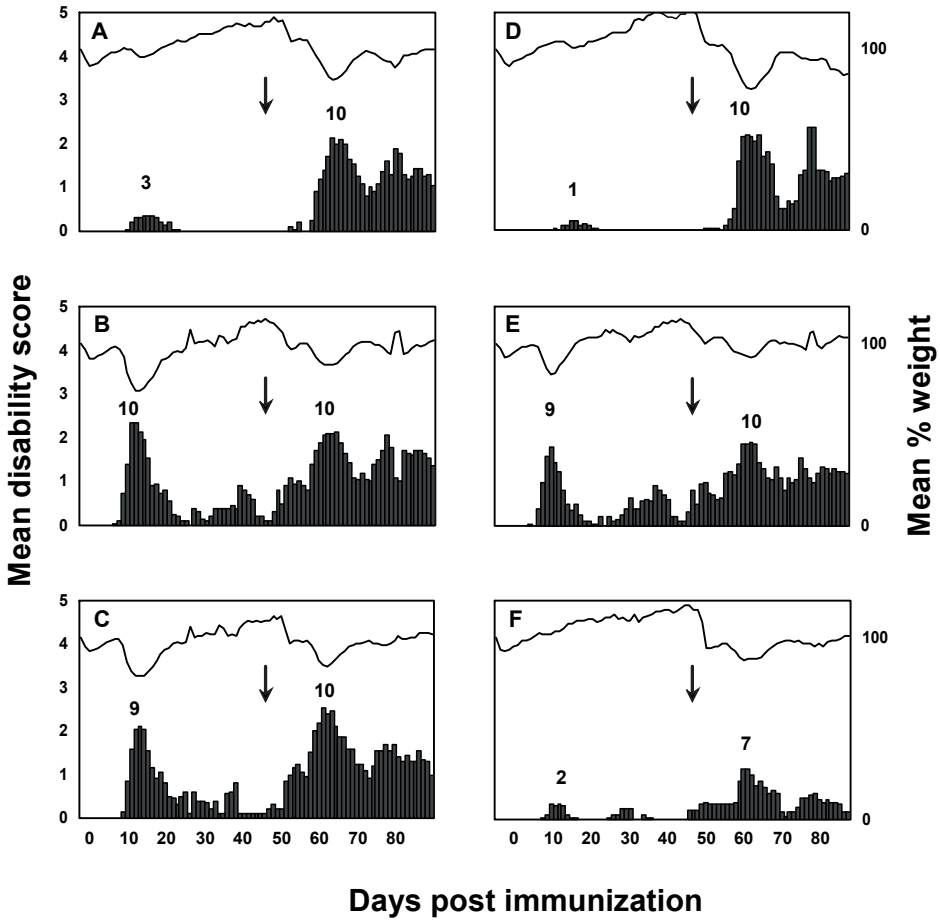

Figure 1. *Mannosylated $PLP_{139-151}$ induces tolerance to EAE. Mice (n=10 per group) were immunized (s.c.) with 0.5 µg (A), 5 µg (B) or 50 µg (C) $PLP_{139-151}$ or with 0.5 µg (D), 5 µg (E) or 50 µg (F) M-$PLP_{139-151}$ in complete adjuvant. On day 49, all mice were re-immunized (s.c.) with 50 µg $PLP_{139-151}$ in complete adjuvant (arrow). Development of disability symptoms and body weight were daily monitored. The results from 2 experiments equal in design were pooled. The numbers of affected mice after the first and second immunization are indicated in the Figure. The mean day of onset, maximal score and cumulative disability score together with the statistical evaluation are indicated in Table 1.*

Results

Mannosylated $PLP_{139-151}$ induces tolerance to EAE

To evaluate if targeting of C-type lectins would induce tolerance to EAE we first compared encephalitogenic $PLP_{139-151}$ and its mannosylated form ($M\text{-}PLP_{139-151}$) with respect to their efficacy to induce EAE when emulsified in complete adjuvant containing *M. tuberculosis* H37RA.

To this end we immunized groups of mice subcutaneously with 0.5, 5 or 50 µg of either peptide emulsified in complete adjuvant. As shown in Figure 1, the severity of EAE during the first 4 weeks after induction was dependent on the dose of peptide used for immunization. Half a µg of either peptide was insufficient to induce the typical disability symptoms of EAE (Figure 1A and 1D). Mice that had received 5 µg of either peptide were comparable in that they developed mild clinical signs of EAE (Figure 1B and 1E). Importantly, mice that were immunized with 50 µg of $M\text{-}PLP_{139-151}$ did not develop significant clinical symptoms (Figure 1F), whereas mice immunized with 50 µg $PLP_{139-151}$ (Figure 1C) showed a disease severity comparable to the 5 µg group. These results are summarized in Table 1: it can be concluded that immunization with 50 µg $M\text{-}PLP_{139-151}$ resulted a lower incidence (20%), a lower maximal score ($p < 0.005$) and a lower severity of disease (cumulative score, $p < 0.005$) during the first phase of disease activity.

Table 1. Mannosylated $PLP_{139-151}$ induces tolerance to EAE [a]

Peptide	Dose (µg)	Incidence (%)	Day of onset (mean ± SEM)	Maximal score (mean ± SEM)	Cumulative score (mean ± SEM) 1st phase [b]	Cumulative score (mean ± SEM) 2nd phase [b]
$PLP_{139-151}$	0.5	30	13.8 ± 1.1	0.6 ± 0.3	2.9 ± 1.7	40.3 ± 7.3
$PLP_{139-151}$	5	100	10.7 ± 0.4	2.6 ± 0.1	20.9 ± 2.9	54.2 ± 8.0
$PLP_{139-151}$	50	90	10.9 ± 0.4	2.4 ± 0.3	22.3 ± 4.9	53.8 ± 6.5
$M\text{-}PLP_{139-151}$	0.5	10	17.0 ± 2.1	0.3 ± 0.2	1.1± 1.0	33.3 ± 5.9
$M\text{-}PLP_{139-151}$	5	80	11.1 ± 0.4	2.3 ± 0.3	15.1 ± 3.7	43.7 ± 8.4
$M\text{-}PLP_{139-151}$	50	20	12.5 ± 0.2	0.6 ± 0.3[c]	3.2 ± 2.4[c]	17.2 ± 4.0[d]

[a] *Results of the experiment are described in Figure 1.*
[b] *For each individual mouse the cumulative disability score (sum of daily disability scores) was calculated from day 1 until 37 (first phase) and from day 49-84 (second phase) and included in the mean.*
[c] *$p < 0.005$ as compared to 50 µg $PLP_{139-151}$.*
[d] *$p < 0.0001$ as compared to 50 µg $PLP_{139-151}$; $p < 0.05$ as compared to 0.5 µg $M\text{-}PLP_{139-151}$.*

To address the possibility that M-$PLP_{139-151}$ had induced a state of tolerance, these mice were re-immunized on day 49 with 50 µg of the non-mannosylated encephalitogenic $PLP_{139-151}$ in complete adjuvant (see arrow in Figure 1). Although an increased disease incidence was observed, the mice that had been pre-immunized with 50 µg M-$PLP_{139-151}$ developed only mild signs of EAE (Figure 1F), despite the presence of a strong adjuvant. The severity of the disease during this second phase was significantly less than in mice pre-immunized with 50 µg $PLP_{139-151}$ ($p < 0.0001$), or in mice pre-immunized with 0.5 µg M-$PLP_{139-151}$ which had an equally low disease activity during the first phase of the disease ($p < 0.05$). Based on these observations we conclude that the mannosylated peptide induced a state of tolerance to EAE.

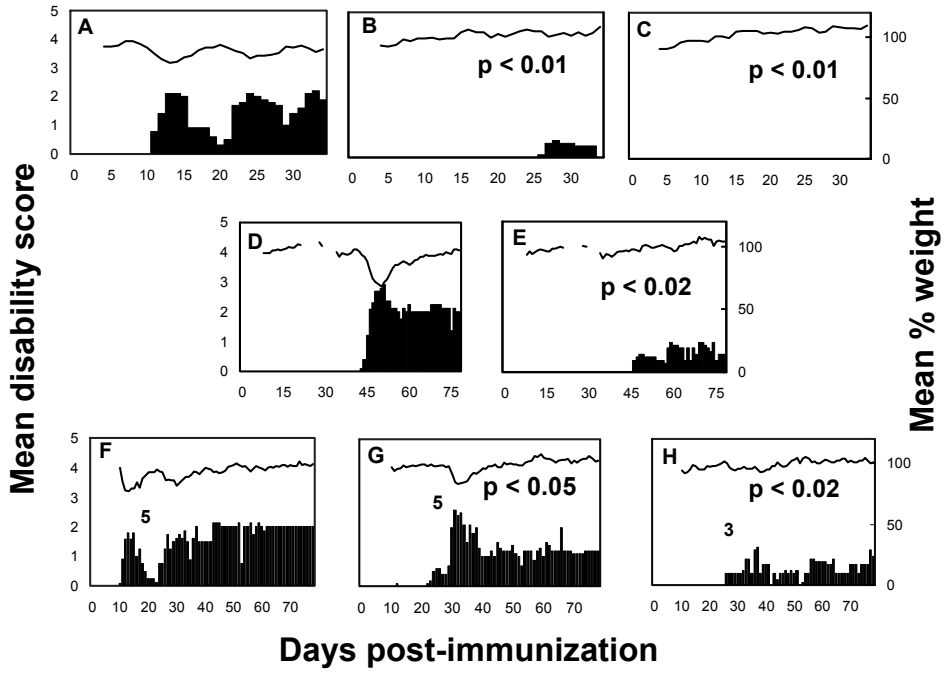

Figure 2. *M-$PLP_{139-151}$ suppresses EAE induction by encephalitogenic peptide. Mice (n=5 per group) were immunized with 50 µg PLP139-151 (Figure 2A), 50 µg M-$PLP_{139-151}$ (Figure 2B) or a mixture of 50 µg $PLP_{139-151}$ and 50 µg M-$PLP_{139-151}$ (Figure 2C) emulsified in complete adjuvant. (Co-)immunization with M-$PLP_{139-151}$ resulted in a significantly lower ($p < 0.01$) cumulative EAE score as compared to mice immunized with $PLP_{139-151}$. In a separate experiment mice (n=5 per group) were treated subcutaneously with 50 µg soluble $PLP_{139-151}$ (Figure 2D) or M-$PLP_{139-151}$ (Figure 2E), separate from a subcutaneous depot of complete adjuvant. On day 32, all mice were immunized with 50 µg $PLP_{139-151}$ emulsified in complete adjuvant. Mice pretreated with M-$PLP_{139-151}$ showed a significantly lower ($p < 0.02$) cumulative EAE score than mice pretreated with $PLP_{139-151}$. In a third experiment, mice (n=5 per group) were immunized on day 0 with 50 µg $PLP_{139-151}$ emulsified in complete adjuvant and treated on day 8 by i.v. injection of PBS (Figure 2F), 50 µg $PLP_{139-151}$ (Figure 2G) or 50 µg M-$PLP_{139-151}$ (Figure 2H). The number of affected animals is indicated in the Figure. Mice treated with either peptide showed a delay in onset of disease and consequently a significantly lower ($PLP_{139-151}$, $p < 0.05$; M-$PLP_{139-151}$, $p < 0.02$) cumulative EAE score than PBS-treated mice. In this respect, M-$PLP_{139-151}$ was more effective than $PLP_{139-151}$ ($p = 0.07$).*

In a separate experiment we demonstrated that these observations were not due to poor induction of disease by mannosylated peptide but rather to an active mechanism, because 50 µg M-$PLP_{139-151}$ abrogated the ability of 50 µg non-mannosylated $PLP_{139-151}$ to induce EAE (Figures 2A-C ; cumulative score, $p < 0.01$). To determine whether the tolerization with 50 µg M-$PLP_{139-151}$ required its presence in a depot in emulsion with complete adjuvant, we also evaluated the effect of systemic administration of soluble M-$PLP_{139-151}$ compared to the non-mannosylated peptide. As expected, neither peptide induced disease when injected subcutaneously as a soluble peptide (Figures 2D and 2E). Of interest, as opposed to mice that had received soluble $PLP_{139-151}$ (100% incidence), mice that had received soluble M-$PLP_{139-151}$ were largely resistant to EAE-induction one month later, evident from a lower cumulative EAE-score ($p < 0.02$).

We also evaluated the efficacy of M-$PLP_{139-151}$ to suppress the development of disease when administered 8 days after immunization with $PLP_{139-151}$ in complete adjuvant, thus allowing the initial development of a T_h1 response. Mice received one single intravenous injection of 50 µg M-$PLP_{139-151}$ or $PLP_{139-151}$ in PBS. Figures 2F and 2G show that $PLP_{139-151}$ treatment resulted in 100% incidence of disease, albeit with delayed onset of symptoms ($p < 0.01$). Consequently these mice had a lower cumulative score ($p < 0.05$) than control mice. However, these mice were not different with respect to their maximal disability score ($p = 0.73$). M-$PLP_{139-151}$ treatment resulted in a decrease of disease incidence to 60%, a delay of onset of disease (Figure 2F vs. 2H ; $p < 0.01$), a lower cumulative score ($p < 0.02$) and a lower maximal disability score ($p < 0.05$). M-$PLP_{139-151}$ was more effective than $PLP_{139-151}$ in the suppression of EAE (Figure 2G vs. 2H; day of onset, $p < 0.05$; cumulative score, $p = 0.07$; maximal score, $p = 0.06$). These data indicate that the effects of M-$PLP_{139-151}$ are less pronounced when administered after the priming of peptide-specific T cells.

Decreased mononuclear cell infiltration of the CNS in mice immunized with M-$PLP_{139-151}$

It has been demonstrated previously that the encephalitogenicity of myelin-specific T cells is dependent on re-activation within the CNS (18, 19). Therefore, we investigated whether immunization with M-$PLP_{139-151}$ resulted in infiltration of the CNS by mononuclear cells and focused on cerebellum and spinal cord representing the major sites of inflammation in this EAE model. As shown in Figure 3, immunization with $PLP_{139-151}$ resulted in substantial numbers of infiltrating mononuclear cells in cerebellum and spinal cord. By contrast, no or only few cells were found in mice immunized with the mannosylated peptide. These data

suggest that immunization with M-PLP$_{139-151}$ did not result in sufficient numbers of effector cells capable to infiltrate the CNS or may have caused a delay in their migration into the CNS.

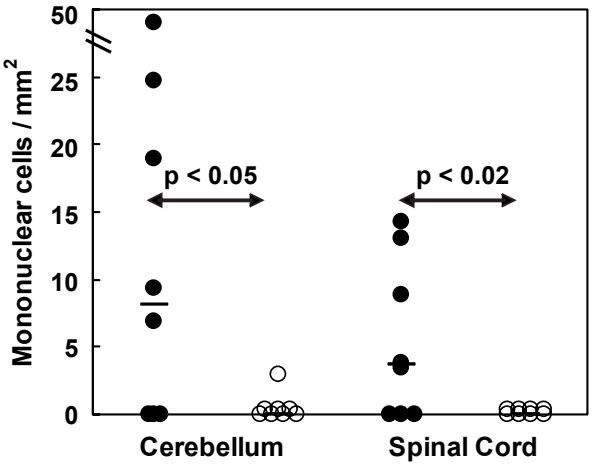

Figure 3. *Diminished_CNS-infiltration by mononuclear cells in M-PLP$_{139-151}$ immunized mice. Mice (n=8) immunized with 50 μg PLP$_{139-151}$ (filled circles) or 50 μg M-PLP$_{139-151}$ (open circles) emulsified in complete adjuvant were sacrificed on day 20. Cerebellum and spinal cord were analyzed for infiltration of mononuclear cells. Each symbol represents the result of an individual mouse.*

Th1 activity may be impaired in mice immunized with mannosylated peptide

To evaluate if immunization with mannosylated peptide resulted in qualitatively different T cell responses we determined the ability of M-PLP$_{139-151}$ immunized mice to mount a delayed-type-hypersensitivity response to the non-mannosylated peptide. As shown in Figure 4A, M-PLP$_{139-151}$ immunized mice have significantly less ear swelling after challenge with PLP$_{139-151}$ as compared to mice that had been immunized with the non-mannosylated peptide. This is suggestive for an impaired ability of T_h1 cells to migrate to the site of challenge and mediate a local inflammatory response, or an impaired development of encephalitogenic T cells.
Although lymph node cells isolated from mice primed with the mannosylated peptide and re-immunized with PLP$_{139-151}$ in complete adjuvant showed significantly lower peptide-specific T cell proliferation (Figure 4B), the cells did not display differences in terms of IFN-γ, IL-10 or IL-4 secretion (Figure 4B) and this was confirmed by similar levels of mRNA encoding these cytokines (data not shown).

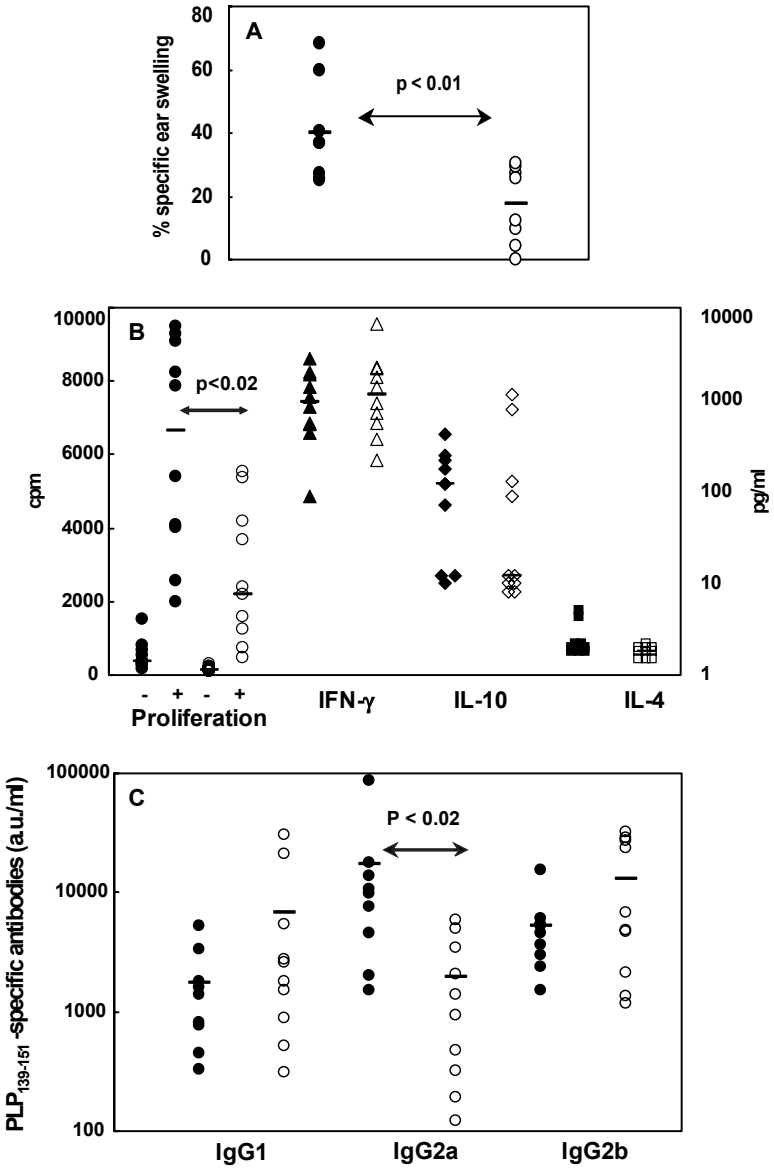

Figure 4. *Effects of M-PLP$_{139-151}$ on Th1 effector functions.* (A) *Mice were immunized with 50 µg PLP$_{139-151}$ (filled circles) or 50 µg M-PLP$_{139-151}$ (open circles) in complete adjuvant. After 15 days, mice were challenged by the injection of 13.5 nMol PLP$_{139-151}$ in 10 µl saline into the dorsal side of the right ear; as a control, 10 µl saline was injected into the left ear. For each individual mouse the percentage swelling was determined after 24 hours. Peptide-specific swelling was obtained by subtracting the % swelling of the control ear. (B) Lymph node cells were collected 58 days after immunization of mice with 50 µg PLP$_{139-151}$ (filled symbols) or 50 µg M-PLP$_{139-151}$ (open symbols) and 23 days after a boost with 50 µg PLP$_{139-151}$ in complete adjuvant. Cells were cultured with 10 µg/ml PLP$_{139-151}$ (+) or with medium (-) for a period of 4 days, the last 18 hours in the presence of 0.5 µCi [^{3}H]-Thymidine. Cytokines were measured by ELISA in culture supernatants collected on day 3.(C) Serum of individual mice treated as described under 4B was collected on day 58 and the subclass of antibodies specific for PLP$_{139-151}$ was analyzed. Each symbol represents the result of one individual mouse.*

Support for a decreased T_h1 response came from the analysis of the subclass of $PLP_{139-151}$-specific antibodies. As shown in Figure 4C mice immunized with mannosylated peptide and re-immunized with $PLP_{139-151}$ had lower levels of peptide-specific IgG2a antibodies, but normal levels of IgG1 or IgG2b antibodies. These data suggest that M-$PLP_{139-151}$ immunization induced a less vigorous T_h1 response.

Because it was uncertain whether these 2 peptides were comparably efficient in the induction of a peptide-specific T cell response, we also evaluated antigen-specific T cell responses early after immunization. As shown in Figure 5A, we did not obtain evidence that the early T cell response was different between mice that were immunized with $PLP_{139-151}$ versus M-$PLP_{139-151}$. Moreover, these data did not provide evidence that *in vitro* responses to M-$PLP_{139-151}$ were different from the responses to $PLP_{139-151,}$ which suggests that T cells recognized the two peptides equally efficient (Figure 5A). We substantiated this by demonstrating that purified CD4+ T cells from $PLP_{139-151}$ immunized mice responded similarly to mannosylated and non-mannosylated peptide presented by bone-marrow derived DC (Figure 5B). These data suggest that M-$PLP_{139-151}$ is well able to induce T cell proliferation.

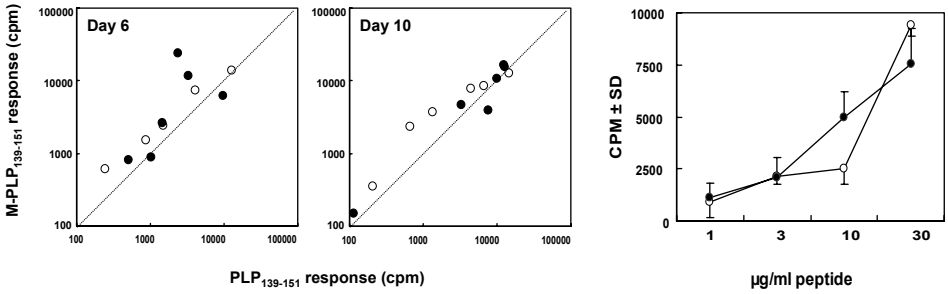

Figure 5. *No major differences in early T cell responses between M-$PLP_{139-151}$ and $PLP_{139-151.}$ Mice were immunized with 50 µg $PLP_{139-151}$ (filled circles) or M-$PLP_{139-151}$ (open circles) in complete adjuvant. Top panels: 6 and 10 days after immunization draining lymph node cells from individual mice were stimulated in vitro with 10 µg/ml $PLP_{139-151}$ (abscissa) or 10 µg/ml M-$PLP_{139-151}$ (ordinate). Each symbol represents one individual mouse. Statistical analysis did not reveal differences in the proliferative response between the two peptides used for immunization or restimulation in vitro. Bottom panel: CD4+ T cells were isolated from pooled lymph nodes of mice 15 days after immunization with 50 µg $PLP_{139-151}$ in complete adjuvant and stimulated with $PLP_{139-151}$ (filled circles) or M-$PLP_{139-151}$ (open circles) presented by bone-marrow derived DC. Results representative of three experiments are shown. T cell proliferation was measured on day 4 by labeling the cells with 0.5 µCi ^{3}H-Thymidine during the last 6 hours of culture.*

M-$PLP_{139-151}$ induced tolerance is antigen-specific

Because ligand binding to mannose receptors may cause down-regulation of IL-12 (20), we studied if tolerance was peptide-specific or due to a nonspecific suppressive mechanism. We immunized mice with two H-2^s binding peptides, i.e. M-$PLP_{139-151}$ or M-$OVA_{371-386}$, and a mannosylated control peptide incapable of H-2^s binding, each emulsified in complete adjuvant. As shown in Figure 6A, pre-treatment with M-$PLP_{139-151}$ rendered mice largely

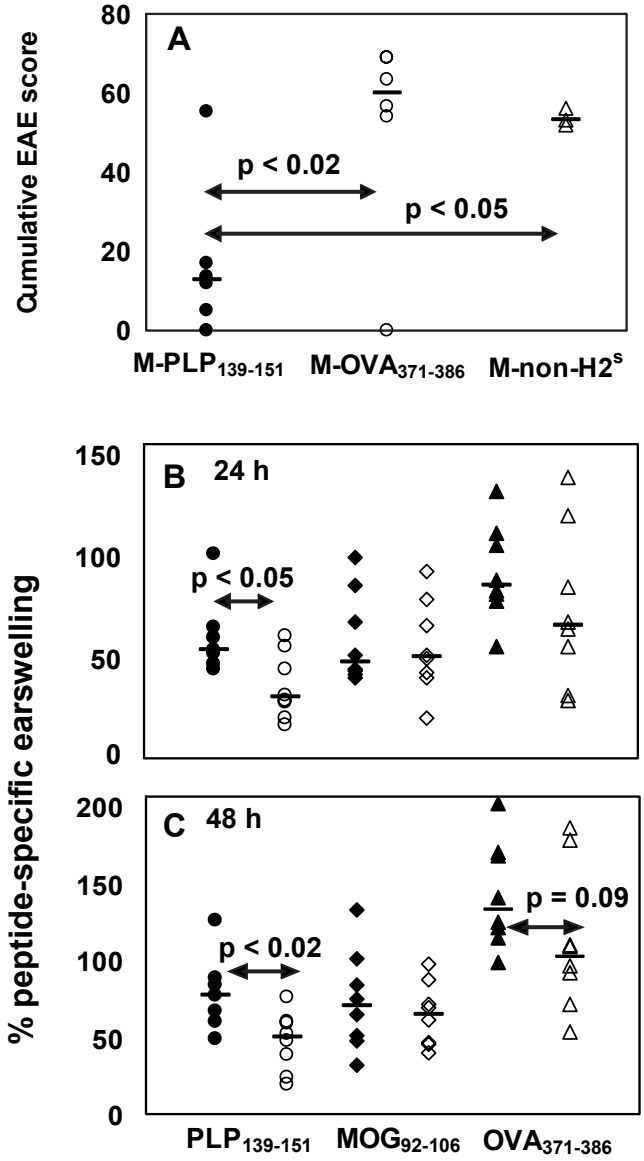

Figure 6. *Peptide-specificity of M-PLP$_{139-151}$ induced tolerance induction. (A) Mice were immunized (s.c.) with two H-2^s binding peptides, i.e. 50 µg M-PLP$_{139-151}$ (n=6) or M-OVA$_{371-386}$ (n=6), or with 50 µg of a mannosylated random peptide incapable of H-2^s binding (n=3). All peptides were emulsified in complete adjuvant. On day 31 all mice were re-immunized with 50 µg M-PLP$_{139-151}$ in complete adjuvant. Each symbol represents the cumulative EAE-score of an individual mouse from day 31 until endpoint (day 67). ANOVA, p < 0.05 ; p-values of post-hoc analysis are indicated in the Figure. (B) Mice were immunized (s.c.) with 50 µg PLP$_{139-151}$, 200 µg OVA$_{371-386}$ or 200 µg MOG$_{92-106}$ emulsified in complete adjuvant, in the absence (filled symbols) or presence (open symbols) of an equimolar amount of M-PLP$_{139-151}$. After 12 days, mice were challenged by the injection of 13.5 nMol of the non-mannosylated peptide used for immunization in 10 µl saline into the dorsal side of the right ear; as a control, 10 µl saline was injected into the left ear. For each individual mouse the percentage swelling was determined after 24 hours (Figure 6B) and after 48 hours (Figure 6C). Peptide-specific swelling was obtained by subtracting the % swelling of the control ear.*

resistant to subsequent EAE induction as opposed to pre-treatment with M-$OVA_{371-386}$ ($p < 0.02$) or M-non-H-2^s ($p < 0.05$). It is concluded that tolerance induction in this experimental setting is antigen-specific.

We also studied whether M-$PLP_{139-151}$ would suppress the immune response to a different myelin peptide, i.e. MOG_{92-106}, or to an irrelevant H-2^s binding peptide $OVA_{371-386}$. We immunized mice with these peptides or with $PLP_{139-151}$ emulsified in complete adjuvant, in the absence or presence of an equimolar amount M-$PLP_{139-151}$, and studied the effects on delayed-type-hypersensitivity by ear challenge with the immunizing non-mannosylated peptides twelve days later. As shown in Figures 6B and 6C, M-$PLP_{139-151}$ suppressed the DTH-response to $PLP_{139-151}$ but not to MOG_{92-106}; the DTH response to $OVA_{371-386}$ was not suppressed after 24 hours although a trend ($p=0.09$) towards significant suppression was observed after 48 hours. It is therefore likely that the effects of M-$PLP_{139-151}$ on EAE are largely peptide-specific although a slight non-specific mechanism can not be excluded.

Discussion

C-type lectins have gained a lot of interest in view of their role as receptors that facilitate the interaction between DC and microorganisms and the subsequent induction of a protective immune response. In contrast, it has also been shown that such interactions may result in the induction of tolerance. Targeting of ovalbumin to DEC205, a mannose-receptor expressed on immature DC, induces unresponsiveness *in vivo* (12, 13). However, as yet, no data are available to show that such an approach would induce tolerance in an autoimmune setting.

We have explored this possibility in EAE employing a mannosylated form of the encephalitogenic peptide $PLP_{139-151}$ that is known to induce EAE in SJL mice. While the non-mannosylated form of $PLP_{139-151}$ indeed induced disease, mannosylated $PLP_{139-151}$ was a poor inducer of EAE, despite the fact that this peptide was emulsified in adjuvant containing *M. tuberculosis*. This phenomenon was most likely not due to poor or supra-optimal recognition of the peptide, since a ten-fold lower amount of M-$PLP_{139-151}$ was capable of inducing a normal EAE response, whereas a hundred-fold lower amount of either peptide did not induce disease. Most likely, the intermediate dosage was high enough to induce an immune response in draining lymph nodes but too low to mediate suppression. We speculate that the high dosage of mannosylated peptide induced an optimal T cell response in the draining lymph nodes - as confirmed by our proliferation data -, but simultaneously elicited a suppressive mechanism due to a higher bioavailability.

As opposed to $PLP_{139-151}$-immunized mice, the mice immunized with M-$PLP_{139-151}$ did not show

significant inflammation of the CNS when evaluated shortly after the peak of disease activity. Possibly, such cells appear at a later time point albeit without causing severe symptoms at least until day 49 (Figure 1). In addition, T cells in M-$PLP_{139-151}$ immunized mice mediate a poor DTH response and may therefore be impaired either in their ability to migrate to the site of challenge or in their ability to mediate local inflammation.

Our observation that mannosylated peptide prevented EAE induction by the non-mannosylated encephalitogenic peptide when administered as a mixture, suggests that the mannosylated peptide induced active suppression of the disease. Indeed, mice that had been pre-treated with M-$PLP_{139-151}$ - as opposed to mice that were pre-treated with irrelevant mannosylated peptides - were largely resistant to induction of EAE with the encephalitogenic peptide in complete adjuvant. Because M-$PLP_{139-151}$ inhibited DTH induced by $PLP_{139-151}$ and not by MOG_{92-106} we conclude that tolerance induction in this system is largely peptide-specific. However, in view of a slight effect on the $OVA_{371-386}$ specific DTH response a minor non-specific suppressive effect can not be excluded.

Several mechanisms may play a role in the inhibition of disease development by M-$PLP_{139-151}$, including poor induction of T_h1 responses, anergy or deletion, immune deviation, induction of a suppressive mechanism, e.g. regulatory T cells, and limitation of disease by a local effect within the CNS.

Although shortly after immunization $PLP_{139-151}$ specific T cell proliferation was comparable in $PLP_{139-151}$ and M-$PLP_{139-151}$ immunized mice, the latter mounted a poor DTH response as compared to mice immunized with the encephalitogenic peptide. These data are in agreement with observations by Hawiger *et al.* (12) who showed that targeting of DEC205 on immature DC leads to a normal or even improved T cell proliferation without subsequent development of effector T cells. Alternatively, such cells may be impaired in their ability to migrate to the CNS or site of challenge. Further experiments are needed to establish whether the T cells that develop in response to immunization with mannosylated peptide show decreased expression of chemokine receptors and homing receptors impairing their migration into the CNS.

Importantly, after re-immunization with $PLP_{139-151}$ in complete adjuvant, lymph node cells from M-$PLP_{139-151}$ primed mice showed less vigorous $PLP_{139-151}$-specific T cell proliferation *in vitro*. Although this diminished proliferation was not reflected by differences in secretion of IFN-γ, IL-10 or IL-4, lower levels of peptide-specific IgG2a in serum may suggest that M-$PLP_{139-151}$ is less capable of inducing T_h1 effector cells.

To evaluate whether T cell recognition of the mannosylated peptide differed from the non-mannosylated peptide we studied presentation of the two peptides by bone marrow-derived DC to $PLP_{139-151}$-specific CD4+ T cells. Our observation that these responses were similar

does not support the possibility that differences in affinity for MHC class II and T cell receptor binding account for the tolerizing effect of mannosylated peptide *in vivo*. Indeed, this is in line with the finding that immunization with 5 μg of either peptide resulted in a similar disease pattern.

Importantly, the presence of a danger signal induced maturation of the DC and abrogated tolerance favoring a fully competent T cell response (12). Several agonists of toll-like receptors including mycobacterial cell wall components can drive maturation of DC and as a consequence induce a potent T_h1 response (21). On the other hand, binding of the mycobacterium-derived mannose-capped cell-wall component ManLAM to DC-SIGN may down-regulate T_h1 responses in favor of a T_h2 response (2). In line with these observations it is of interest to note that cross-linking of the mannose-receptor caused down-regulation of IL-12 (20) and up-regulation of IL-10 in human DC (22). The fact that we observed tolerance induction despite the presence of complete adjuvant containing (heat-killed) *M. tuberculosis* indicates that in our system peptide-binding to mannose receptors or other C-type lectins results in a powerful suppressive mechanism that is not easily abrogated by DC-activating signals. On the other hand, we found that the induction of tolerance with mannosylated peptide can be abrogated by including *Bordetella pertussis* or pertussis toxin (data not shown). Previously, pertussis toxin was found to interfere with the induction of antigen-induced T cell anergy (23). Moreover, it was recently demonstrated that pertussis toxin abrogated the induction of tolerance to EAE whereas the binding to G-proteins on antigen-presenting cells was suggested as a potential mechanism resulting in the activation of antigen-presenting cells in lymphoid tissues and the CNS (24). This suggests that the induction of tolerance versus immune responsiveness is dependent on a delicate balance between tolerizing and maturation signals delivered to dendritic cells.

Because irrelevant mannosylated peptides did not block EAE induction and since M-$PLP_{139-151}$ significantly inhibited DTH induced by $PLP_{139-151}$ but not by MOG_{92-106} we conclude_that tolerance induction in this system is largely peptide-specific. Although a minor non-specific suppressive effect can not be excluded in view of a slight suppressive effect of M-$PLP_{139-151}$ on the $OVA_{371-386}$ specific DTH response, it is tempting to speculate that mannosylated peptide induced active suppression or deletion of encephalitogenic $PLP_{139-151}$ specific T cells. So far we have been unsuccessful in demonstrating active suppression by transfer of spleen cells to naïve recipients (data not shown). Alternatively, mannosylated peptide may interfere with re-activation of T cells within the CNS, a process required for full development of disease (18, 19). Indeed, mannose-receptors have been demonstrated on a variety of cells including microglia (24, 25).

We observed that both $PLP_{139-151}$ and $M\text{-}PLP_{139-151}$ delayed the onset of clinical symptoms when administered shortly before the onset of disease and that the mannosylated peptide was more effective in suppressing the incidence and severity of EAE. Although preliminary data suggest that mannosylated peptide may also inhibit ongoing EAE, further studies are needed to reveal in more detail the mechanism by which mannosylated peptides alter the immune response and in particular the nature of the APC involved. Irrespective of the outcome of such studies our current results open new avenues towards the treatment of autoimmune diseases.

Acknowledgements

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3

An ELISA method for evaluation of the *in vivo* distribution of intact peptides

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Abstract

Immunization with mannosylated myelin peptide induces tolerance instead of disease in experimental autoimmune encephalomyelitis (EAE), an animal model for Multiple Sclerosis. Binding of this peptide to receptors that can recognize the attached mannose groups may result in a specific *in vivo* distribution pattern. Therefore, monitoring the distribution of mannosylated peptide *in vivo* may hint towards the mechanism underlying tolerance induction in EAE. For this purpose, mannosylated myelin peptide and its non-mannosylated counterpart were synthesized with tracers conjugated at both terminal ends. The development of an ELISA method based on the detection of these two conjugated tracers enables the detection of intact peptide in organ homogenates. In a first pilot experiment both peptides were injected intravenously into mice that were sacrificed thirty minutes later and the presence of either peptide in organ homogenates was evaluated. Applying this new method, both peptides could be detected in organ homogenates of mice, but so far no differences between the *in vivo* distribution of mannosylated myelin peptide and its non-mannosylated counterpart were observed.

Introduction

In experimental autoimmune encephalomyelitis (EAE), a model for Multiple Sclerosis, rodents are immunized with myelin fragments in complete Freund's adjuvant, resulting in disability due to inflammation in the central nervous system (1;2). Importantly, we have demonstrated that immunization of SJL mice with a mannosylated peptide from proteolipid protein (M-$PLP_{139-151}$) mediates tolerance induction to EAE instead of disease, despite the presence of a strong adjuvant (3). We hypothesize that targeting of M-$PLP_{139-151}$ towards mannose-recognizing receptors, for example C-type lectin receptors (CLR), may contribute to tolerance induction. Terminal mannose structures can bind with high affinity to the CLR family members DEC-205 and the Mannose Receptor (MR). Both these receptors are expressed by different subsets of antigen presenting cells (APC); in mice the expression of DEC-205 is restricted to dendritic cells (DC) (4;5), while the MR is primarily expressed by macrophages (6-8). Ligation of these receptors results in very efficient endocytosis and antigen presentation (9-11). In addition to membrane-bound receptors, also soluble mannose-binding structures are present in rodents and in humans, such as mannose binding lectins (MBL) in the blood stream (12;13).

In the field of drug delivery and gene therapy, targeting of liposomes containing a cargo of interest towards CLR has gained considerable interest. The receptor-mediated uptake of liposomes by APC is selective and very efficient; therefore this approach is regarded safe to be used as an adjuvant (14-17). For example, Kawakami et al. has shown that galactosylated, mannosylated and fucosylated liposomes were rapidly cleared from the blood stream and accumulated in the liver after intravenous injection due to CLR-mediated uptake (18). Specific modification of the liposome to induce more selective targeting and increased stabilization of the cargo inside contributed to further improvement of this method (19).

To evaluate the *in vivo* distribution of a compound of interest, radioactive labelling of this compound is most frequently applied (20;21). A major drawback of this technique is, that detection of the attached tracer not necessarily reflects the presence of the intact compound of interest. Because only intact epitopes are well-recognized by the immune system and the majority of proteins and peptides show low metabolic stability *in vivo* (22), a reliable tracing method is of particular concern to evaluate the distribution pattern of peptides.

Here, the development of a new method is described that can be used to monitor the *in vivo* biodistribution of intact peptides. This method was used to compare the *in vivo* distribution pattern of mannosylated myelin peptide (M-$PLP_{139-151}$) and its non-mannosylated counterpart ($PLP_{139-151}$). For this purpose, both $PLP_{139-151}$ and M-$PLP_{139-151}$ were conjugated at the terminal

ends; biotine was attached to the N-terminal side, while a dinitrophenyl-group (DNP) was conjugated to the C-terminus. To demonstrate the presence of intact peptide an ELISA system was set up, based on the simultaneous detection of both tracers. Pilot experiments were performed in which mice were intravenously injected with bis-labelled $PLP_{139-151}$ or M-$PLP_{139-151}$ and the presence of peptide in organ homogenates was subsequently evaluated.
Comparing the *in vivo* biodistribution of both peptides, we observed no obvious differences so far that may hint towards a mechanism underlying tolerance induction by mannosylated myelin peptide.

Materials and Methods

Animals

Female SJL mice, 6-10 weeks old were purchased from Janvier (Le Genest, France). All studies were performed with approval of the Animal Ethical Committee and in compliance with Dutch governmental regulation of Animal Experimentation.

Peptide synthesis

Peptides were synthesized by solid phase strategies on a SyroII automated multiple peptide synthesizer (MultiSyntech, Witten, Germany). TentagelS AC (Rapp, Tübingen, Germany), a graft polymer of polyethyleneglycol and polystyrene, was used as a resin (loading 0.2 meq, particle size 90μm) (23). Repetitive coupling cycles to sequentially introduce the various Fmoc amino acids (Nova Biochem, Läufelfingen, Switserland), carrying acid labile side chain protections when required, were performed basically as described using NMP as a solvent (24). In short, couplings were performed with 6-fold excess amino acid, activation was with PyBOP (Nova Biochem) / NMM (Aldrich, Zwijndrecht, The Netherlands) 1:2 (mol: mol) and removal of the Fmoc group was with piperidine/NMP 1:4 (v:v). The DNP tag was introduced into the peptides by application of Fmoc-Lys(DNP)-OH and biotin by coupling with Fmoc-Lys(biotin)-OH. The mannose groups were introduced N-terminally using Nα,Nε-bismannosylated lysine, Fmoc removal, and subsequent coupling with an acetyl-protected mannose building block. The control peptide carrying acetyl groups instead of mannose groups was prepared by N-terminal coupling with Fmoc-Lys(Fmoc)-OH, followed by Fmoc removal and acetylation using acetic acid/PyBOP/NMM.

After synthesis the peptidyl resins were washed extensively with NMP, dichloromethane, dichloromethane/ ether 1:1 (v:v) and ether respectively, and air dried. Peptidyl resins were treated for 2.5 hours with TFA containing 5 vol% water and 5 vol% ethanethiol. The solution was discolored by the addition of a few drops of triethylsilane and the peptide was precipitated with about 10 volumes ether/pentane 1:1 (v:v). After cooling for 1 hour at -20°C the peptides were isolated by centrifugation (-20°C, 2,500 x g, 10 min). After trituration and vortexing of the pellet with ether/pentane 1/1 (v/v) and isolation by the same procedure, the peptides were air dried at room temperature for 1 hour. The peptides were dissolved in water/ectic acid and lyophilized. Further purification occurred by RP-HPLC on a Vydac 218TP1010 column (Grace Vydac, Hesperia, CA, USA), using a water to acetonitrile gradient system containing 0.1% TFA, and lyophilized. Analyses of the purified peptides by Maldi-Tof mass spectrometry on a Voyager DE-Pro (Applied Biosystems) using 4-ACH (Aldrich) as a matrix and synthetic peptides of known molecular mass as internal reference revealed the expected masses. Peptide stock solutions were prepared by dissolving in DMSO (Merck, Amsterdam, The Netherlands).

Peptide sequences:

DNP-$PLP_{139-151}$-biotin:
Ac-K(Ac)-A-K**(biotin)**-R-H-S-L-G-K-W-L-G-H-P-D-K-F-R- K**(DNP)**-amide

DNP-$PLP_{139-151}$ (Competing peptide):
Ac-K(Ac)-A-K-R-H-S-L-G-K-W-L-G-H-P-D-K-F-R- K**(DNP)**-amide

DNP-M-$PLP_{139-151}$-biotin:
Man-K(Man)-A-K**(biotin)**-R-H-S-L-G-K-W-L-G-H-P-D-K-F-R-K**(DNP)**-amide

DNP-M-$PLP_{139-151}$ (Competing peptide):
Man-K(Man)-A-K-R-H-S-L-G-K-W-L-G-H-P-D-K-F-R- K**(DNP)**-amide

In vivo administration

Mice were injected i.v. with 50 or 100 µg of peptide diluted in 200 µl PBS. Control mice were injected with 200 µl PBS only and mice were sacrificed 30 minutes after injection, using CO_2.

Isolation and homogenisation of organs

Immunologically relevant organs, CNS tissue and organs in which CLR expression has been described, were isolated in standardized order as listed in Table 1. Blood was collected in EDTA immediately after sacrifice and plasma was obtained by centrifugation. All isolated organs were weighed and immediately snap frozen in cryogenic vials containing liquid nitrogen. The organs were stored at –80°C for homogenisation.

The whole homogenisation procedure was performed on ice to prevent peptide breakdown. All organs were homogenized in a fixed volume of ice-cold TX-100 homogenisation buffer (Table 1). The TX-100 buffer was prepared in milliQ and contained 20 mM Tris-HCl pH 7.4 (USB Corporation, Cleveland OH, USA), 137 mM NaCl, 10% glycerol (Invitrogen, Paisley, UK), 1% Triton X-100 (Fluka, Zwijndrecht, The Netherlands) and 2 mM EDTA (Fluka, Zwijndrecht, The Netherlands). Protease inhibitor cocktail tablets (Roche Diagnostics, Almere, The Netherlands) were added just before use. Frozen organs were cut into small pieces, transferred to a tube containing TX-100 buffer and homogenized with an Ultra turrax (IKA Labortechnik, Staufen, Germany). Organ homogenates were stored at -80°C for ELISA.

To dissociate protein complexes that may prevent the detection of bis-labelled peptides, organ homogenates were treated with a pH shock. The pH of homogenates was lowered to pH 1 with 5M HCl and subsequently the pH was restored to neutral levels with 5M NaOH.

ELISA

Flat-bottom 96 well maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 µl/well 2 µg/ml of Rat-anti-DNP antibody (Biogenesis, Poole, UK) in 0.1 M $NaHCO_3$ buffer pH 9.5. After a triple washing step with PBS containing 0.05% Tween-20 (Sigma, Zwijndrecht, The Netherlands), plates were blocked for two hours at room temperature with PBS containing 3% BSA (Invitrogen, Paisley, UK) and 0.05% Tween-20. Standard curves were prepared in TX-100 buffer, starting at 1000 pg/ml DNP-(M)-$PLP_{139-151}$-biotin, followed by two-fold dilution steps. After thorough washing, organ homogenate samples obtained from peptide-treated mice were incubated for two hours at room temperature (50 µl/well). To evaluate organ-specific background staining, control homogenates obtained from PBS-injected mice were included. In addition, all organ homogenate samples were incubated in the presence of competing peptide to determine the peptide specificity of ELISA signals. After another washing step, streptavidine-polyHRP (Sanguin, Amsterdam, The Netherlands)

diluted 1:10.000 in PBS containing 0.05% Tween and 0.02% gelatin (Merck, Amsterdam,The Netherlands) was added for one hour to bind biotinylated peptides. The plates were developed with 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine (TMB; Sigma, Zwijndrecht, the Netherlands), dissolved in sodium acetate pH 5.5 containing 0.003% H_2O_2 (Merck, Amsterdam, The Netherlands). The color reaction was stopped with 2M H_2SO_4 (Fluka, Zwijndrecht, The Netherlands) and plates were read at 450 nm using a Versamax microplate reader.

Table 1. Isolation of organs and preparation of organ homogenates

Organ isolation	TX-100 buffer (ml)	Isolation characteristics
Plasma	-	Analyze blood volume, collect plasma
Skin	1	From the back, shave hairs
Cervical LN	0.5	All
Axilliary and inguinal LN	0.5	All
Lungs	1	Both
Thymus	0.5	Whole
Pancreas	1	Whole
Spleen	1	Whole
Liver	6	Whole
Intestine	3	Part of the small intestine
Kidney	2	Both
Mesenteric LN	0.5	All
Spinal cord	1	Whole
Brain	3	Whole

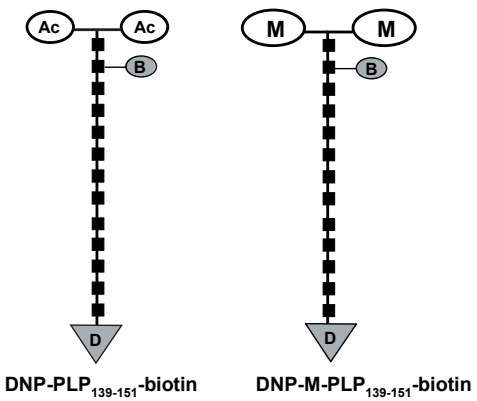

Figure 1. *Schematic representation of bis-labelled PLP139-151 and M-PLP139-151. ■ = Amino acid(s), B = biotin, D = DNP, Ac = Acetyl group and M = Mannose cluster.*

Results

Development and validation of the ELISA method

Initial experiments to develop an optimal peptide-design revealed that N-terminal attachment of tracers hampered the interaction between the coating antibody and the tracer due to the presence of mannose groups (data not shown). Therefore the peptides designed for *in vivo* distribution studies contained a biotine as well as two mannose clusters at the N-terminus and DNP at the C-terminus (Figure 1). A schematic representation of the developed ELISA method is depicted in Figure 2, in which detection of intact peptides is enabled by simultaneous detection of C-terminal DNP by Rat-anti-DNP coated on the plates and binding of streptavidine to N-terminal biotin.

To validate this ELISA method, dilution series of purified bis-labelled peptides were tested and in Figure 3 both standard curves are depicted. The detection limit of the designed ELISA was 60-120 pg peptide/ml for both peptides and this ELISA signal could be completely inhibited by a 10.000-fold excess of competing peptide.

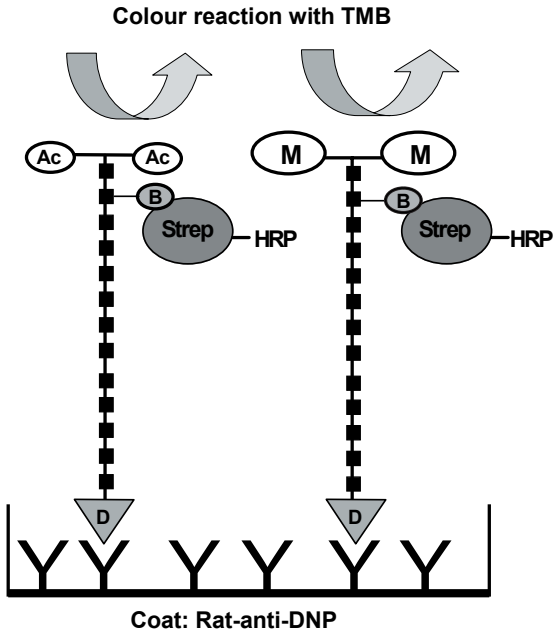

Figure 2. Schematic representation of the developed ELISA method. Plates were coated with Rat-anti-DNP antibodies (Y). Captured peptide was visualized with streptavidin-polyHRP ('Strep-HRP') and TMB was used as a substrate to develop the ELISA plates. ■ = Amino acid(s), B = biotin, D = DNP, Ac = Acetyl group and M = Mannose cluster.

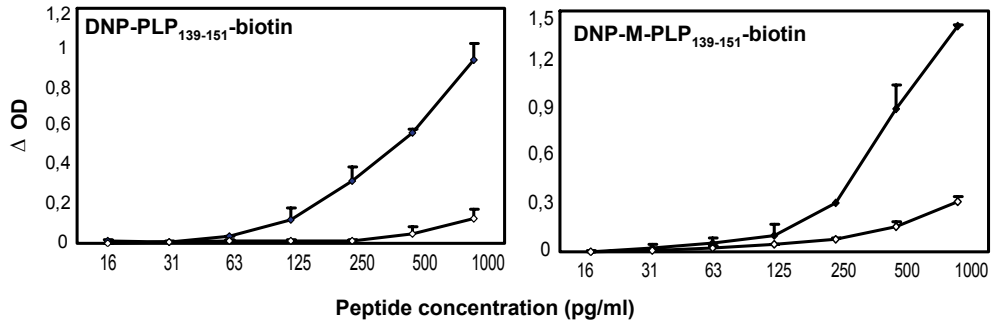

Figure 3. *Standard curves for ELISA. Standard dilution series of DNP-PLP139-151-biotine and DNP-M-PLP139-151-biotine were tested in duplicate with ELISA, starting at 1000 pg/ml followed by 2-fold step dilutions (filled symbols). The sensitivity was 60-120 pg/ml for either peptide. Peptide signals were blocked by a 10.000-fold excess of competing DNP-PLP139-151 or DNP-M-PLP139-151 (open symbols). The optical densities after subtraction of background signals are presented (ΔOD) and standard deviations are indicated.*

Detection of PLP $_{139-151}$ and M-PLP $_{139-151}$ in organ homogenates

To evaluate whether the developed ELISA was suitable for the detection of bis-labelled peptides in organ homogenates of mice, we intravenously inoculated either peptide, which results in a quick systemic distribution of the peptides. Two mice were injected with 100 µg DNP-PLP$_{139-151}$-biotine in 200 µl PBS, two mice with 50 µg of DNP-M-PLP$_{139-151}$-biotine and as a control mice were injected with PBS only. We could not inject higher doses of the peptides, because toxic effects of DNP-conjugated peptides were observed.

The mice were sacrificed after 30 minutes and homogenates were prepared from all isolated organs (Table 1). The homogenates from the two PBS-injected mice were used to evaluate the organ-specific background ELISA signals. Considerable differences between background signals of the isolated organs were observed. As expected, these background signals of control homogenates were not affected by incubation in the presence of competing peptide (Figure 4). The signals were independent from binding to coating antibodies (data not shown).

Despite this considerable background, peptide-specific signals could be detected in organ homogenates of mice, as demonstrated by incubation in the presence of competing peptide. In this way it was determined that thirty minutes after intravenous inoculation of DNP-PLP$_{139-151}$-biotin or DNP-M-PLP$_{139-151}$-biotin both peptides were primarily detectable in the liver, but also in plasma, lungs and spleen. Moreover, DNP-PLP$_{139-151}$-biotin was also detectable in the kidneys (Figure 5).

Unfortunately, only small amounts of the peptide could be retrieved from organ homogenates and we estimated this to be around 5‰ of the injected dose. To evaluate whether bis-labelled

peptides were complex-bound in organ homogenates, which would prevent their detection by ELISA, we additionally tested the organ homogenates after pH-treatment. This procedure only slightly increased in the detectable amount of peptide in the organ homogenates (data not shown).

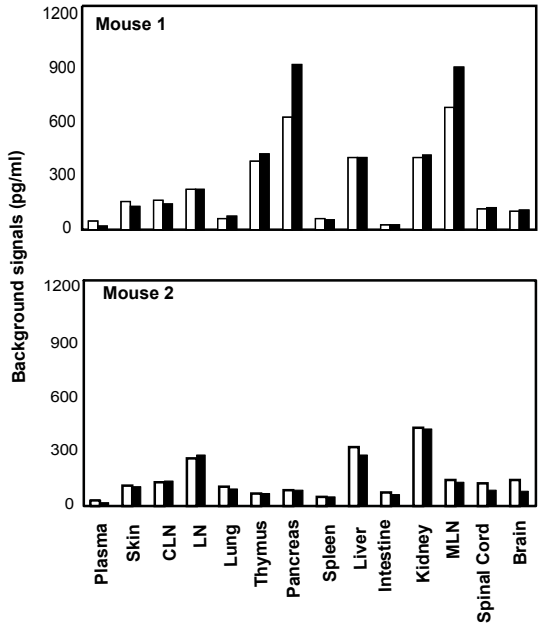

Figure 4. *Organ-specific background signals. Two mice were intravenously injected with PBS and sacrificed after thirty minutes to collect organs. Homogenates of these organs were tested by ELISA and all organs showed considerable background staining (open bars) that could not be blocked by competing peptides (filled bars).*

Discussion

Insight in the *in vivo* distribution of mannosylated self-peptide may provide a hint towards the mechanism underlying tolerance induction to EAE that has been observed after immunization with this peptide. Therefore, a method was developed to evaluate the *in vivo* distribution of intact peptides, consisting of a new peptide-labelling technique in combination with a compatible ELISA method. A intact peptide sequence is required for recognition of the epitope by immune cells and particularly this aspect is addressed in the newly developed assay.

To ensure a systemic distribution of the peptide which would facilitate peptide detection in organ homogenates, the intravenous route of inoculation was chosen for the initial pilot studies. Applying this new method the presence of intact peptide in organ homogenates of

mice thirty minutes after inoculation was demonstrated, despite the presence of considerable background signals of organ homogenates derived from sham-injected mice. Nevertheless, the peptide recovery rates were very low, which may be ascribed to the low metabolic stability of small peptides *in vivo*. Probably, the largest part of peptide is rapidly broken down upon intravenous injection, despite the terminally attached tracers that may slow down degradation. This again underlines the importance of monitoring the distribution of intact peptide. To further elucidate this phenomenon, additional *in vivo* tracing studies with less degradable peptides are suggested.

Besides the breakdown of peptide, the actual presence of peptide *in vivo* might well be underestimated, because only organs of interest were analysed. Moreover, we hypothesized that complex formation between bis-labelled myelin peptide and endogenous proteins in organ homogenates hampered the detection of peptide by ELISA. Indeed, we observed that spiking of bis-labelled peptides in different organ homogenates for thirty minutes at 37°C resulted in a loss of signal measured by ELISA (data not shown). These results suggest that complex formation by endogenous proteins contribute to a reduced detection of bis-labelled peptide by ELISA. To release complex-bound peptides an acidic pH treatment can be applied and this method has been successfully applied to dissociate MHC-peptide complexes (25;26). Although treatment of organ homogenates with such a pH-shock considerably increased the recovery of bis-labelled peptide after spiking, still part of the peptide remained undetectable. In a similar way, organ homogenates of intravenously injected mice were treated with a pH shock to release complex-bound peptide. Analysis of these pH-treated homogenates with ELISA revealed that the peptide recovery was only slightly increased (unpublished observations). To further improve the detection of labelled peptide in organ homogenates the application of other peptide dissociation strategies might be useful, for example dissociation with a basic pH shock or dissociation in the presence of competing peptide, to prevent re-aggregation of complexes.

Despite these technical drawbacks an initial picture of the *in vivo* distribution pattern of mannosylated and non-mannosylated peptide was obtained. In line with previous studies by others, we observed that the majority of mannosylated peptide was recovered from the liver (27;28). However, also non-mannosylated peptide accumulated in the liver, indicating that mechanism(s) independent from glycosylation mediated binding or uptake of the peptide. Both peptides were retrieved from sanguinary organs such as the lungs and the spleen, but specific uptake via mannose-recognizing receptors was not apparent. Glycosylation has been described as a useful tool to improve transport across the blood brain barrier (22). However, we found no indications that either peptide crossed the blood brain barrier and accumulated

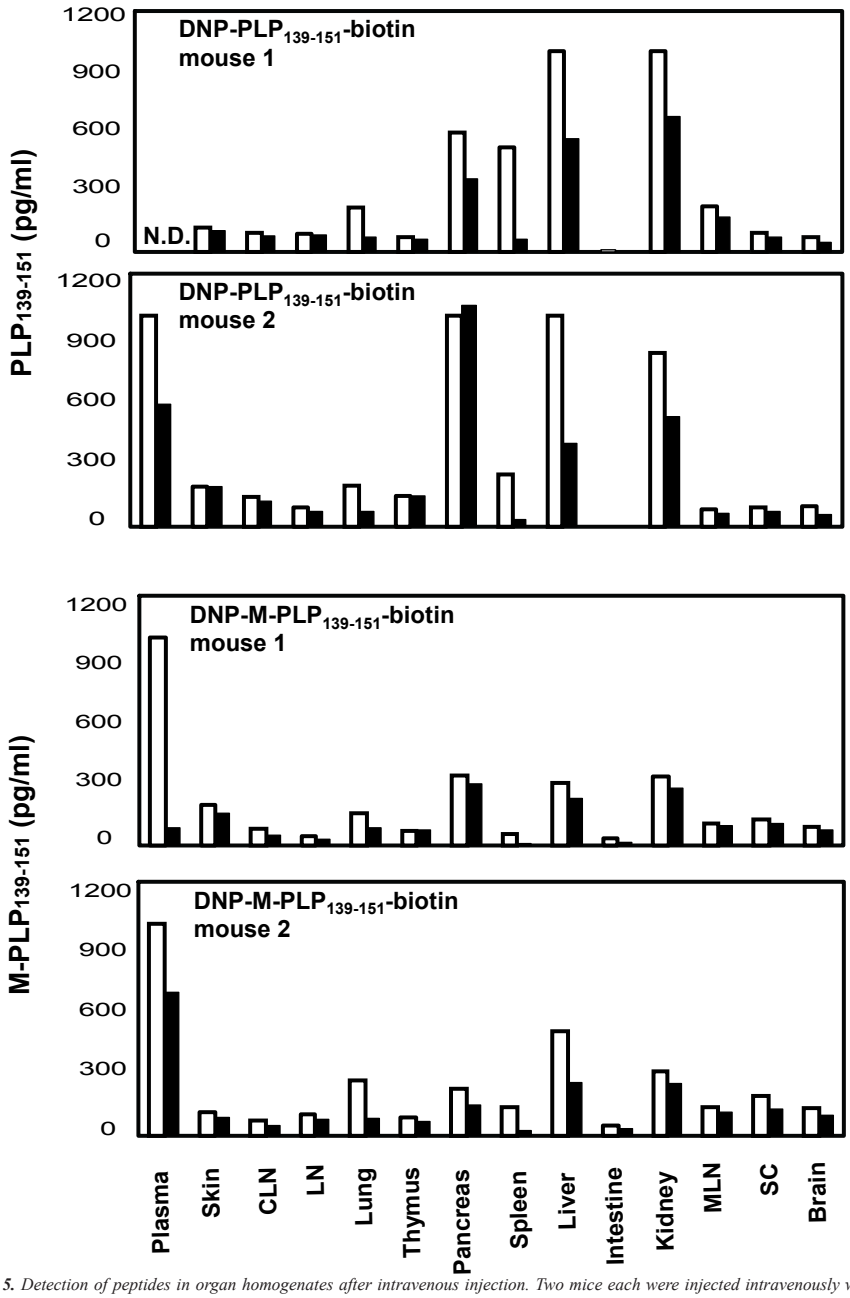

Figure 5. *Detection of peptides in organ homogenates after intravenous injection. Two mice each were injected intravenously with 100 µg DNP-PLP139-151-biotin or with 50 µg DNP-M-PLP139-151-biotin and were sacrificed after 30 minutes to collect organs. Homogenates of these organs were tested by ELISA in the absence (open bars) or presence (filled bars) of competing peptide. The difference between these two conditions represents peptide-specific signals.*

in spinal cord or brain tissue within thirty minutes. The results are suggestive for a reduced accumulation of mannosylated self-peptide in the kidneys, which may indicated that clearance of mannosylated peptide is delayed or decreased due to binding to mannose-recognizing structures *in vivo*. Altogether, these preliminary results indicate no striking differences between the *in vivo* distribution patterns of the two peptides.

Although it cannot be excluded that attachment of (small) tracers influences the *in vivo* distribution of the compound under investigation, Kessler et al clearly showed that dinitrophenylation does not alter the physiochemical properties of DNP-tagged molecules (29). Unfortunately, the inoculated dose of DNP-conjugated peptides could not be further increase to facilitate detection, because toxic side effects were observed. To our knowledge, tracing of DNP-conjugated whole proteins in rodents after intravenous injection was successfully applied by others, without toxic side effects (30) and the dose of DNP-coupled peptide used was below the known LD50 of 2,4-dinitrophenol in mice. However, the very same peptides conjugated to other tracers showed no such side effects (unpublished observations) and the observed toxic effects indeed matched the disturbance of energy processes in mitochondria that have been described for 2,4-dinitrophenol (31).

Although the aspects described above and the limited numbers of tested mice so far demand a careful interpretation of the data, we found no indications that the *in vivo* distribution of mannosylated self-peptide differed from its non-mannosylated peptide, which would hint towards a mechanism underlying the tolerance that was observed in EAE after immunization with M-$PLP_{139-151}$. However, targeting of mannosylated peptide towards a small population of specialized (antigen-presenting) cells may remain undetected in this newly developed assay due to sensitivity limits. A functional immunological assay, as described in chapter 5 of this thesis, may therefore be more suitable to locate antigen presentation in organs.

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4

IL-10 is not essential for tolerance induction with mannosylated self-peptide

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Introduction

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that is involved in immune regulation (1-3). It can be produced by antigen presenting cells (APC) (4-6), but also by regulatory T cells (7) and other lymphocytes such as B cells (8). Especially regulatory T cells have been described as key players in tolerance induction against autoimmune responses (9;10).
The importance of IL-10 in suppression of autoimmunity has been demonstrated by induction of experimental autoimmune encephalomyelitis (EAE) in IL-10 deficient mice (IL-$10^{-/-}$ mice). These mice developed more severe disease than wild type mice and also showed minimal recovery (11;12). Besides, it has been shown that treatment with IL-10 during EAE can ameliorate clinical disease in SJL mice (13).
We have previously shown that immunization with mannosylated self-peptide is a powerful way to induce antigen-specific tolerance to EAE (14). Injection of mannosylated antigens probably results in their targeting to C-type lectins, a family of sugar-recognizing receptors expressed on APC (15). Ligation of C-type lectins has been described as a way to induce anti-inflammatory pathways in APC, for example via IL-10 production (16;17).
To address the possibility that immunization with mannosylated self-peptide induces tolerance via an IL-10-dependent mechanism, EAE development in IL-$10^{-/-}$ mice and wild type mice was compared after immunization with mannosylated self-peptide. This immunization resulted in tolerance towards EAE in IL-$10^{-/-}$ mice and in reduced delayed type hypersensitivity responses. Therefore it can be concluded that IL-10 is not essential for tolerance induction by mannosylated self-peptide.

Materials and Methods

Animals

Female and male SJL mice were purchased from Janvier (France). IL-10 deficient mice (IL-$10^{-/-}$ mice) on a C57Bl6 background were obtained from Jackson Laboratories (Maine, US) and back-crossed to a SJL background for at least 10 generations. Animals were housed in individually ventilated cages with unlimited access to food and water. All experimental procedures were approved by the Animal Welfare Committee.

Peptides and immunization

$PLP_{139-151}$ (HSLGKWLGHPDKF) and its mannosylated form (M-$PLP_{139-151}$) were synthesized as described elsewhere (14). Mice (8-10 weeks old) were immunized subcutaneously (s.c.) with 50 µg $PLP_{139-151}$ or M- $PLP_{139-151}$ dissolved in PBS and emulsified in an equal volume of complete adjuvant supplemented with 1 mg/ml *Mycobacterium tuberculosis* (H37RA, Difco Laboratories, Detroit, MI, USA).

Delayed type hypersensitivity (DTH)

DTH responses were evaluated on day 18 by intradermal injection of 25 µg $PLP_{139-151}$ dissolved in 10 µl saline into the right ear of mice. As a control for non-specific ear swelling, saline was injected into the left ear. Ear thickness was measured before and 24 hours after injection. The results are expressed as the percentage specific ear swelling, obtained by subtracting the percentage non-specific ear swelling.

Statistical analysis

Statistical analysis was performed with a Mann-Whitney test.

Results and Discussion

To compare EAE development in wild type and IL-$10^{-/-}$ animals, mice were immunized with 50 µg $PLP_{139-151}$ or M- $PLP_{139-151}$ in complete adjuvant supplemented with 1 mg/ml *Mycobacterium tuberculosis*. Because IL-$10^{-/-}$ mice frequently suffer from spontaneous colitis (18), limited numbers of mice were available for long-term EAE studies. Therefore, equal numbers of male and female IL-$10^{-/-}$ mice were included in the studies, although gender differences with regard to EAE susceptibility have been described (19).

Importantly, the injection of heat-killed *Bordetella pertussis* (BP) bacteria or Pertussis toxin (PTX) was omitted in the EAE experiments, although it can be used to facilitate EAE development (20). However, this abrogates tolerance induction using mannosylated self-peptide, as described in chapter 6 of this thesis. This mild EAE induction protocol may explain why immunization of WT mice with $PLP_{139-151}$ in adjuvant containing *M. Tuberculosis* induced EAE in only 1 out of 6 WT mice during the first phase of disease (Figure 1A and Table 1). However, 5 out of 6 IL-$10^{-/-}$ mice developed clinical symptoms after immunization

with $PLP_{139-151}$ (Figure 1C), confirming that IL-$10^{-/-}$ mice are more susceptible to develop autoimmunity. Accordingly, IL-$10^{-/-}$ mice showed a high maximal and cumulative EAE score, compared to WT mice during the first phase of disease. Importantly, immunization with M-$PLP_{139-151}$ in adjuvant neither induced EAE in WT mice nor in IL-$10^{-/-}$ mice (Figure 1B and D).

Table 1. EAE development in WT and IL10 $^{-/-}$ mice

Phenotype	Immunization	EAE Incidence		Mean maximal score		Mean cumulative score	
		Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
WT	$PLP_{139-151}$	1/6	3/5 *	0.7 ± 1.2	1.4 ± 1.3	4.3 ± 8.7	12.2 ± 13.2
WT	M-$PLP_{139-151}$	0/6	1/6	0	0.3 ± 0.8	0	1.9 ± 4.7
IL10 $^{-/-}$	$PLP_{139-151}$	5/6	4/5 **	1.8 ± 1	2.2 ± 1.4	12.2 ± 6.5	25.5 ± 14.5
IL10 $^{-/-}$	M-$PLP_{139-151}$	0/5	2/5	0 #	0.5 ± 0.8 ##	0 ###	3.6 ± 4.7 #

*Phase I : Day 0-21. Phase II : Day 22-40. * One mouse died directly after the 2nd immunization (not due to EAE) and was excluded from further analysis. ** One mouse developed colitis during phase II and was sacrificed and excluded from further analysis. # P < 0.05 compared to IL10-/- mice immunized with $PLP_{139-151}$. ## P = 0.09 compared to IL10-/- mice immunized with $PLP_{139-151}$. ### P< 0.01 compared to IL10-/- mice immunized with $PLP_{139-151}$.*

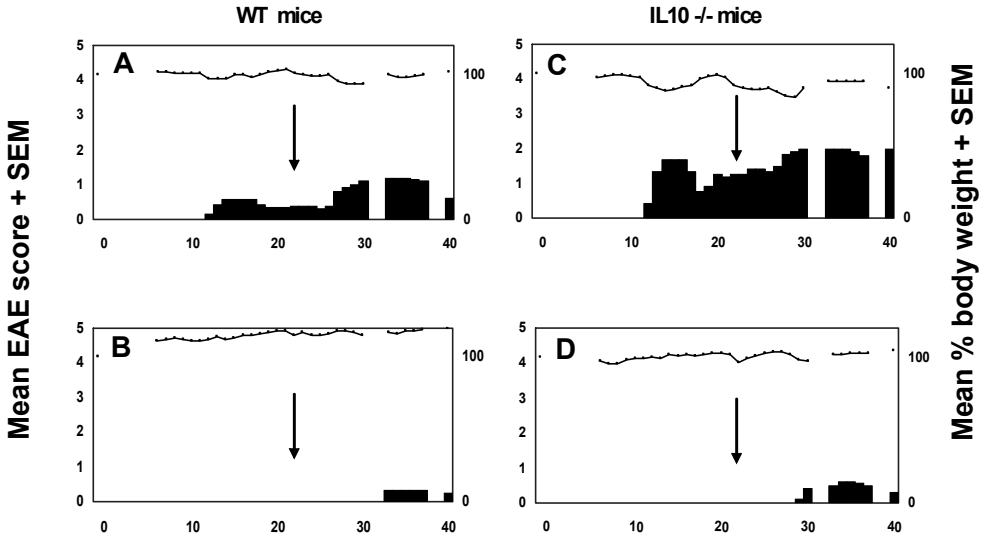

Figure 1. *Tolerance in WT and IL10 -/- mice after immunization with M-$PLP_{139-151}$. Male and female WT and IL10-/- mice were immunized with 50 µg $PLP_{139-151}$ (A and C) or M-$PLP_{139-151}$ (B and D) in complete adjuvant supplemented with 1 mg/ml Mycobacterium tuberculosis. On day 22 all mice were re-immunized with 50 µg $PLP_{139-151}$ in complete adjuvant (indicated with an arrow). EAE development and body weight were monitored daily. IL10-/- mice were more susceptible to EAE induction with $PLP_{139-151}$ (comparing panel A and C). Immunization of both WT mice (B) and IL10-/- mice (D) with M-$PLP_{139-151}$ resulted in the absence of EAE and resistance to re-immunization with $PLP_{139-151}$.*

To study whether mice were rendered tolerant, a second immunization with 50 µg $PLP_{139-151}$ in complete adjuvant supplemented with 1 mg/ml *Mycobacterium tuberculosis* was performed on day 22 (Indicated with an arrow in Figure 1). Three out of five available WT animals initially immunized with $PLP_{139-151}$ developed EAE symptoms upon secondary challenge. Clinical symptoms in $IL10^{-/-}$ mice worsened upon re-immunization, resulting in a further increase of the mean maximal EAE score (See Table I). The majority of WT and $IL10^{-/-}$ mice initially immunized with M-$PLP_{139-151}$ did not develop EAE upon challenge with $PLP_{139-151}$ and we therefore conclude that tolerance was induced in both WT and $IL10^{-/-}$ mice.

EAE development as well as DTH responses are highly dependent on T_h1 effector cells (21). In a second experiment, the role of IL-10 during local inflammation in the periphery was determined by studying DTH responses. Ear challenge of WT mice immunized with $PLP_{139-151}$ induced a mean ear swelling of 29 ± 12% after 24 hours and this swelling sustained until 48 hours after injection (See figure 2A). As expected, WT mice immunized with M-$PLP_{139-151}$ showed poor DTH responses 24 hours after ear challenge (19 ± 14%) and this became significantly reduced after 48 hours (18 ± 5%, P = 0.05). DTH responses in $IL10^{-/-}$ mice that were immunized with $PLP_{139-151}$ were similar to those in WT mice (23 ± 11%, Figure 1B) 24 hours after ear challenge, although a further increased DTH response was observed 48 hours after injection (40 ± 25%). Importantly, immunization of $IL10^{-/-}$ mice with M-$PLP_{139-151}$ resulted in significantly decreased DTH responses 24 hours after ear challenge (Figure 2B: 6 ± 0.4%, P = 0.03). After 48 hours DTH responses still showed a tendency towards a reduced response (15 ± 4%, P = 0.1). Male and female mice showed comparable responses in this experiment.

Summarizing, these data indicate that IL-10 does not play a crucial role in tolerance induction to EAE applying mannosylated self-peptide.

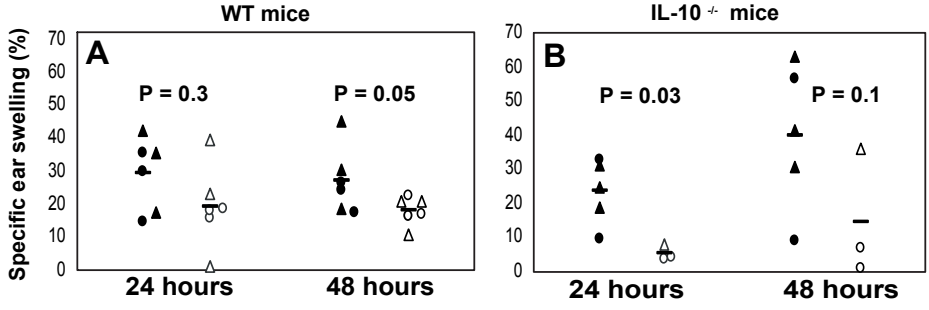

Figure 2. *Poor DTH responses in WT and $IL10^{-/-}$ mice after immunization with M-$PLP_{139-151}$. Male (Δ) and female (O) WT and $IL10^{-/-}$ mice were immunized with 50 µg $PLP_{139-151}$ (filled symbols) or M-$PLP_{139-151}$ (open symbols). On day 18 $PLP_{139-151}$ was injected into the right ear and saline into the left ear. Ear swelling was measured 24 hours and 48 hours later and peptide-specific ear swelling was calculated. WT mice immunized with M-$PLP_{139-151}$ showed a poor DTH response (A), which was significantly reduced compared to $PLP_{139-151}$-immunized mice. $IL10^{-/-}$ mice immunized with $PLP_{139-151}$ developed a DTH response that was comparable to WT mice and also in these mice immunization with M-$PLP_{139-151}$ induced a significantly reduced DTH response.*

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5

Immunization with mannosylated peptide induces poor T cell effector functions despite enhanced antigen presentation

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Abstract

In this study we investigated the development of T cell responses in mice after administration of a mannosylated ovalbumin-peptide (M-$OVA_{323-339}$). Immunization with M-$OVA_{323-339}$ in complete adjuvant resulted in enhanced antigen presentation in draining lymph nodes. Monitoring the fate of CFSE-labeled OVA-specific TCR transgenic $CD4^+$ T cells revealed that immunization with M-$OVA_{323-339}$ induced normal clonal expansion, recirculation and CD62L-expression of antigen-specific T cells *in vivo*. However, these T cells developed only poor effector functions, reflected by minimal IFN-γ production, low IgG2a levels in serum and poor peptide-specific Delayed Type Hypersensitivity (DTH) responses. This diminished inflammatory response was associated with decreased infiltration of T cell blasts and macrophages. Importantly, also mice with functional effector T cells did not mount a robust DTH response after a challenge with M-$OVA_{323-339}$ in the ear, although their T cells responded normally to M-$OVA_{323-339}$ *in vitro*. In conclusion, mannosylated peptide induces proliferation of T cells with impaired T_h1 cell effector functions and additionally abrogates the activity of pre-existing effector T cells.

Introduction

Antigen-presenting cells (APC) are well-equipped to discriminate between self and non-self via the expression of pathogen recognition receptors. The balance between stimulatory and inhibitory signals evoked by triggering these receptors enable APC to fine-tune immune responses (1). Toll-like receptors (TLR) can recognize a wide range of conserved molecular motifs of pathogens, which can result in APC maturation and the induction of a powerful immune response (2). Recognition of glycosylated self- and non-self antigens is mediated by C-type lectin receptors (CLR) (3). These receptors are highly expressed on immature APC and several studies indicate that CLR targeting may result in immune suppression and tolerance instead of immunity.

Studies by Apostolopoulos et al. have revealed that a tumor antigen conjugated to mannan is efficiently internalized by the mannose receptor, expressed on murine dendritic cells (DC) and macrophages, resulting in MHC class I or MHC class II restricted presentation, depending on the applied form of mannan (4;5). In line with this, we have previously shown *in vitro* that endocytosis of bis-mannosylated peptides by human DC is mediated via the mannose receptor, resulting in very efficient uptake and presentation in MHC II (6). However, applying this strategy of receptor-mediated uptake *in vivo* did not result in the development full-blown immunity. Instead, immunization with mannosylated self-peptide induced antigen-specific tolerance to experimental autoimmune encephalomyelitis (EAE) in mice. The presence of complete adjuvant containing *M. tuberculosis* could not break this tolerance, indicating that this might be a very powerful strategy to silence autoimmunity. Immunization with mannosylated self-peptide resulted in poor T cell effector functions, resulting in reduced DTH responses and the absence of EAE symptoms. Paradoxically, antigen-specific T cells were present *in vivo* and were responsive to antigen-specific stimulation *in vitro* (7). Several others have shown that members of the CLR family can modulate the immune response. For example, *in vivo* targeting of immature mouse DC can be achieved by antigen delivery via DEC-205. Fusion proteins consisting of an anti-DEC-205 antibody and a T cell epitope of Ovalbumin induced immunological unresponsiveness both to class I and class II restricted T cell epitopes (8;9). Similarly, targeting of myelin antigens towards DEC-205 was used to induce peripheral tolerance in EAE (10). It has also been shown that various pathogens can target DC-SIGN, expressed on DC, to circumvent intracellular degradation and antigen presentation, and prevent their elimination by the host (11-14).

Here, we studied the immune reaction in response to a mannosylated ovalbumin peptide, employing TCR transgenic $CD4^+$ T cells specific for $OVA_{323-339}$ (15;16). Immunization with

mannosylated $OVA_{323-339}$ (M-$OVA_{323-339}$) induced enhanced antigen presentation in draining lymph nodes and resulted in apparently normal expansion and recirculation of $OVA_{323-339}$-specific T cells. However, the effector functions of T cells were impaired, evident from poor DTH responses in conjunction with little infiltration of T cell blasts and macrophages at the site of antigen challenge.

Materials and Methods

Animals

Female C57BL/6 mice and B6.SJL mice were purchased from Charles River (Maastricht, Netherlands). OTII and DO11.10 mice on a C57BL/6 background were bred in our own facility. Animals were housed under standard conditions with free access to food and water. All experimental procedures were approved by the Animal Welfare Committee.

Peptide synthesis

$OVA_{323-339}$ (ISQAVHAAHAEINEAGR) and its mannosylated form (M-$OVA_{323-339}$) were synthesized as described elsewhere (6;17). In short, the peptides were prepared using solid phase synthesis. Mannosylation was accomplished by N-terminal elongation of the peptide with a building block containing lysine coupled to two tetra-acetyl protected mannose groups. The $OVA_{323-339}$ peptide was elongated with *bis*-acetyl lysine only. The acetyl protecting groups on the mannose moieties were removed using Tesser's base. All peptides were analyzed with Maldi-Tof mass spectrometry and showed the expected masses.

In vitro cultures

Spleens were isolated from OTII or DO11.10 mice and single cell suspensions were prepared through a 40 µm filter (BD Falcon, Bedford, MA). For CFSE-labeling, the cells were incubated with 5µM CFSE in RPMI 1640 (Cambrex, East Rutherford, NJ) for 10 minutes and subsequently washed twice with ice-cold PBS, as has been published by Lyons et al (18). To study *in vitro* proliferation, cells were resuspended in RPMI 1640 containing 5% FCS (Cambrex), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM ultraglutamine and 50 µM β-mercaptoethanol and seeded in 96-well flat bottom plates (Costar, Cambridge, MA) at a density of 2 x 10^5 cells per well. The OTII cells were stimulated with 0.1 – 100 µg/ml peptide

for 4 days. For long-term cultures OTII and DO11.10 cells were stimulated with 3-30 µg/ml peptide for 3 days and subsequently expanded in the presence of 15 U/ml IL-2 for 7 days. The recall response of LN cells isolated from immunized C57BL/6 mice was determined after 3 days of stimulation with 30 µg/ml $OVA_{323-339}$ or M-$OVA_{323-339}$.
For localization of *in vivo* antigen presentation C57BL/6 mice were euthanized and single cell suspensions were prepared from spleen and lymph nodes. To isolate cells from ears, skin layers were separated and incubated for one hour at 37°C with collagenase type IV (Worthington, Lakewood, NJ) before preparing a single cell suspension. Either of these cells were irradiated (30 Gy) and used as APC presenting endogenous antigen to naive $CD4^+$ OTII cells for 3 days at a ratio of 2 to1, without further addition of peptide. Naïve $CD4^+$ OTII cells were isolated from the spleen via negative selection with Dynal beads (Dynal Biotech, Oslo, Norway), resulting in a 80-85% pure $CD4^+$ T cell population. Supernatants were collected from the cultures and proliferation was assessed by addition of 0.5 µCi per well of ^{3}H-Thymidine (2Ci/mmol; Amersham biosciences, Buckinghamshire, UK) for 6 hours.

In vivo transfer of OTII cells

OTII mice were sacrificed and spleens and lymph nodes were isolated. A single cell suspension was prepared in RPMI medium and incubated on a nylon wool column for 45 minutes. This procedure depleted 50% of B cells and macrophages and resulted in an enriched cell suspension containing 50% $CD4^+$ T cells. Cells were washed extensively with PBS before transfer into C57BL/6 or B6.SJL mice. In some experiments OTII cells were labeled with CFSE before transfer as described above. Intravenous injection of 5 x 10^6 CFSE-labeled OTII cells into mice was performed one day before immunization.

Immunization protocol

Mice (7 to 10 weeks old) were immunized subcutaneously with 100 µg $OVA_{323-339}$ or M-$OVA_{323-339}$ dissolved in PBS and emulsified in an equal volume of complete adjuvant supplemented with 1 mg/ml *Mycobacterium tuberculosis* (H37RA, Difco laboratories, Detroit, MI). Control mice were immunized with PBS in adjuvant only.

Delayed Type hypersensitivity (DTH)

The DTH response was evaluated by injecting 12 nmol of $OVA_{323-339}$ or M-$OVA_{323-339}$,

dissolved in 10 µl saline, into the dorsal side of the right ear of mice using a Hamilton syringe fitted with a 30 gauge needle. As a control for non-specific ear swelling, 10 µl of saline was injected into the left ear. Ear thickness was measured before and 24 hours after intradermal injection using a Mitutoyo micrometer. Results are expressed as the percentage specific ear swelling, obtained by subtracting the percentage non-specific ear swelling.

Flow cytometry

For FACS analysis cells were collected from *in vitro* cultures, or single cell suspensions were prepared from isolated organs. Blood samples were treated with lysing solution (Pharmingen, San Diego, US) before staining. To isolate cells from ears, these were incubated with collagenase type IV (Worthington) for 1 hour before homogenization. Lungs and livers were isolated after perfusion of mice with PBS and incubated with collagenase type IV as described above. Subsequently a Ficoll gradient was used to isolate cells from these organ homogenates. Cells were stained with different rat-anti-mouse antibodies obtained from Pharmingen (CD3-FITC, CD4-PE, CD4-PerCP, CD25-biotine, CD45.2-PerCP, CD62L-APC, IFN-γ-Alexa647, IL10-FITC, IL-4 Alexa647, IL5-PE and F4/80 APC). Where needed isotype-matched controls were included (rat-anti-mouse IgM-biotin, rat-anti-mouse IgG1-PE and APC, rat-anti-mouse IgG2a-APC and rat-anti-mouse IgG2b-Alexa647). Biotinylated antibodies were visualized with Streptavidin-APC. For intracellular FACS staining cells were incubated at 4-6 hours with 10 µg/ml $OVA_{323-339}$ or PMA/ionomycin in the presence of Brefeldin A (Sigma, Zwijndrecht, The Netherlands). Fixation and permeabilization reagents (Caltag Laboratories, Burlingame, CA) were used for intracellular staining.

ELISA

IFN-γ, IL-4 and IL-10 production was measured in supernatants by ELISA. For IFN-γ antibody R46A2 (ATCC HB170) was used as capture antibody and biotinylated AN18 (kindly provided by Dr. Anne O'Garra, DNAX) as detection antibody. For IL-4 antibody 11B1 (ATCC HB188) was used as capture antibody and biotinylated BVD4 (kindly provided by Dr. Jon Abrams, DNAX) as detection antibody. For IL-10 antibody 2A5 was used as capture antibody and biotinylated SXC-1 as detection antibody (both obtained from Pharmingen, San Diego, US). ELISA plates with high binding capacities (Nunc, Roskilde, Denmark) were coated overnight with antibodies properly diluted in carbonate buffer pH 9.5 or phosphate buffer pH 6.5. After blocking with PBS containing 0.05% Tween and 0.02%

gelatin (PTG), plates were incubated with supernatant samples (diluted in culture medium). After washing, detection antibodies diluted in PTG were added to the plates. Subsequently, plates were incubated with Streptavidin-polyHRP (Sanquin, Amsterdam, The Netherlands) and developed using 3,3',5,5'-tetramethylbenzidine (TMB, Sigma). The color reaction was stopped with 2M H_2SO_4 and plates were read at 450 nm using a Versamax microplate reader. Standard curves were prepared using recombinant cytokines.

The detection of OVA-specific IgG antibodies in serum was performed as described elsewhere (19) and for in this study plates with high binding capacities were coated overnight with 2 µg/ml $OVA_{323-339}$ dissolved in PBS.

Statistics

Statistical analysis of data was performed with the Mann-Whitney U-test.

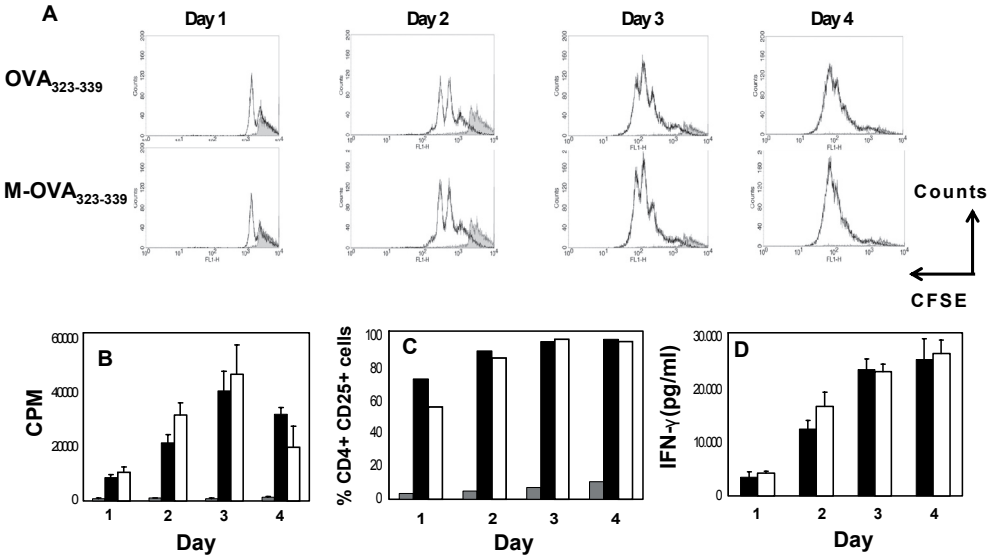

Figure 1. *Mannosylated peptide induces normal in vitro proliferation of OTII splenocytes. OTII splenocytes were cultured for 4 days in the presence of 10 µg/ml $OVA_{323-339}$ or M-$OVA_{323-339}$ or with medium alone. Cell division of $CD4^+$ T cells was evaluated via dilution of CFSE signal (Fig. 1A; the grey histograms represent the medium control) and incorporation of ^{3}H-Thymidine (B; ■ $OVA_{323-339}$, □ M-$OVA_{323-339}$, medium contol in grey).. CD25 expression by $CD4^+$ OTII cells was evaluated and numbers of positive cells are indicated (Fig. 1C; ■ $OVA_{323-339}$, □ M-$OVA_{323-339}$, medium contol in grey). IFN-γ production levels in supernatants collected on days 1 to 4 was evaluated in duplicate by ELISA (Fig. 1D). Similar results were obtained at the other peptide concentrations tested. Representative data from 4 different experiments are shown.*

Results

Mannosylated peptide is well-recognized by OTII splenocytes in vitro

To study whether peptide mannosylation influenced antigen recognition by OTII cells *in vitro*, we cultured CFSE-labelled OTII splenocytes for 4 days with different concentrations (0.1-100 µg/ml) of $OVA_{323-339}$ or M-$OVA_{323-339}$. Cell division of gated $CD4^+$ T cells was monitored daily by dilution of CFSE signal (Figure 1A) and by ^{3}H-Thymidine incorporation (Figure 1B). In general, OTII splenocytes proliferated equally to $OVA_{323-339}$ or M-$OVA_{323-339}$ at all peptide concentrations tested. Results after stimulation with 10 µg/ml peptide are depicted. Maximal proliferation was observed after 3 days of culture and no peptide-related differences were found. As depicted, medium-stimulated OTII cells showed no detectable proliferation.
We studied the activation state of $CD4^+$ OTII cells via their CD25 expression and as presented in Figure 1C similar CD25 expression was detected after stimulation with either peptide, reaching up to 90% positive cells on day 4. ELISA on culture supernatants revealed that both *in vitro* stimulation with $OVA_{323-339}$ and M-$OVA_{323-339}$ induced considerable IFN-γ production (Figure 1D). The levels of IL-4 were below the detection limit (< 8 pg/ml) under all conditions; stimulation with the highest concentration of $OVA_{323-339}$ induced some IL-10 production, while the mannosylated peptide did not (data not shown). Staining for Annexin-V revealed that stimulation with either peptide did not induce apoptosis of OTII cells (data not shown).
To address the possible development of T_h2 cells, which requires T cell progression through multiple cell divisions for full differentiation (20), we cultured CFSE-labeled OTII and DO11.10 splenocytes for a prolonged period of time. After 3 days of antigen-specific stimulation, we observed that DO11.10 cells had responded more vigorously to both peptides, as compared to OTII cells (Figure 2A). We further expanded the cells with IL-2 for one more week and on day 7 (data not shown) and day 10 cytokine production was determined by intracellular FACS staining. Both OTII and DO11.10 cells developed substantial numbers of IFN-γ-producing cells regardless of stimulation with mannosylated or non-mannosylated peptide; IL-4, IL-5 and IL-10 production was undetectable (Figure 2B and C).
Altogether, we conclude that i*n vitro* stimulation with $OVA_{323-339}$ or M-$OVA_{323-339}$ induced equal proliferation of $OVA_{323-339}$ specific $CD4^+$ T cells and resulted in the development of a T_h1 phenotype.

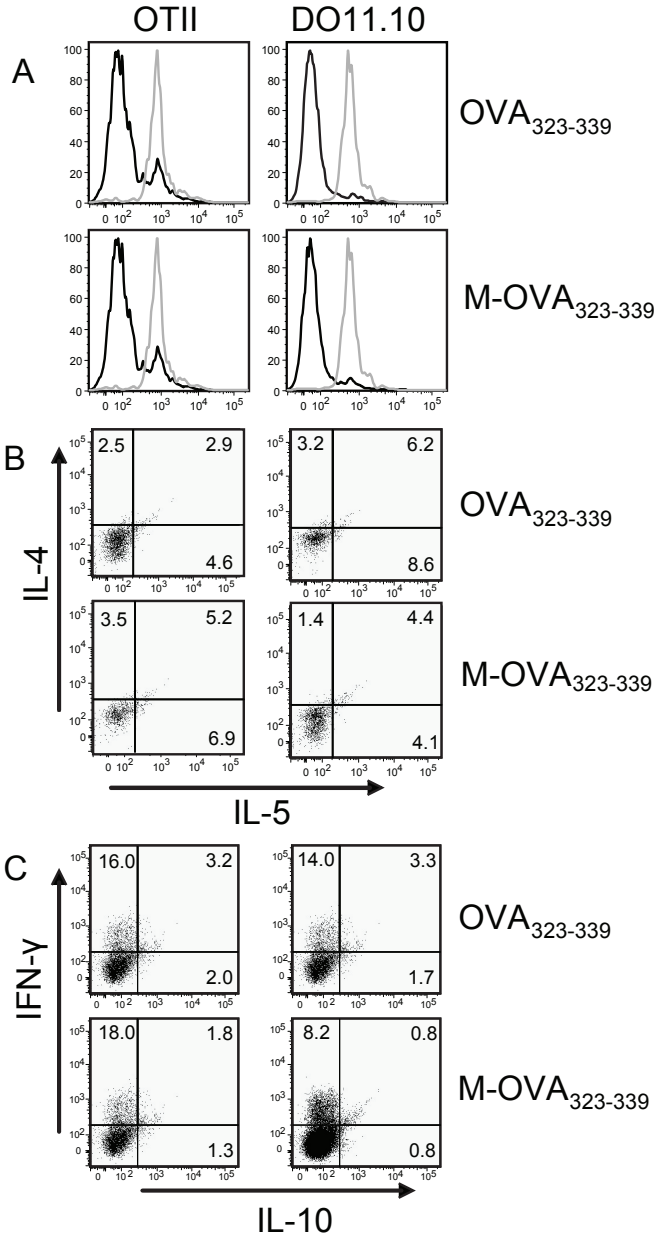

Figure 2. *Stimulation with mannosylated peptide does not support T_h2 differenation. CFSE-labeled DO11.10 and OTII splenocytes were cultured for 3 days in the presence of 10 μg/ml $OVA_{323-339}$ or M-$OVA_{323-339}$ or medium alone. Cell division of $CD4^+$ T cells was evaluated via dilution of CFSE signal on day 3 (Fig. 2A; the grey histogram represents the control). Subsequently the cells were expanded in the presence of IL-2 for another week and on day 10 the production of cytokines was determined by intracellular FACS staining. DO11.10 cells as well as OTII cells showed no IL4 and IL-5 staining (Fig. 2B), while considerable numbers of IFN-γ-producing cells were observed in the absence of IL-10 production (Fig. 2C). Similar results were obtained at other peptide concentrations tested.*

Immunization with mannosylated peptide results in an impaired T_h1 response

To evaluate whether immunization with mannosylated peptide induced qualitatively different T cell responses *in vivo*, the frequency of naïve $OVA_{323-339}$-specific T cells was first increased in C57BL/6 mice by injection of enriched OTII cells. One day later, these mice were immunized with $OVA_{323-339}$ or M-$OVA_{323-339}$ in CFA. Five days after immunization the ability of the mice to mount a DTH response to soluble $OVA_{323-339}$ was determined. We observed a tendency towards decreased ear swelling in M-$OVA_{323-339}$-immunized mice as compared to $OVA_{323-339}$-immunized mice ($P = 0.12$). A significant difference was observed when the mice were challenged again on day 26 (Figure 3A, $P < 0.01$).

After sacrifice on day 35 the lymph nodes of the mice were collected and the *in vitro* recall response to both peptides was determined. Despite the reduced DTH reactivity *in vivo*, equal proliferation was observed in mice immunized with $OVA_{323-339}$ or M-$OVA_{323-339}$, independent from the peptide used for stimulation (Figure 3B).

Analysis of the levels of $OVA_{323-339}$-specific IgG antibodies in the sera of these mice revealed that significantly less peptide-specific IgG was detectable in M-$OVA_{323-339}$-immunized mice ($P<0.05$), due to lower levels of IgG2a and IgG2b (Figure 3C).

Also in mice that were sacrificed on day 16 after immunization with M-$OVA_{323-339}$ equal *in vitro* responses of LN cells were observed and significantly lower levels of $OVA_{323-339}$-specific IgG2a, although the overall response at this earlier time point was less vigorous (data not shown). These data are supportive for decreased T_h1 reactivity in the absence of an increased T_h2 response.

Mannosylated peptide induces enhanced antigen presentation in vivo

Antigen presentation after immunization with $OVA_{323-339}$ or M-$OVA_{323-339}$ in adjuvant containing *M. tuberculosis* was next evaluated in C57BL/6 mice. For this purpose lymph nodes (LN) draining the immunization site, (non-draining) mesenteric LN and spleens were isolated one to ten days after immunization of these mice. The isolated cells were irradiated and used as APC presenting endogenous peptide to naive $CD4^+$ OTII cells without further addition of antigen. $CD4^+$ OTII cells showed minimal proliferation (<1000 CPM) when co-cultured with irradiated cells from sham-immunized mice (data not shown). Particularly, cells from the draining LN induced proliferation of naïve OTII cells, regardless of the peptide used for immunization, indicating that this was a major site of antigen presentation *in vivo*.

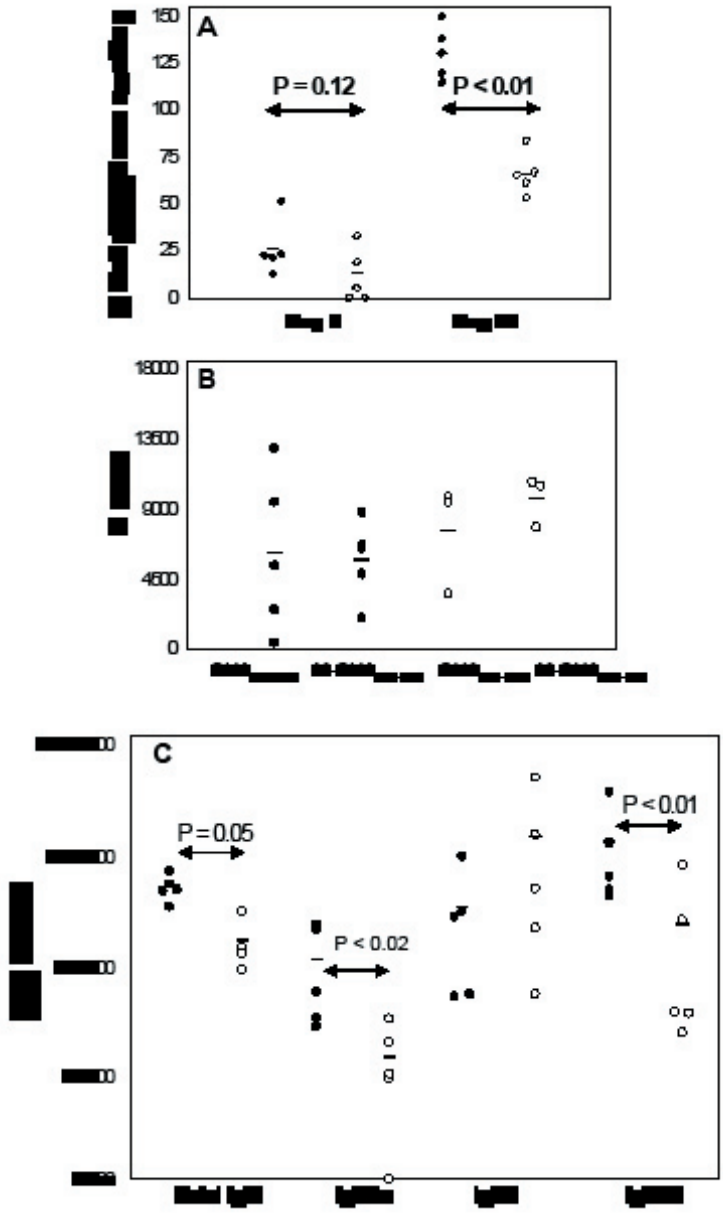

Figure 3. *Immunization with mannosylated peptide induces poor T_h1 responses. C57BL/6 mice were immunized with $OVA_{323-339}$ (filled symbols) or M-$OVA_{323-339}$ (open symbols) and DTH responses to $OVA_{323-339}$ were evaluated on day 5 and day 26 (Fig. 3A). Immunization with M-$OVA_{323-339}$ resulted in a significantly reduced DTH response on day 26 ($P < 0.01$). Mice were sacrificed on day 35 and draining lymph node cells were cultured in vitro with 10 µg/ml $OVA_{323-339}$ or M-$OVA_{323-339}$ (Fig. 3B). All animals showed similar proliferation, regardless of the peptide used for stimulation. $OVA_{323-339}$-specific IgG antibodies levels in serum were determined by ELISA (Fig. 3C). Immunization with M-$OVA_{323-339}$ resulted in significantly lower IgG titers ($P<0.05$), because IgG2a levels ($P < 0.02$) and IgG2b levels ($P < 0.01$) were reduced. Group means are indicated.*

Immunization with M-$OVA_{323-339}$ induced enhanced antigen presentation on days 1 and 3 (Figure 4A).

In a similar way antigen presentation after local administration of soluble mannosylated peptide was evaluated. For this purpose, soluble $OVA_{323-339}$ or M-$OVA_{323-339}$ or PBS was injected into the ears of C57BL/6 mice and one day later the draining (cervical) LN, (non draining) inguinal LN and the ears were used as a source of APC. After irradiation these cells were co-cultured with naive $CD4^+$ OTII cells without further addition of peptide. Injection of soluble M-$OVA_{323-339}$ into the ear induced increased antigen presentation in the draining LN 24 hours after administration (Figure 4B, $P < 0.05$), as opposed to injection of $OVA_{323-339}$. Part of the mannosylated antigen remained localized in the ear and was presented there by local APC to the same extent as $OVA_{323-339}$ (Figure 4C, $P < 0.05$ and $P = 0.08$ respectively, as compared to PBS controls).

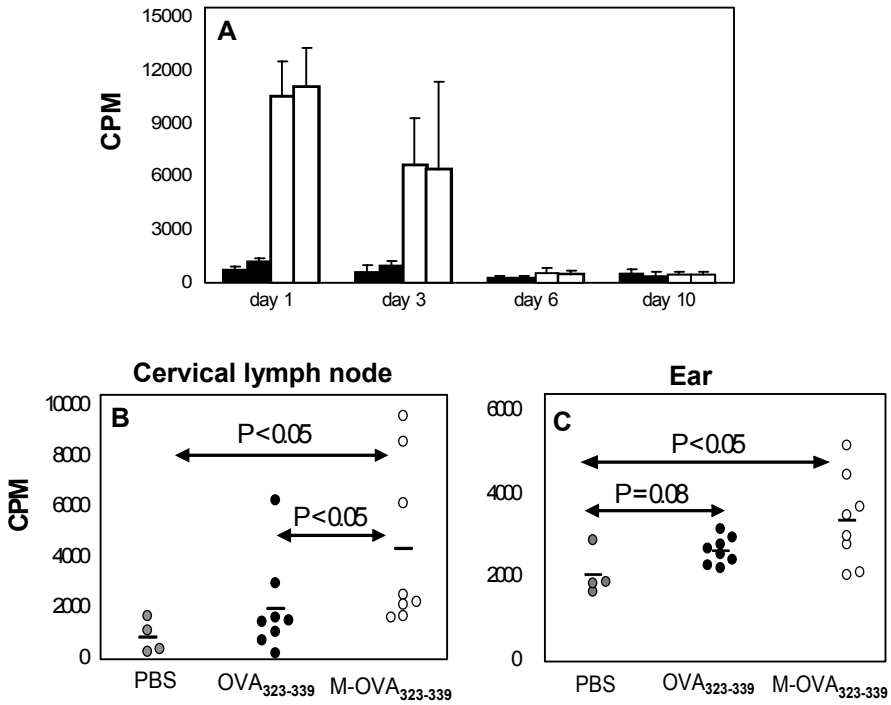

Figure 4. *Mannosylated peptide induces enhanced antigen presentation in vivo. C57BL/6 mice were immunized with $OVA_{323-339}$ or M-$OVA_{323-339}$ and on each indicated time point two mice were sacrificed (represented by individual bars). To detect antigen presentation, the draining LN were isolated, irradiated and co-cultured with naive $CD4^+$ OTII cells that were isolated via negative selection with Dynal beads and were 80-85% pure. No further antigen was added (Fig. 4A; ■ $OVA_{323-339}$, □ M-$OVA_{323-339}$) Mean data from quadruplicates are presented as CPM ± SD. One out of 3 similar experiments is presented. Figures 4B and 4C show the responses of C57BL/6 mice that received an intradermal injection with PBS, $OVA_{323-339}$ or M-$OVA_{323-339}$ into the ear. One day later cell suspensions were prepared from the cervical LN and ears. The cells were irradiated and co-cultured with naive $CD4^+$ OTII cells to detect antigen presentation. Proliferation was assessed in triplicates and evaluated by ^{3}H-thymidine incorporation (CPM ± SD). Results from two combined experiments are presented and group means are indicated.*

In both experiments described above substantial presentation of mannosylated peptide, but not the normal peptide, was observed in non-draining LN (data not shown).
In conclusion, both peptides were equally presented in tissue, while presentation of the mannosylated peptide in the (draining) LN was enhanced for a prolonged period of time.

Similar expansion and circulation of OTII cells in vivo after immunization with mannosylated peptide

To evaluate the clonal expansion of T cells in response to immunization with $OVA_{323-339}$ or M-$OVA_{323-339}$, CFSE-labelled OTII cells were transferred into B6.SJL mice ($CD45.1^+$), which enabled retrieval of OTII cells based on expression of the congenic marker CD45.2. Control mice were immunized with PBS in complete adjuvant.
Draining LN and spleens were isolated on days 4, 5 and 6 after immunization. Representative data obtained on day 4 are presented in Figure 5. Immunization with $OVA_{323-339}$ or M-$OVA_{323-339}$ resulted in the recruitment of similar numbers of OTII cells both to draining LN and spleens (Figure 5A). The division pattern of these OTII cells is depicted in Figure 5B and we observed that highly divided OTII cells were particularly situated in the draining LN,

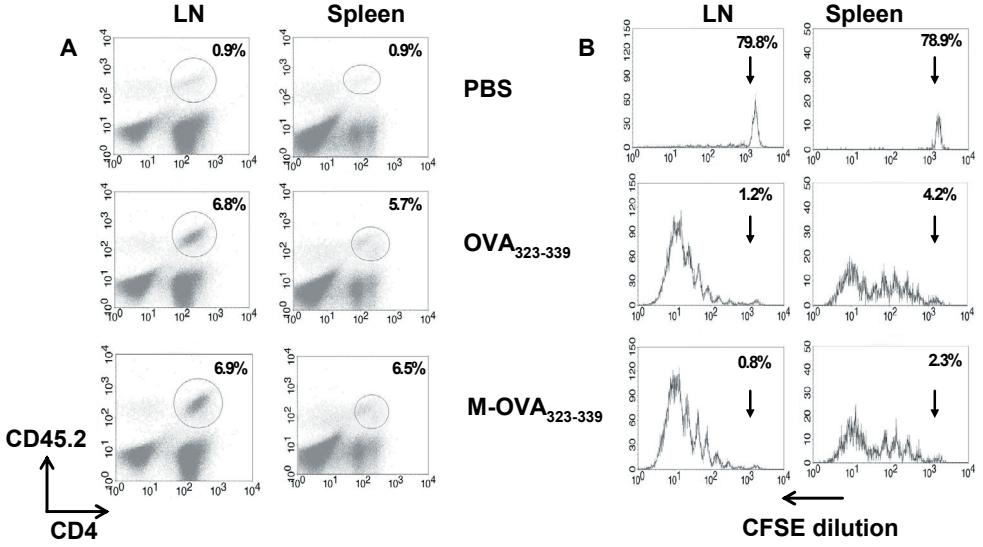

Figure 5. *Normal in vivo expansion of OTII cells after immunization with M-$OVA_{323-339}$. CFSE-labeled OTII cells were transferred into B6.SJL mice and these were immunized one day later with PBS, $OVA_{323-339}$ or M-$OVA_{323-339}$. Mice were sacrificed on day 4 after immunization and draining LN and spleens were isolated. The percentage of $CD45.2^+$ OTII cells within the population of $CD4^+$ T cells was determined by flow cytometry. These cells were gated and percentages are presented (Fig. 5A). Cell division was visualized via dilution of the CFSE signal. The percentages of undivided cells are indicated (Fig. 5B). Similar results were obtained on day 5 and 6. Three mice per group were studied and representative data are shown.*

while these cells in the spleen were less proliferative. No peptide-related differences were observed. Upon sham-immunization only low numbers of OTII cells (< 1%) were found in lymphoid organs and these were mainly undivided. Comparable results were obtained on day 5 and 6, although OTII cell division proceeded over time (data not shown).

Activation of T cells leads to downregulation of CD62L, allowing T cells to exit the LN and enter the circulation (21;22). We studied CD62L expression on $CD4^+$ OTII cells that were transferred into B6.SJL mice on day 5 and 6 after immunization with peptide or PBS. Five days after immunization with $OVA_{323-339}$ draining LN (Figure 6A) and spleen (Figure 6B) showed partial downregulation of CD62L and its expression further decreased on day 6 (data not shown). After immunization with M-$OVA_{323-339}$ similar results were obtained and the downregulation of CD62L was even slightly accelerated. Sham-immunization resulted in minimal CD62L downregulation.

Blood samples were collected during the first week after immunization to monitor the circulation of OTII cells. Immunization with either peptide resulted in the reduced presence of OTII cells in the blood stream on day 2 as compared to sham-immunization (Figure 6C; 0.4% versus 0.8%). On days 4 and 6 considerable numbers of OTII cells were present in

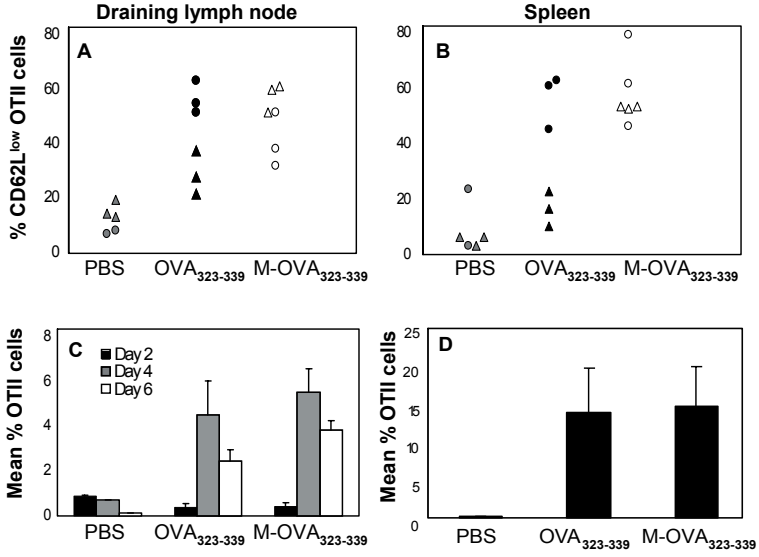

Figure 6. *Similar recirculation of OTII cells after immunization with M-$OVA_{323-339}$. CFSE-labeled OTII cells were transferred into B6.SJL mice and these were immunized one day later with PBS, $OVA_{323-339}$ or M-$OVA_{323-339}$. Five (triangles) and six days (circles) after immunization draining LN (Fig. 6A) and spleens (Fig. 6B) were isolated and CD62L expression on $CD4^+$ $CD45.2^+$ OTII cells was analyzed. Combined data from two experiments are shown. Blood samples were collected on days 2, 4 and 6 after immunization to monitor the presence of OTII cells. The numbers of $CD45.2^+$ OTII cells within the $CD4^+$ population were analyzed. Mean results (±SD) from at least three individual mice per time point are shown (C). Cells were isolated from the liver on day 4 and numbers of OTII cells within the live gate were analyzed; percentages of $CD45.2^+$ OTII cells within the $CD4^+$ population were calculated and mean data (+SD) from three individual mice are depicted (Fig. 6D).*

the blood stream after immunization with $OVA_{323-339}$ or M-$OVA_{323-339}$, but no peptide-related differences were observed. The majority of circulating OTII cells went through at least 6 cell divisions and highly expressed CD62L (data not shown).

Circulation of OTII cells was also evaluated by their presence in peripheral tissue, such as the liver. As depicted in figure 6D, similar numbers of OTII cells were isolated from the liver on 4 days after immunization with either peptide, while no OTII cells were detectable after sham-immunization. Isolation of OTII cells from the lung showed comparable results (data not shown).

Summarizing, these data indicate that mannosylated peptide induced normal clonal expansion and activation of OTII cells that were able to circulate through lymphoid as well as non-lymphoid tissue.

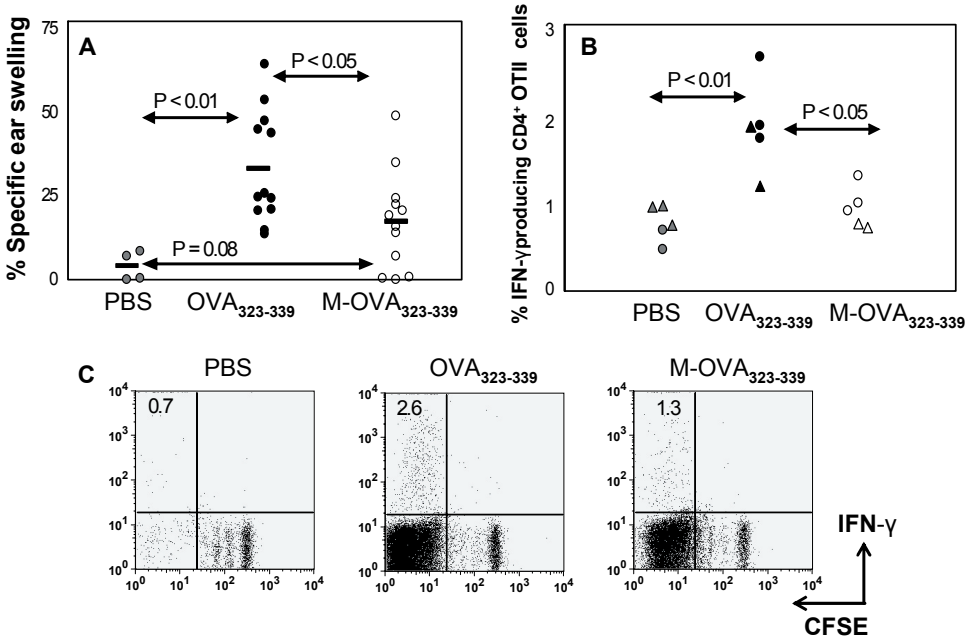

Figure 7. *Immunization with M-$OVA_{323-339}$ induces poor effector T cells in congenic mice. CFSE-labeled OTII cells ($CD45.2^+$) were transferred into B6.SJL mice ($CD45.1^+$) and these were immunized one day later with PBS, $OVA_{323-339}$ or M-$OVA_{323-339}$. On day 3 $OVA_{323-339}$ was injected intradermally into the dorsal site of the ear and swelling was evaluated after 24 hours (A). Combined data from three experiments are presented. Five (triangles) and six (circles) days after immunization draining LN were isolated and IFN-γ production by the injected OTII cells was evaluated by flow cytometry, after gating for $CD4^+$ $CD45.2^+$ cells. The combined data from two experiments are presented (Fig. 7B). In figure 7C a representative dot plot of each experimental group is depicted (gated on $CD4^+$ $CD45.2^+$) to show that in particular multiple-divided OTII cells produce IFN-γ.*

Immunization with mannosylated peptide induces poor T_h1 effector T cells

We evaluated T cell effector functions also in congenic recipient mice of CFSE-labeled OTII cells by means of DTH responses to soluble $OVA_{323-339}$, injected into the ear 3 days after immunization. As expected, immunization with $OVA_{323-339}$ in adjuvant induced functional effector T cells that elicited a substantial DTH response (32 ± 16%), as compared to sham-immunized mice (Figure 7A, $P < 0.01$). In contrast, immunization with M-$OVA_{323-339}$ in adjuvant induced a significantly lower DTH response (16 ± 14%, $P < 0.05$). The cell cycle plays an important role in helper T cell differentiation and it has been described previously that IFN-γ expression is increased with each cell division (20). Therefore, we evaluated the *in vivo* production of IFN-γ by CFSE-labeled OTII cells in the draining LN after transfer into congenic recipient mice was evaluated 5 and 6 days after immunization. In response to immunization with $OVA_{323-339}$, IFN-γ production by $CD4^+$ OTII cells that went through multiple cell divisions was detectable. However, in M-$OVA_{323-339}$-immunized mice the IFN-γ production by multiple-divided $CD4^+$ OTII cells hardly surpassed the level observed in sham-immunized mice (Figure 7B and 7C).

Local administration of soluble mannosylated peptide inhibits inflammation

Because immunization with M-$OVA_{323-339}$ in adjuvant induced poor DTH responses, we also characterized the cells that infiltrated the site of antigen challenge. For this purpose C57BL/6 mice were reconstituted with OTII cells, immunized with $OVA_{323-339}$ or M-$OVA_{323-339}$ in adjuvant and subjected to an ear challenge with soluble $OVA_{323-339}$ on day 5. Mice that were immunized with M-$OVA_{323-339}$ showed significantly decreased DTH responses as compared to mice that were immunized with $OVA_{323-339}$ (Figure 8A, $P < 0.05$). FACS analysis revealed that similar numbers of $CD3^+$ T cells were present in the ears of mice (data not shown), but that the absence of ear swelling was associated with a lower fraction of blast cells within this T cell population (Figure 8B, $P < 0.05$). As macrophages are considered indispensable effector cells in DTH responses, we analyzed the numbers of F4/80 positive cells and we observed that the numbers of macrophages were considerably, though not significantly, reduced (Figure 8C).

To evaluate whether mannosylated peptide could trigger an inflammatory response mediated by pre-formed effector T cells, mice immunized with $OVA_{323-339}$ were also challenged with

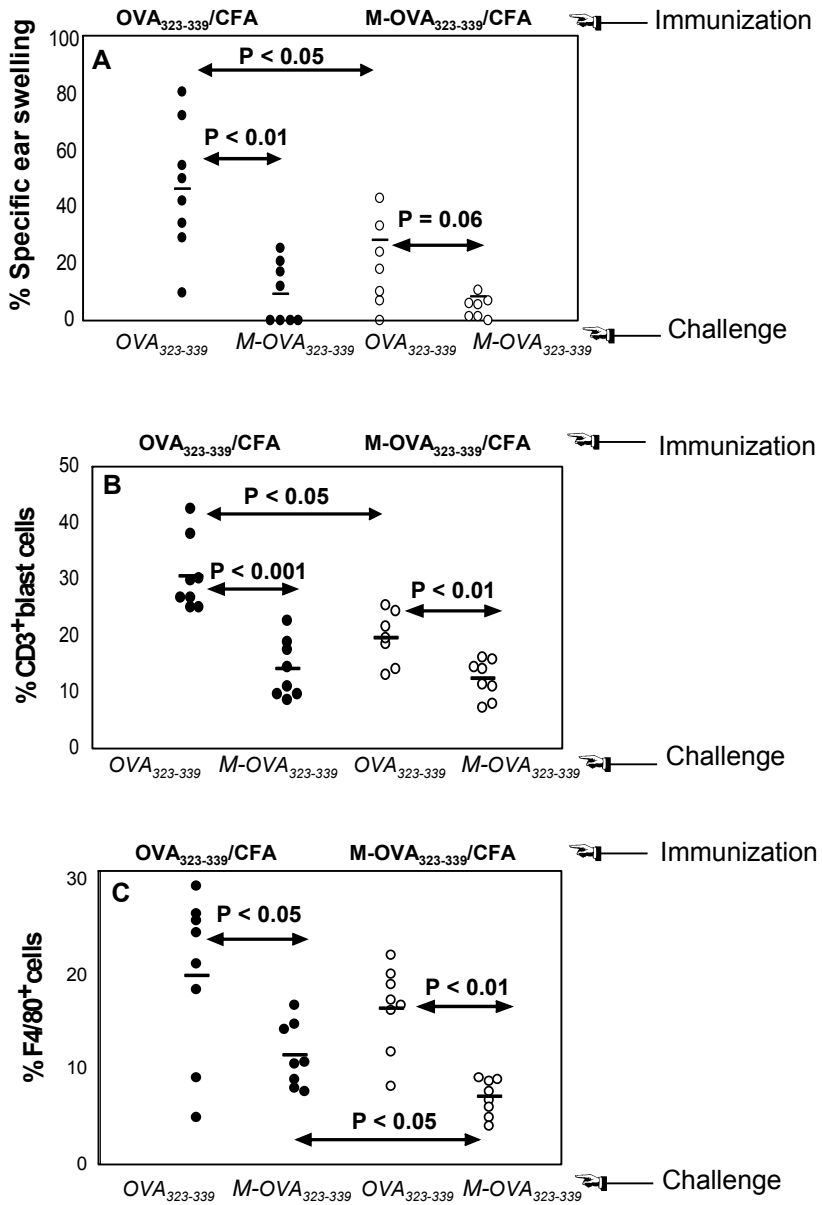

Figure 8. *Local injection of soluble M-OVA$_{323-339}$ inhibits DTH responses. C57BL/6 mice were reconstituted with OTII cells and immunized with OVA$_{323-339}$ (filled symbols) or M-OVA$_{323-339}$ (open symbols) and on day 5 the in vivo recall response of T cells was tested by intradermal challenge with 12 nmol OVA$_{323-339}$ or M-OVA$_{323-339}$ into the ear. Ear thickness was measured 24 hours later (Fig. 8A). Subsequently, the mice were sacrificed and the ears were collected to isolate the cells that accumulated in response to peptide injection. The numbers of blastoid cells within the CD3$^+$ T cell population (Fig. 8B) and the numbers of F4/80$^+$ macrophages (Fig. 8C) were determined by flow cytrometry. The reduced swelling of ears was associated with significantly decreased numbers of T cell blasts and macrophages. Combined data from two experiments are presented and group means are indicated.*

soluble M-$OVA_{323-339}$. Importantly, ear swelling was significantly lower after injection of soluble M-$OVA_{323-339}$, as compared to soluble $OVA_{323-339}$ ($P < 0.01$). The reduced ear swelling was reflected by lower numbers of blastoid T cells (Figure 8B, $P < 0.001$) and macrophages (Figure 8C, $P < 0.05$). M-$OVA_{323-339}$-immunized mice challenged with soluble M-$OVA_{323-339}$ showed the lowest DTH response of all groups (Figure 8A, $P = 0.06$ compared to challenge with $OVA_{323-339}$), which was associated with reduced blastoid T cells and macrophages (Figure 8B and 8C, $P < 0.01$).

Thus, in addition to poor development of T_h1 effector cells in response to immunization with M-$OVA_{323-339}$ in adjuvant, local downregulation of T_h1 effector functions by soluble M-$OVA_{323-339}$ contributes to immune suppression.

Discussion

We here show that immunization with mannosylated OVA-peptide results in immunological unresponsiveness of TCR transgenic OTII cells. Although the mannosylated peptide caused robust T cell proliferation *in vitro* and *in vivo*, immunized mice showed decreased DTH responses, diminished IgG2a levels in serum, reduced blastogenesis and minimal IFN-γ production, all indicative for impaired T_h1 cell effector functions. These data indicate that the proliferative cells did not develop full effector functions in response to mannosylated peptide or were unable to migrate into inflamed tissue. This finding is in line with previous studies in which we have demonstrated that mannosylated self-peptide induced antigen-specific tolerance to EAE (7).

The immunological unresponsiveness did not result from poor recognition of the mannosylated peptide, as *in vitro* stimulation of OTII and DO11.10 splenocytes with both peptides induced equal expansion of T cells with a T_h1-phenotype (Figure 1).

The possibility that immunization with mannosylated OVA-peptide caused a disturbed formation of memory T cells *in vivo* was addressed by monitoring the fate of CFSE-labeled OTII cells and this approach revealed normal expansion of OTII cells, which seemed well-activated based on the downregulation of CD62L. Targeting of antigen towards DEC-205 induced enhanced early expansion of $CD4^+$ T cells, followed by immunological unresponsiveness due to deletion or the induction of $CD5^+$ T cells (8;10). Therefore, it might be that major differences *in vivo* in response to mannosylated peptide occurred at earlier time points (before day 4) , which have not been evaluated in the present studies.

Another possibility might be that effector T cells do not mediate their effects, because they have impaired migratory capacities, as previously described Mirenda et al (23). However,

OTII cells were detectable in peripheral organs as well as in the blood (Figure 6). On the other hand, it has been described that only fully differentiated T_h1 cells are able to infiltrate inflamed peripheral tissue, while non-polarized T cells are not (24;25). Therefore, we cannot exclude that the differentiation state of OTII cells after immunization with mannosylated OVA-peptide limited their extravasation into local tissue during inflammation, resulting in poor DTH responses. Future studies will focus on the gene expression profile of T cells in response to mannosylated peptide to gain more insight into the mechanism of T cell unresponsiveness.

Besides the fact that mannosylated OVA-peptide induced poor T cell effector functions, pre-existing effector functions of fully polarized $CD4^+$ T were also inhibited by soluble mannosylated OVA-peptide. $OVA_{323-339}$-immunized mice, with competent effector T cells, displayed impaired DTH responses to an intradermal ear challenge with soluble mannosylated OVA-peptide. These poor DTH responses were associated with reduced recruitment of T cell blasts and fewer infiltrating macrophages. This phenomenon cannot be explained by a lack of antigen presentation mediated by local APC, as co-culture experiments revealed presentation of mannosylated OVA-peptide in the ear (Figure 4). Therefore, it is likely that the cells presenting mannosylated OVA-peptide in the dermis or epidermis suppressed the effector functions of infiltrating T cells. In line with this, we have previously shown that treatment with soluble mannosylated self-peptide during ongoing autoimmunity can ameliorate clinical symptoms in EAE (26). Our findings extend previous observations by Higgins et al who described that $CD4^+$ effector T cells can be tolerized in lymphoid and non-lymphoid organs after injection of soluble antigen, resulting in abrogation of IFN-γ and IL-2 production and reduced blastogenesis (27).

The presentation of mannosylated peptide *in vivo* itself was not impaired, but rather improved, according to the very efficient antigen presentation in the skin and draining lymph nodes (Figure 2). These results are in line with *in vitro* studies in which bis-mannosylated peptides were targeted to the mannose receptor, resulting in more efficient uptake and presentation of antigens (28-30). Although the exact nature of its receptor remains to be elucidated, it is likely that mannosylated peptides are targeted towards a member of the CLR family, which are widely expressed by APC in both lymphoid and non-lymphoid organs (31-33). It might well be that ligation of CLR by these bis-mannosylated peptides induces an anti-inflammatory program in APC. Although previous studies by Sheng et al indicated that cross-linking of CLR by mannan results in DC activation to some extent, this signal alone was insufficient for full DC maturation (34). It becomes increasingly clear that (pathogen-mediated) signaling by CLR can modulate the immune response via the inhibition of proinflammatory NFkB-

mediated signaling pathways (35-37). The existence of endogenous CLR ligands (38-40) is suggestive for self-control of immune responses; therefore it might well be that our findings reflect physiological mechanisms that are indispensable for immune regulation and the prevention of autoimmunity.

Especially immature APC are considered to be tolerogenic (41;42) and Hawiger et al showed that tolerance induction employing targeting of CLR was abrogated by ligation of CD40 (8;9). Therefore, it is striking that mannosylated OVA-peptide induced a poor T_h1 response despite the presence of complete adjuvant containing *M. tuberculosis*. Mannosylated peptides can thus be considered as a very powerful tool to control the effector functions of T cells.

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Mannosylated self-peptide inhibits the development of experimental autoimmune encephalomyelitis via expansion of non-encephalitogenic T cells

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Abstract

Tolerance to experimental autoimmune encephalomyelitis (EAE) in SJL mice can be induced by immunization with a mannosylated form of $PLP_{139-151}$ ($M\text{-}PLP_{139-151}$), despite the presence of complete Freund's adjuvant. The state of tolerance is characterized by poor DTH responses and the absence of clinical EAE symptoms. *In vivo* monitoring of CFSE-labeled $PLP_{139-151}$-specific TCR transgenic (5B6) T cells revealed that immunization with $M\text{-}PLP_{139-151}$ increases the clonal expansion of 5B6 T cells that do not develop full effector functions. Moreover, non-functional T cells obtained from $M\text{-}PLP_{139-151}$-immunized mice showed poor blastogenesis and were unable to transfer EAE to naïve recipients. Nevertheless, the *in vitro* production of cytokines and chemokines associated with EAE was unaffected. Importantly, tolerance induced by $M\text{-}PLP_{139-151}$ was abrogated by the administration of pertussis toxin, resulting in EAE development. Our results suggest that $M\text{-}PLP_{139-151}$ inhibits EAE development by affecting the differentiation of T cells into encephalitogenic effector cells.

Introduction

Multiple Sclerosis (MS) is an inflammatory disease that affects the central nervous system (CNS) and although little is known about its etiology, a role for autoimmune responses against myelin has been suggested (1-3). Inflammation of the CNS - as observed in MS patients - can be mimicked in rodents by immunization with myelin components in adjuvant or by adoptive transfer of myelin-specific T cells, resulting in development of experimental autoimmune encephalomyelitis (EAE) (4;5). EAE is mediated by autoreactive $CD4^+$ T cells that are primed in lymphoid organs after immunization and subsequently infiltrate the CNS to mediate inflammation (6;7). Frequently, pertussis toxin (PTX) is applied to facilitate EAE development, because it affects blood brain barrier integrity (8;9). Moreover, PTX affects immune regulatory mechanisms (10) and activates antigen presenting cells, resulting in enhanced T_h1 immunity (11-13).

Reestablishment of self-tolerance can ameliorate symptoms in autoimmune disease and it has recently been demonstrated that signaling via C-type lectin receptors (CLR) can result in the suppression of immunity in an antigen-specific manner. CLR-family members were first discovered as pathogen recognition receptors, expressed on antigen presenting cells (APC). Ligation of these receptors by sugar moieties present in pathogenic cell walls can contribute to the induction of immunity (14;15). However, CLR-targeting may also contribute to immune suppression. For example, various pathogens circumvent their elimination by the host via binding to DC-SIGN, a CLR family member expressed on DC (16-18). In addition, targeting of immature DC by fusion proteins consisting of a T cell epitope and an anti-DEC-205 antibody mediated immunological unresponsiveness to class I and class II restricted T cell epitopes (19;20). A similar targeting strategy resulted in tolerance induction to EAE, which was associated with increased CD5 expression of T cells (21). In line with this, we have shown previously that immunization with a mannosylated self-peptide induces antigen-specific tolerance to EAE in SJL mice, despite the presence of complete adjuvant. This state of tolerance was characterized by poor DTH reactivity, absence of clinical EAE symptoms and reduced inflammation in the CNS (22). In addition, treatment with this mannosylated self-peptide during ongoing autoimmune responses can ameliorate EAE symptoms (23).

Here, we investigate the fate of autoreactive T cells after immunization with a mannosylated peptide derived from proteolipid protein ($M\text{-}PLP_{139\text{-}151}$). Employing TCR transgenic $PLP_{139\text{-}151}$-specific $CD4^+$ T cells (5B6 cells (24)), we show that immunization with $M\text{-}PLP_{139\text{-}151}$ enhances the clonal expansion of T cells, that do not develop effector functions. Because pertussis toxin abrogated tolerance, resulting in mil EAE, our study favors the idea that a

delicate balance between pro- andanti-inflammmatory signals determins the development of autoimunity.

Materials and Methods

Animals

5B6 mice (kindly provided by Dr. V.K. Kuchroo and Dr. D.C. Wraith) were bred and housed in our own animal facilities in individually ventilated cages, with unlimited access to food and water. Female SJL mice were obtained from Janvier (France). All experimental procedures were approved by the Animal Welfare Committee of TNO Quality of Life, The Netherlands.

Peptide synthesis

$PLP_{139-151}$ (HSLGKWLGHPDKF) and its mannosylated counterpart ($M\text{-}PLP_{139-151}$) were produced as described elsewhere (25;26). In short, peptides were synthesized using solid phase synthesis. Mannosylation was accomplished by N-terminal elongation of the peptide with a building block containing lysine coupled to two tetra-acetyl protected mannose groups. $PLP_{139-151}$ was elongated with *bis*-acetyl lysine only and the acetyl protecting groups on the mannose moieties were removed using Tesser's base. All peptides were analyzed with reversed phase HPLC and Maldi-Tof mass spectrometry and showed the expected masses.

Cell separation and in vitro cultures

Spleens were isolated from 5B6 mice and single cell suspensions were prepared using a 40 µm filter (BD Falcon, Bedford, MA). For CFSE labeling, splenocytes were incubated with 5 µM CFSE in RPMI1640 (Cambrex, NJ) for 10 minutes and subsequently washed twice with ice-cold PBS, as described by Lyons et al (27). To study *in vitro* proliferation, these cells were resuspended in RPMI1640 containing 5% FCS (Cambrex), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 mM glutamine and 50 µM β-mercaptoethanol, and seeded in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at a density of 2×10^5 cells per well. The 5B6 cells were stimulated with 0.1 – 100 µg/ml $PLP_{139-151}$ or $M\text{-}PLP_{139-151}$ for a period of 4 days.

For co-culture experiments naive $CD4^+$ 5B6 cells were isolated from spleens via negative isolation using Dynal beads (Dynal Biotech, Oslo, Norway). Naive SJL spleens were used

for the isolation of $CD11c^+$ dendritic cells, $CD11b^+$ macrophages and $CD19^+$ B cells, using MACS beads and columns according to the manufacturer's protocol (Miltenyi Biotec, Auburn, US). The APC populations were irradiated (30 Gy) and co-cultured with 5 x 10^4 $CD4^+$ 5B6 cells (ratio 2:1 and 1:4) in the presence of 30 µg/ml $PLP_{139-151}$ or M-$PLP_{139-151}$ for 3 days. Supernatants were collected from all cultures and proliferation was assessed by addition of 0.5 µCi [^{3}H]-labeled thymidine for 6 hours (2 Ci/mmol; Amersham Biosciences, Buckinghamshire, UK).

In vivo transfer of 5B6 cells

Female 5B6 mice were sacrificed and lymph nodes and spleens were isolated. A single cell suspension was prepared in RPMI medium and incubated for 45 minutes on a nylon wool column. This procedure depleted 50% of B cells and macrophages and resulted in an enriched cell suspension containing 50% $CD4^+$ T cells. The 5B6 cells were CFSE-labeled as described above and extensively washed with PBS before injection. Intravenous injection of 10 x 10^6 5B6 cells into naïve female SJL mice occurred one day before immunization.

EAE induction

Female SJL mice (8 to 12 weeks old) were immunized s.c. with 50 µg $PLP_{139-151}$ or M-$PLP_{139-151}$ dissolved in PBS and emulsified in an equal volume of complete adjuvant supplemented with 1 mg/ml *Mycobacterium tuberculosis* (H37RA, Difco laboratories, Detroit, MI, USA). Control mice were immunized with PBS in adjuvant. Animals were weighed daily and monitored for EAE development. Clinical EAE was graded as follows: 0: no symptoms, 0.5: partial loss of tail tonus, 1: complete loss of tail tonus or partial limb weakness, 1.5: limb weakness and partial tail paralysis, 2: limb weakness and complete tail paralysis, 2.5: partial paresis, 3: complete paralysis of hind limbs, 3.5: complete paralysis from diaphragm and hind limbs, 4: moribund and 5: death due to EAE. Where indicated, 1 x 10^9 heat-hilled *Bordetella pertussis* bacteria or 200 ng Pertussis toxin (Toxin Technology, Sarasota, Fl) was injected i.v. on day 3.

Adoptive transfer EAE

Donor mice were immunized with 75 µg $PLP_{139-151}$ or M-$PLP_{139-151}$ in adjuvant as described above. After 14 days these mice were sacrificed and draining lymph nodes were isolated. Single cell suspensions were prepared and lymph node cells were cultured with 30 µg/ml $PLP_{139-151}$ or M-$PLP_{139-151}$ for 3 days in the presence of 50 U/ml IL-2. Subsequently, cells were harvested and washed with PBS before i.p. injection into naive recipient mice (15 x 10^6 cells /mouse).

Delayed type hypersensitivity (DTH)

The DTH response was evaluated by injecting 12 nmol $PLP_{139-151}$ dissolved in 10 µl saline into the dorsal side of the right ear, using a Hamilton syringe fitted with a 30-gauge needle. As a control for non-specific ear swelling, 10 µl of saline was injected into the left ear. Ear thickness was measured before and 24 hours after intradermal injection, using of a Mitutoyo micrometer. Results are expressed as the percentage specific ear swelling, obtained by subtracting the percentage non-specific ear swelling.

Flow cytometry

For FACS analysis cells were collected from *in vitro* cultures or single cell suspensions were prepared from isolated organs. Blood samples were treated with lysing solution (Pharmingen, San Diego, US) before staining. Cells were stained with different rat-anti-mouse antibodies obtained from BD Biosciences (CD4-PerCP, Vβ6-PE, CD19-FITC, CD8-APC-Cy7, CD11c-Alexa647, CD11b-biotin, CD25-biotin, CD25-APC, CD62L-APC, CD44-PE, FoxP3-PE, IL-4-PE and IFNγ-APC). Where needed isotype-matched controls were included (rat-anti-mouse-IgM-biotin, rat-anti mouse-IgG1-APC, rat-anti-mouse-IgG2a-PE and rat-anti-mouse-IgG2a-APC). Biotinylated antibodies were visualized with Streptavidin-APC or Streptavidin-PE-Cy7. For intracellular FACS staining cells were incubated for 4 hours with Brefeldin A (Sigma) and fixation and permeabilization reagents (Caltag Laboratories, Burlingame, CA) were used for staining.

Antibody arrays

The undiluted supernatants of the LN cultures used for transfer experiments were incubated on Mouse Inflammation Antibody array I (Raybiotech, Norcross, GA, USA), according to the manufacturer's instructions. The presence of cytokines was evaluated by chemoluminescent detection, using a chemiDoc-it imaging system (UVP Inc, Upland, CA, USA) and the signal intensities were quantified with Labworks software. On the array duplicate spots for each cytokine were present, as well as positive and negative controls. For analysis, the optical density (OD) of all spots was quantified, the background was subtracted and the mean signal of duplicate cytokine spots was calculated (mean background: 500). All results were normalized towards the positive control to enable comparison of different arrays.

Statistics

Statistical analysis of data was performed with a Mann-Whitney test and a Chi-square test.

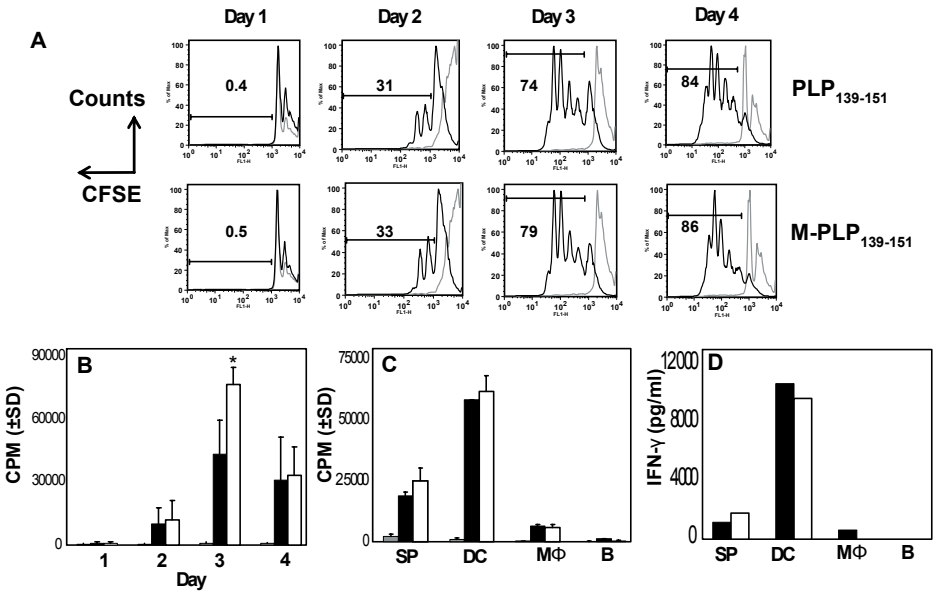

Figure 1. *In vitro stimulation of 5B6 splenocytes with $PLP_{139-151}$ and M-$PLP_{139-151}$. Naïve CFSE-labeled 5B6 splenocytes were cultured for 4 days with 10 µg/ml $PLP_{139-151}$ (filled bars) or M-$PLP_{139-151}$ (open bars) or medium (gray bars). Division of CD4+ 5B6 cells was evaluated via dilution of CFSE signal (A; medium control in dotted histogram). Proliferation of 5B6 cells was also assessed via the incorporation of ^{3}H-thymidine (B, * $P < 0.05$). Similar results were obtained with other peptide concentrations and one out of three similar experiments is shown. $CD4^+$ enriched 5B6 T cells were co-cultured with irradiated splenocytes (SP), $CD11c^+$ enriched cells (DC), CD11b-enriched cells (MΦ) or CD19-enriched cells (B cells) in the presence of 30 µg/ml $PLP_{139-151}$ or M-$PLP_{139-151}$ or medium. T cell proliferation was assessed by incorporation of ^{3}H-thymidine (C). IFNγ-levels in the supernatant of these co-cultures were determined by ELISA (D).*

Results

Mannosylated self-peptide is well-recognized by antigen-specific T cells in vitro

To study the fate of myelin-specific T cells in response to immunization with M-$PLP_{139-151}$ 5B6 cells were used. First, it was studied whether mannosylated peptide influenced antigen recognition by 5B6 cells *in vitro*. Therefore, CFSE-labeled 5B6 splenocytes were cultured for four days in the presence of 0.1-100 µg/ml $PLP_{139-151}$ or M-$PLP_{139-151}$ or medium. Cell division of $CD4^+$ 5B6 cells was monitored daily by flow cytometric evaluation of CFSE dilution (Figure 1A) and by ^{3}H-Thymidine incorporation (Figure 1B). Although comparable results for the two peptides were obtained with all tested concentrations, CD4+ T cells incidentally responded better to the mannosylated form of $PLP_{139-151}$. Furthermore, flow cytometric analysis revealed similar activation of $CD4^+$ 5B6 cells in response to either peptide, based on CD25 expression and IFN-γ production. ELISA of culture supernatants confirmed that equal amounts of IFNγ were secreted, in the absence of IL-4 and IL-10 production (data not shown). C type lectins are widely expressed by innate immune cells (28;29) and to evaluate whether targeting of such receptors resulted in their proliferation, the CFSE dilution pattern of CD4-negative 5B6 splenocytes was studied, which revealed that it was primarily the CD4-positive T cell population that was responsible for the proliferative response to either peptide (data not shown).

To study whether APC populations discriminated between normal peptide and mannosylated peptide, $CD4^+$ enriched 5B6 cells were stimulated for three days with irradiated spleen cells, CD11c-enriched dendritic cells, CD11b-enriched macrophages or CD19-enriched B cells in the presence of $PLP_{139-151}$, M-$PLP_{139-151}$ or medium. Results from co-cultures in a ratio of 2:1 are presented, but similar results were obtained with a 1:4 ratio (APC:T). In particular, dendritic cells induced proliferation (Figure 1C) and IFN-γ-production (Figure 1D) by 5B6 T cells and we obtained no indications that the APC populations under investigation differentially presented the two peptides. We conclude that both peptides were equally presented and recognized *in vitro,* resulting in the expansion of $CD4^+$ T cells with a T_h1 phenotype.

Table 1. EAE development after 5B6 cell transfer into SJL mice

Immunization *	Incidence	Day of onset (Mean ± SEM)	Maximal score (Mean ± SEM)	Cumulative score ** (Mean ± SEM)
None	0/3	n.a.	0	0
PBS/CFA	0/4	n.a.	0	0
$PLP_{139-151}$/CFA	9/10	10.6 ± 1.9	3.2 ± 1.4	39.8 ± 16
M-$PLP_{139-151}$/CFA	7/14 [1]	32.6 ± 12.3 [2]	1.2 ± 1.1 [2]	8.6 ± 9.3 [3]

* *SJL mice were injected i.v. with 10 x 10^6 5B6 cells and 2 days later subjected to immunization as indicated. Combined data from 3 experiments are presented.*

** *The evaluation of cumulative EAE scores is based on one experiment.*

[1] *P = 0.08 compared with $PLP_{139-151}$-immunized mice*

[2] *P < 0.01 compared with $PLP_{139-151}$-immunized mice*

[3] *P < 0.05 compared with $PLP_{139-151}$-immunized mice*

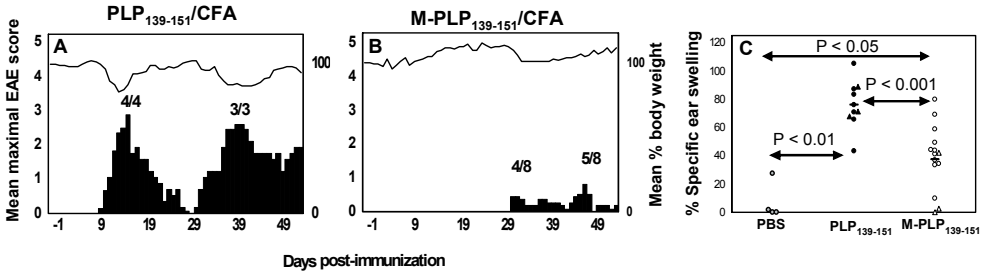

Figure 2. *Immunization with M-$PLP_{139-151}$ induces poor effector T cells in vivo. CFSE-labeled 5B6 cells were transferred into naïve recipient mice and these were immunized one day later with PBS (no EAE development), $PLP_{139-151}$ (A) or M-$PLP_{139-151}$ (B). Representative data from one experiment are shown, combined data and statistics are presented in Table 1. The numbers of animals with clinical EAE are indicated in the graphs. On day 3 (circles) or 6 (triangles) after immunization $PLP_{139-151}$ was injected into the right ear and saline was injected in the left ear. Antigen-specific ear swelling was determined 48 hours later (C). Combined data from 3 experiments are presented.*

Increased expansion of T cells after immunization with M-$PLP_{139-151}$

To evaluate the clonal expansion of $PLP_{139-151}$-specific T cells in response to immunization to $PLP_{139-151}$ or M-$PLP_{139-151}$, CFSE-labeled 5B6 cells were injected into naive recipient mice that were immunized one day later with either peptide or PBS in complete adjuvant. Transfer of 5B6 cells itself or sham-immunization did not induce EAE (Table 1). After immunization with $PLP_{139-151}$ EAE developed around day 11 and one animal died due to the disease (Table 1 and Figure 2A). In contrast, only 50% of the mice developed EAE after immunization with M-$PLP_{139-151}$ (Table 1 and Figure 2B, P = 0.08) and the disease onset was significantly delayed (P < 0.01). In affected mice, we observed a mild disease pattern, resulting in a significant reduction of the maximal EAE score (P < 0.01) and the cumulative EAE score (P < 0.05).
DTH responses towards $PLP_{139-151}$ were evaluated on day three and six after immunization and

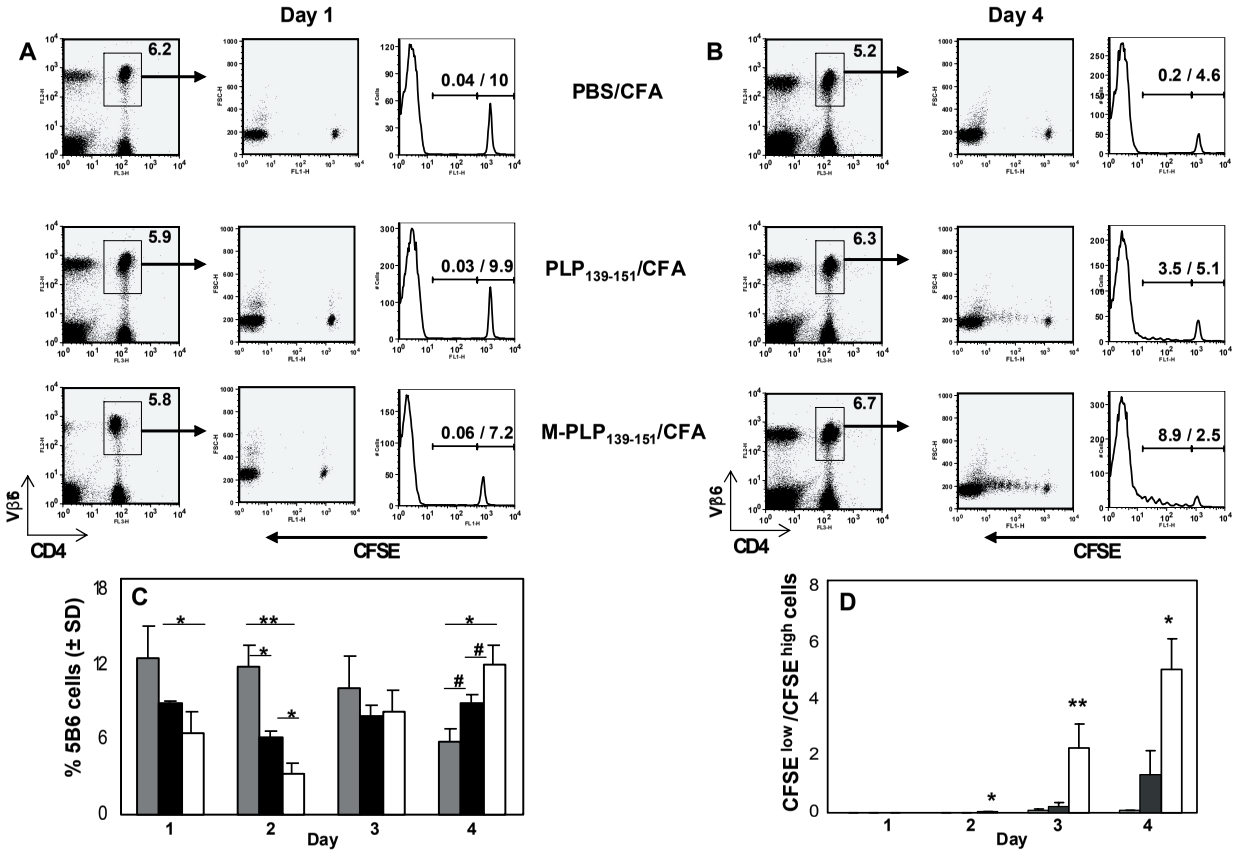

Figure 3. *Expansion of 5B6 cells in vivo after immunization with M-PLP$_{139-151}$. CFSE-labeled 5B6 cells were transferred to naïve recipient mice and these were immunized one day later with PBS (grey bars), PLP$_{139-151}$ (filled bars) or M-PLP$_{139-151}$ (open bars). The presence of 5B6 cells in the blood was monitored on days 1 to 4 after immunization. After gating on the CD4$^+$Vβ6$^+$ population, the CFSE signal was used to identify the donor T cells from 5B6 origin. At least three individual mice per time point were analyzed and representative data obtained on day 1 (A) and day 4 (B) are presented. The percentages of 5B6 cells in the blood were monitored in time and within the CD4$^+$ population the mean percentages are shown (C). To visualize the expansion of 5B6 cells the ratio between CFSE^{low} (divided) and CFSE^{high} (undivided) cells was calculated (D). * P < 0.01, ** P < 0.001, # P < 0.05, compared to both PLP$_{139-151}$-immunized and sham-immunized mice.*

poor responses were measured in mice that had been immunized with M-$PLP_{139-151}$ (Figure 2C, $P < 0.001$). Transfer of 5B6 cells itself and sham-immunization were insufficient to elicit a DTH response (data not shown).

The presence of $CD4^+$ 5B6 cells in the blood was monitored one to four days after immunization. Retrieval of the injected 5B6 cells was based on CFSE signal within the $CD4^+$ $V\beta6^+$ population. Representative results from the analysis on day 1 (Figure 3A) and on day 4 (Figure 3B) are presented. Immunization with either peptide resulted in reduced numbers of 5B6 cells in the blood stream up to two days after immunization, compared to sham-immunized mice. Subsequently, the numbers of 5B6 cells increased substantially, in particular in response to M-$PLP_{139-151}$ (Figure 3C). With respect to the division pattern of the 5B6 cells in the blood, we observed that the ratio between divided and undivided cells was significantly increased after immunization with M-$PLP_{139-151}$ (Figure 3D). In all mice 5B6 cells in the blood highly expressed CD62L (data not shown). After four days multiple-divided 5B6 cells had lost their CFSE signal and could no longer be visualized due to the endogenous expression of Vβ6 in SJL mice.

Together, the data indicate that immunization with M-$PLP_{139-151}$ increased the early *in vivo* expansion and circulation of $PLP_{139-151}$-specific T cells that develop poor effector functions.

Reduced encephalitogenicity of T cells after immunization with M-$PLP_{139-151}$

Adoptive transfer EAE experiments were performed to study whether the intrinsic encephalitogenic capacities of autoreactive T cells were affected by immunization with M-$PLP_{139-151}$. For this purpose, donor mice were immunized with 75 µg $PLP_{139-151}$ or M-$PLP_{139-151}$ and sacrificed after 14 days when initial EAE symptoms in $PLP_{139-151}$-immunized mice were present. Draining lymph nodes were isolated and cultured with the corresponding peptide for three days in the presence of IL-2. Although the viability in the pooled LN cell cultures was similar for both peptides, M-$PLP_{139-151}$-stimulated cells showed less blastogenesis and reduced cell recovery after three days of culture (Figure 4A, $P < 0.05$). Equal numbers of expanded cells were injected into naive recipient mice. Transfer of $PLP_{139-151}$-expanded T cells resulted in EAE development in five out of six recipient mice (mean day of onset 15.4 ± 7.0; maximal EAE score 1.8 ± 1.1), while M-$PLP_{139-151}$-expanded T cells were unable to transfer EAE (Figure 4B). Moreover, these cells mounted significantly reduced DTH responses in the recipient mice after an ear challenge with $PLP_{139-151}$ (Figure 4C, $P < 0.01$).

Supernatants from the LN cell cultures were incubated on cytokine antibody arrays. As

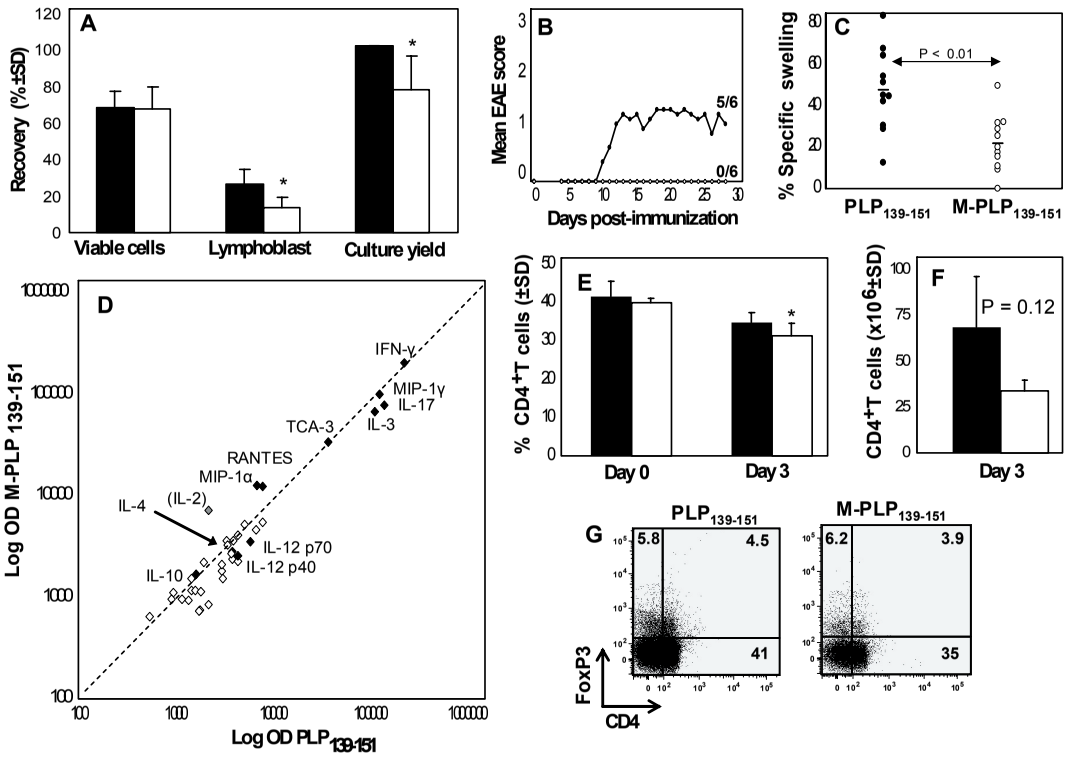

Figure 4. *Reduced encephalitogenicity after immunization with M-PLP$_{139-151}$. Donor mice were immunized with PLP$_{139-151}$ or M-PLP$_{139-151}$ and after 14 days the draining LN were isolated and cultured for 3 days with corresponding peptide and IL-2. M-PLP$_{139-151}$-stimulated cells showed similar viability after culture, but a significantly decreased yield and blastogenesis (A, * P < 0.05). Transfer of M-PLP$_{139-151}$-stimulated cells into naïve recipient mice did not result in EAE development (B) and a poor DTH response after ear challenge with PLP$_{139-151}$ (C). The numbers of mice with clinical EAE are indicated in the graph and the DTH results were combined from three similar experiments. The supernatants of the transferred cells were analyzed with cytokine antibody arrays. The chemo luminescent signals (OD) of supernatants obtained from PLP$_{139-151}$- and M-PLP$_{139-151}$-stimulated LN cell cultures were plotted against each other to show that a similar cytokine production profile was observed (D). One out of two similar experiments is presented and factors of interest are indicated by filled symbols. Because IL-2 is added to the cultures it was excluded from the analysis (indicated in gray). In a separate experiment 3 individual mice per group were immunized with PLP$_{139-151}$ or M-PLP$_{139-151}$ and their LN cells were cultured in a similar way for flow cytometric analysis. The percentage (E) as well as the absolute number (F) of CD4$^+$ T cells was decreased after three days of culture with M-PLP$_{139-151}$ (* P < 0.05). No differences in FoxP3 expression were observed (G). Filled bars and symbols: PLP$_{139-151}$; Open bars and symbols: M-PLP$_{139-151}$*

depicted in Figure 4D, a similar spectrum of cytokines and chemokines was produced by $PLP_{139-151}$- and M-$PLP_{139-151}$-stimulated cells. Surprisingly, the T cells that were rendered non-encephalitogenic after immunization with M-$PLP_{139-151}$, were not impaired with respect to the production of IFN-γ and IL-17 *in vitro* and also the secretion levels of the chemokines TCA-3 (CCL1), MIP1α (CCL3) and RANTES (CCL5) were similar. Furthermore, high levels of IL-3 and MIP-1γ (CCL9) were detected, while IL-4 and IL-10 levels were low.

In a separate experiment mice were immunized with $PLP_{139-151}$ or M-$PLP_{139-151}$ and sacrificed on day 10. For flow cytometric analysis the draining LN cells from individual mice were cultured for three days in the presence of corresponding peptide and IL-2. Although the LN suspensions obtained from mice immunized with either peptide contained similar percentages of $CD4^+$ T cells (i.e. results obtained on day 0), the percentage of $CD4^+$ T cells was significantly lower after three days of culture in the presence of M-$PLP_{139-151}$ (Figure 4E, $P < 0.05$) and accordingly the absolute numbers of $CD4^+$ T cells recovered from the LN cultures tended to be lower (Figure 4F). However, the T cells seemed equally activated, based on the expression of CD44 and CD25 (data not shown). In addition, we observed similar FoxP3 expression by $CD4^+$ T cells after 3 days of culture (Figure 4G).

These data suggest that the inability of LN cells of M-$PLP_{139-151}$-immunized mice to transfer EAE may be partially due to less expansion of $CD4^+$ T cells, although these T cells appear well-activated and not impaired with respect to the *in vitro* production of cytokines and chemokines that are associated with EAE development.

Pertussis toxin abrogates tolerance induction by M-$PLP_{139-151}$

Immunization with M-$PLP_{139-151}$ resulted in poor EAE development, despite the presence of complete adjuvant (Figure 5A and B). To increase the efficacy of EAE induction heat-killed *B. pertussis* bacteria (BP) or pertussis toxin (PTX) are frequently used as an additional adjuvant (8). Importantly, treatment with BP and PTX abrogated the induction of tolerance associated with M-$PLP_{139-151}$. Immunization with M-$PLP_{139-151}$ combined with intravenous BP (Figures 5C and 5D) or PTX injection (Figures 5E and 5F) resulted in disease development, although EAE symptoms were mild compared with $PLP_{139-151}$-immunized mice that received the same treatment (Table 2).

To further investigate why PTX abrogates tolerance induction by M-$PLP_{139-151}$, mice were immunized with $PLP_{139-151}$ or M-$PLP_{139-151}$ in CFA with or without additional administration of PTX, and sacrificed on day 10. At this time point clinical EAE was only observed in $PLP_{139-151}$-immunized mice that had received PTX (data not shown). The draining LN of

Table 2. The effect of pertussis toxin on EAE development

Immunization	Incidence	Day of onset (Mean ± SEM)	Maximal score (Mean ± SEM)	Cumulative score (Mean ± SEM)
$PLP_{139-151}$	4/4	8.3 ± 4.9	3.0 ± 0.4	53.3 ± 17.1
$PLP_{139-151}$/B. Pertussis	4/4	10.3 ± 0.6	3.0 ± 0.5	45.9 ± 23.6
$PLP_{139-151}$ / PTX *	4/4	12.5 ± 0.6	3.6 ± 1.6	51.5 ± 23
M-$PLP_{139-151}$	0/4 [1]	-	0 [2]	0.1 ± 0.3 [3]
M-$PLP_{139-151}$/B. Pertussis	3/4	14.0 ± 0 [4]	1.6 ± 1.1 [4]	13.5 ± 16.7 [4]
M-$PLP_{139-151}$ / PTX	4/4	15.5 ± 1.3	1.8 ± 1	19.4 ± 8.8 [5]

** Two mice died during the experiment.*
[1] P < 0.01 compared with all other groups.
[2] P < 0.05 compared with all other groups.
[3] P < 0.05 compared with $PLP_{139-151}$-immunized mice and M-$PLP_{139-151}$-immunized mice that received PTX.
[4] P < 0.05 compared with $PLP_{139-151}$-immunized mice that received B. Pertussis
[5] P = 0.08 compared to $PLP_{139-151}$-immunized mice that received PTX.

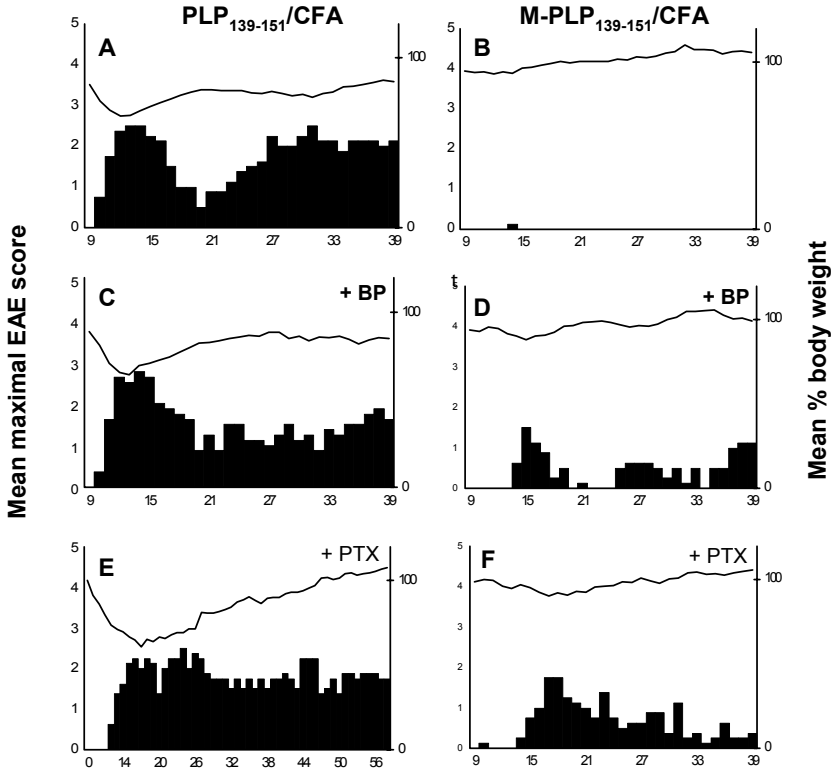

Figure 5. *PTX abrogates tolerance induction by M-$PLP_{139-151}$. Mice were immunized with 50 µg $PLP_{139-151}$ or M-$PLP_{139-151}$ and were injected i.v. with PBS (A and B), heat-killed B. Pertussis bacteria (C and D) or PTX (E and F) on day 3. Body weight and clinical scores were assessed daily. Mean data of 4 mice per group are shown and statistics are described in Table 2.*

individual mice were collected and a significantly increased number of LN cells was found in mice that were immunized with M-PLP$_{139-151}$, compared to PLP$_{139-151}$-immunized mice (Figure 6A, $P < 0.001$). Administration of PTX in M-PLP$_{139-151}$-immunized mice decreased the number of LN cells ($P < 0.01$), although the LN cellularity was still increased compared to mice that were immunized with the non-mannosylated peptide and PTX ($P < 0.05$).

Flow cytometric analysis revealed that the increased cellularity was due to increased numbers of $CD4^+$ and $CD8^+$ T cells as well as B cells (Figure 6B, $P < 0.05$). Based on the expression of CD49b only small numbers of NK cells were present (data not shown). As shown in Figure 6C, significantly increased numbers of $CD11b^+$ cells were found in draining LN of M-PLP$_{139-151}$-immunized mice, and these comprised both the $CD11c^{high}$ and the $CD11c^{low}$ population ($P < 0.05$).

Altogether, these data suggest that the inability M-PLP$_{139-151}$ to induce EAE is associated with the accumulation of lymphocytes in draining LN. Tolerance was abrogated by PTX, possibly by driving DC maturation in conjunction with the development of effector T cells capable of migration to target tissues.

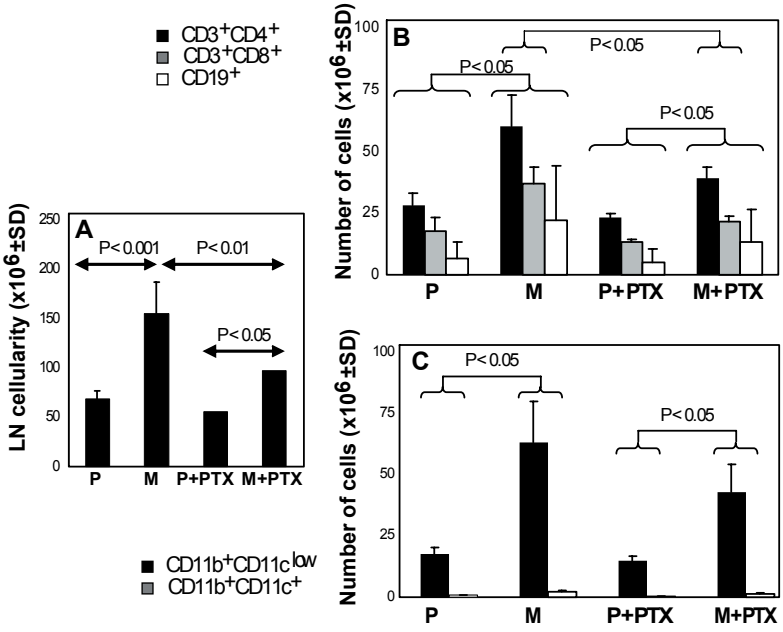

Figure 6. **Immunization with M-PLP$_{139-151}$ increases cell numbers in the lymph node.** *Mice were immunized with PLP$_{139-151}$ or M-PLP$_{139-151}$ with or without i.v. PTX injection on day 3. Ten days later the draining LN were isolated and the numbers of LN cells were counted (A). Flow cytometric analysis revealed that the LN of M-PLP$_{139-151}$-immunized mice contained increased numbers of T cells and B cells (B) as well as increased numbers of $CD11b^+CD11c^{low}$ and $CD11b^+CD11c^{high}$ cells (C). Injection of PTX diminished this effect in the M-PLP$_{139-151}$-immunized mice. Three mice per group were studied (P; PLP$_{139-151}$/CFA, P+; PLP$_{139-151}$/CFA + PTX, M; M-PLP$_{139-151}$/CFA, M+; M-PLP$_{139-151}$/CFA + PTX)*

Discussion

In the search for an improved treatment of patients suffering from autoimmune diseases, such as Multiple Sclerosis, the antigen-specific inhibition of autoreactive T cells has received considerable attention. In this manuscript we substantiated that the development of autoimmunity can be inhibited with high efficiency by immunization with mannosylated self-peptide. The application of TCR transgenic 5B6 cells revealed that the absence of EAE is the result of increased expansion of T cells that develop poor effector functions.

In vitro experiments using 5B6 cells reveal that T cells respond equally to $PLP_{139-151}$ and M-$PLP_{139-151}$, regardless of the APC population used to present the peptides. This is consistent with our previous *in vitro* experiments employing OTII cells that were stimulated with mannosylated Ovalbumin-peptide (30). Immunization with M-$PLP_{139-151}$ in complete adjuvant results in enhanced early *in vivo* expansion of autoreactive T cells that are impaired with regard to their encephalitogenic properties and the induction of DTH responses. The poor DTH responses suggest that T cells are already non-functional at early time points and may therefore be ineffective in the subsequent induction of EAE. These data are in line with studies by Cua et al who showed a clear association between DTH responsiveness and EAE susceptibility, due to the involvement of the same effector T cells population(s) as well as macrophages (31). Transfer of cells from M-$PLP_{139-151}$-immunized mice into naive recipient mice does not result in EAE development, indicating that the absence of disease is not simply the consequence of a decoy effect mediated by mannosylated self-peptide in donor mice.

The absence of T cell effector functions in recipient mice may be partially explained by less vigorous expansion of $CD4^+$ T cells *in vitro* and the decreased formation of lymphoblasts, suggesting that the T cells do not become fully activated or differentiated. However, the unaffected production of cytokines associated with encephalitogenicity, such as IFNγ, IL-17 and IL-3 (32;33) are supportive for an activated state of the T cells. Moreover, we observed considerable production of TCA-3 (CCL1), MIP-1α (CCL3) and RANTES (CCL5), all chemokines involved in EAE (34). Although MIP-1γ (CCL9) is known as a proinflammatory chemokine, the high levels in culture supernatants of myelin-specific cells were less expected, because a role for MIP-1γ (CCL9) in autoimmunity has not been described so far. The limited production of IL-4 and IL-10 suggests that mannosylated self-peptide does not promote the development of Th2 cells or IL-10-secreting regulatory T cells. Furthermore, the evaluation of the numbers of FoxP3-expressing $CD4^+$ T cells did not reveal that increased numbers of regulatory T cells developed in response to mannosylated peptide. In line with these observations, we found that tolerance induction to EAE by immunization with mannosylated

myelin peptide is successful in IL-10 deficient mice, indicating that IL-10 does not play a pivotal role (data not shown).

In earlier studies we have shown that proliferative responses of T cells isolated from mice that were rendered tolerant by immunization with M-PLP$_{139-151}$ were decreased after day 50, although normal *in vitro* proliferation was observed at earlier time points (22). Unfortunately, the long-term monitoring of 5B6 cells after immunization with mannosylated self-peptide in current studies was hampered by the lack of a congenic marker. The possible deletion of non-functional T cells at later time points can therefore not be excluded.

Interestingly, tolerance induction by mannosylated self-peptide in SJL mice can be established despite strong pro-inflammatory signals provided by complete adjuvant containing *M. tuberculosis* that results in triggering of TLR2 and TLR4 (35). We show here, that the absence of EAE in mice that were immunized with mannosylated peptide is associated with accumulation of lymphocytes in the draining LN, which is suggestive for disturbed migration of lymphocytes towards the periphery. Importantly, disease inhibition was abrogated by PTX injection and in line with this we observed that immunization with mannosylated MOG-peptide in the presence of PTX did not induce tolerance, but severe disease in C57Bl/6 mice (unpublished observations). PTX affects the autoimmune responses in EAE in multiple ways. Administration of PTX prevents T cell anergy (36) and can enhance T_h1-mediated autoimmunity in a TLR-independent way (37). On the other hand PTX affects G-protein-coupled receptor signaling, which promotes blood brain barrier permeability (9), but also disrupts chemokine receptor signaling in lymphocytes in a dose-dependent way (13). At present, the mechanism for the opposing effects of mannosylated myelin peptide and PTX remain unknown.

The balance between inflammatory and tolerizing signals delivered to APC determines the further instruction of T cells (38). Possibly, mannosylated peptide elicits an immune suppressive signal in APC, for example via the inhibition of pro-inflammatory signaling mediated by TLR. Mannosylated antigens may be targeted towards CLR expressed by APC and this can result in the suppression of NFkB-signaling and IL-12 production (39-41). Recent studies have demonstrated that in the mouse both dendritic cells and macrophages can express the mannose receptor (42). Although the *in vitro* experiments suggest that in particular DC are involved in the presentation of mannosylated peptide, increased numbers of $CD11b^{+}CD11c^{high}$ as well as $CD11b^{+}CD11c^{low}$ APC were found in the draining LN of mice that were immunized with mannosylated self-peptide. Therefore, additional studies are required to reveal the identity and the activation status of APC that are targeted by mannosylated self-peptide.

We show here that mannosylated self-peptide inhibits EAE development by affecting the encephalitogenicity of T cells. More research is needed to elucidate whether tolerance induction by M-$PLP_{139-151}$ is the result of early T cell exhaustion, impaired T cell migration or incomplete differentiation. This may further support the development of antigen-specific therapy in autoimmunity based on mannosylated self-antigens.

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7

Soluble mannosylated myelin peptide inhibits the encephalitogenicity of autoreactive T cells during Experimental Autoimmune Encephalomyelitis

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Abstract

We have previously shown that immunization with a mannosylated myelin peptide in complete adjuvant induces tolerance instead of disease in experimental autoimmune encephalomyelitis (EAE), a rodent model for Multiple Sclerosis (MS). In this report we describe that treatment with a soluble mannosylated epitope of proteolipid protein (M-$PLP_{139-151}$) significantly inhibits disease mediated by autoreactive myelin-specific T cells during EAE. Treatment with M-$PLP_{139-151}$ was applied in different EAE models and significantly reduced disease incidence and the severity of clinical symptoms. Delayed type hypersensitivity (DTH) responses were abolished after peptide treatment, emphasizing the impact on peripheral T cell reactivity. Histological analysis of spinal cord tissue from mice treated with M-$PLP_{139-151}$ revealed the presence of only few macrophages and T cells. Moreover, little expression of IFN-γ, IL-23 or MHC class II antigen was detected. Immune modulation by M-$PLP_{139-151}$ was largely antigen-specific, as an irrelevant mannosylated peptide showed no significant effect on DTH responses or the course of EAE. Therefore, mannosylated antigens may represent a novel therapeutic approach for antigen-specific modulation of autoreactive T cells *in vivo*.

Introduction

Experimental autoimmune encephalomyelitis (EAE) serves as an experimental model for human Multiple Sclerosis (MS), reproducing clinical aspects such as inflammation of CNS tissue (1-3). It is depicted as a prototypic $CD4^+$ T_h1-mediated autoimmune disease (4;5) depending on autoreactive T_h1 cells that traffic from the periphery into the CNS (6). EAE can be induced in rodents by immunization with myelin fragments in adjuvant or by transfer of encephalitogenic T cells.

Recently, we have shown that immunization with a mannosylated peptide of proteolipid protein ($M\text{-}PLP_{139\text{-}151}$) is a powerful tool to induce tolerance to EAE in mice, even in the presence of complete adjuvant. Although $PLP_{139\text{-}151}$-specific T cells were present early after tolerance induction, their effector functions were abolished, apparent from inhibited DTH responses and the absence of EAE (7).

C-type lectins have been described as pathogen recognition receptors, that can recognize sugar residues and play a role in the induction of immunity by discriminating between self and non-self (8). However, recent data indicate that certain C-type lectin family members are also involved in inhibition of immune responses. Especially DC-SIGN, DEC-205 and the mannose receptor have been studied to this respect. Binding of several pathogens to DC-SIGN was shown to inhibit dendritic cell (DC) maturation and to promote pathogen survival in the host (9-12). Similarly, targeting of antigens to DEC-205 expressed by immature DC was shown to result in tolerance induction. OVA peptide coupled to a DEC-205 antibody was found to induce rapid early cell division and subsequent deletion of peptide-specific $CD8^+$ T cells (13). Furthermore, activation of $CD4^+$ T cells with an OVA peptide coupled to DEC-205 antibody induced transient T cell responses, lacking IFNγ production, which is a key-feature of T_h1 cells. A systemic challenge with OVA peptide in complete Freund's adjuvant (CFA) revealed that antigen-specific unresponsiveness was induced *in vivo* (13;14). Moreover, treatment of EAE induced with $MOG_{35\text{-}55}$ in CFA with a myelin peptide coupled to a DEC-205 antibody induced tolerance (15).

In Multiple Sclerosis (MS) an ongoing autoimmune response against myelin contributes to chronic CNS inflammation, resulting in tissue damage and clinical symptoms (16). Current treatment of MS patients is based only on non-specific immune suppression. Drugs, such as IFN-β and glatiramer acetate, are often used for prolonged periods of time and bring along considerable side effects (17;18). Therefore, the development of compounds that specifically interfere with autoimmune processes in MS may be a valuable contribution to therapy. In this report we provide evidence that treatment with mannosylated myelin peptide under

conditions where fully activated autoreactive T cells are present significantly ameliorates EAE symptoms. This suggests that antigen-specific interference with T cell effector functions of autoreactive T cells may have therapeutic potential in autoimmune disease.

Materials and Methods

Animals

Female SJL mice were purchased from Harlan (Horst, Netherlands) and Janvier (Le Genest, France). Animals were housed under standard conditions with constant temperature, controlled lightning, and free access to food and water. All experimental procedures were approved by the Animal Welfare Committee.

Peptide synthesis

$PLP_{139-151}$ (HSLGKWLGHPDKF), its mannosylated form (M-$PLP_{139-151}$) and mannosylated $OVA_{371-386}$ (M-$OVA_{371-386}$) (HIATNAVLFFGRSVS) (19;20) were synthesized as described elsewhere (21;22). In short, the peptides were prepared using solid phase synthesis. Mannosylation was accomplished by N-terminal elongation of the peptide with a lysine coupled to two tetra-acetyl protected mannose groups. The $PLP_{139-151}$ peptide was elongated with *bis*-acetyl lysine only. The acetyl protecting groups on the mannose moieties were removed using Tesser's base. All peptides were analyzed with Maldi-Tof mass spectrometry and showed the expected masses.

Active EAE induction

Mice (8 to 10 week old) were immunized subcutaneously (s.c.) with 50 or 75 μg $PLP_{139-151}$ peptide dissolved in PBS and emulsified in an equal volume of complete adjuvant supplemented with 1 mg/ml *Mycobacterium tuberculosis* (H37RA, Difco laboratories, Detroit, MI, USA). The animals were weighed daily and monitored for EAE development. Clinical EAE was graded in 5 scores: 0: no symptoms, 0.5: partial loss of tail tonus, 1: complete loss of tail tonus or partial limb weakness, 1.5: limb weakness and partial tail paralysis, 2: limb weakness and complete tail paralysis, 2.5: partial paresis, 3: complete paralysis of hind limbs, 3.5: complete paralysis from diaphragm and hind limbs, 4: moribund and 5: death due to EAE.

Adoptive transfer EAE

For adoptive transfer, donor mice were immunized with 75 µg $PLP_{139-151}$ in complete supplemented with 1 mg/ml *Mycobacterium tuberculosis*; 1 x 10^9 heat killed *Bordetella pertussis* bacteria were injected intravenously (i.v.) on days 1 and 3. After two weeks the animals were sacrificed and the draining lymph nodes were collected. Lymph node cells were cultured in RPMI 1640 medium (Cambrex) supplemented with 5% FCS (Gibco, Gaitersburg, MD), 30 µg/ml $PLP_{139-151}$ and 50 U/ml IL-2. After 3 days cells were harvested, washed extensively with PBS and were subsequently injected intraperitoneally into naïve recipient mice (3 x 10^6 T cell blasts per mouse).

Peptide treatment protocols

Treatment of mice was performed with 50 µg of $PLP_{139-151}$, M-$PLP_{139-151}$ or M-$OVA_{371-386}$, dissolved in 200 µl PBS. In active EAE experiments one single dosage of peptide was injected i.v. 8, 17 or 24 days after immunization of the animals. In adoptive transfer EAE studies recipient mice were treated subcutaneously (s.c.). Together with the transfer of encephalitogenic cells, peptide was administered one day before the injection of cells and 1 and 3 days afterwards. Peptide treatment after disease onset occurred twice a week. The control mice received PBS only.

Delayed Type Hypersensitivity

The DTH response was evaluated by injecting 25 µg of $PLP_{139-151}$ dissolved in 10 µl saline into the dorsal side of the right ear of mice, using a Hamilton syringe fitted with a 30 gauge needle. As a control for non-specific ear swelling, 10 µl of saline was injected into the left ear. Ear thickness was measured before and 24 or 48 hours after intradermal injection using of a Mitutoyo micrometer. Results are expressed as the percentage specific ear swelling, obtained by subtracting the percentage non-specific ear swelling.

Histological analysis

Brains and spinal cords were collected and frozen in liquid nitrogen for histological analysis. Step serial tissue sections (8 µm) were stained with Haematoxylin to localize inflammatory regions. Cellular composition of infiltrates was analyzed with the following antibodies: Rat-

anti-human-CD3 (polyclonal antibody, DAKO, Glostrup, Denmark), Rat-anti-mouse IFN-γ (XMG1.2), Rat-anti-mouse-CD11b-biotine (M1/70, Pharmingen, San Diego, US), Mouse-anti-rat-MHCII (RT1B, Pharmingen, San Diego, US) and Goat-anti-mouse-IL-23 (R&D systems, Minneapolis, US). Rabbit-anti-rat-IgG-biotine (Vector Laboratories, Burlingame, UK) was applied as a secondary biotinylated antibody where needed. Subsequently, Streptavidin complex (DAKO) was used for detection of biotin and this reaction was visualized with NovaRed (Vector Laboratories).

Splenocyte cultures

Spleens were isolated and single cell suspensions were prepared trough a 40 μm filter (BD Falcon, Bedford, MA). To study *in vitro* proliferation, the cells were resuspended in RPMI 1640 containing 5% FCS (Cambrex), 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM ultraglutamine and 50 μM β-mercaptoethanol and were seeded in 96 well flat bottom plates (Costar, Cambridge, MA) at a density of 2 x 10^5 cells per well. Cells were stimulated with 30 μg/ml $PLP_{139-151}$ for 4 days. Proliferation was assessed by addition of 0.5 μCi ^3H per well for 6 hours.

Statistics

Statistical analysis of data was performed with a Chi-square test or Mann-Whitney test.

Results

Treatment with soluble M-$PLP_{139-151}$ inhibits EAE after active immunization

To study immune modulation by soluble mannosylated myelin peptide after induction of EAE with $PLP_{139-151}$ in complete adjuvant containing *M. tuberculosis* H37RA, a single intravenous treatment was administered on day eight after immunization. Mice were treated with 50 μg soluble $PLP_{139-151}$ or M-$PLP_{139-151}$ or with PBS as a control. All control mice developed clinical symptoms around day 14 (Figure 1A and Table 1). Treatment with soluble $PLP_{139-151}$ on day 8 resulted in 80% EAE incidence (Figure 1B and Table 1) whereas administration of M-$PLP_{139-151}$ resulted in less than 40% incidence (Figure 1C and Table 1, $p < 0.05$ compared to controls). The animals that did develop EAE showed a significantly delayed disease onset

Table 1. Soluble M-PLP$_{139-151}$ inhibits EAE induced by active immunization(1)

Treatment [2]	EAE incidence	Day of onset[3]	Maximal score	Cumulative score
PBS	11/11	13.6 ± 3.1	2.5 ± 1	35.4 ± 17.9
PLP$_{139-151}$	9/11	27.4 ± 11[b]	2.0 ± 1.0	29.5 ± 18.3
M-PLP$_{139-151}$	4/11[a]	27 ± 3.5[b]	0.8 ± 1.1[c]	7.5 ± 11.7[d]

[1] Combined data of two experiments are presented.
[2] Mice were treated i.v. on day 8 with one single dosage of PBS or 50 µg peptide
[3] Only mice with EAE
[a] $p < 0.05$ compared to controls an $p = 0.08$ compared to PLP$_{139-151}$-treated animals
[b] $p < 0.01$ compared to controls
[c] $p < 0.01$ compared to controls and $p < 0.05$ compared to PLP$_{139-151}$-treated animals
[d] $p < 0.01$ compared to controls and PLP$_{139-151}$-treated animals

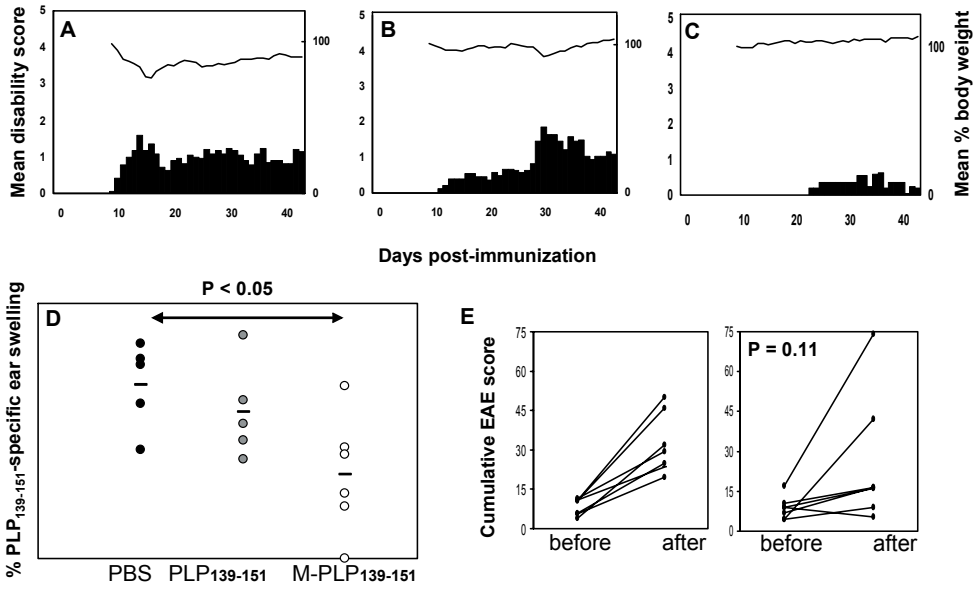

Figure 1. *Soluble M-PLP$_{139-151}$ inhibits EAE after active immunization. Mice were immunized with 50 to 75 µg PLP$_{139-151}$ in complete adjuvant supplemented with 1 mg/ml M. tuberculosis, and treated intravenously with PBS (A), 50 µg PLP$_{139-151}$ (B) or M-PLP$_{139-151}$ (C) eight days later. EAE score and body weight were assessed daily. Combined data from two experiments are shown and statistics are provided in Table 1. In one experiment DTH responses were measured after injection of 25 µg PLP$_{139-151}$ into the ear at day 30 (D). Ear thickness was measured 48 hours later and means are indicated in the graph. Treatment with M-PLP$_{139-151}$ resulted in a significantly decreased DTH response ($p < 0.05$). EAE was induced in mice that were treated on day 17 or 24 (E). The cumulative EAE score of individual mice before and 4 weeks after treatment are connected to visualize disease progression. Treatment with M-PLP$_{139-151}$ resulted in decreased disease progression compared to controls ($P = 0.11$)*

compared to controls after treatment with either peptide ($p < 0.01$). However, only injection of M-$PLP_{139-151}$ resulted in a significantly decreased maximal and cumulative EAE score compared to controls ($p < 0.01$).

Peripheral T cell reactivity was evaluated by measuring DTH responses 30 days after immunization. Single treatment with M-$PLP_{139-151}$ on day 8 significantly reduced the peptide-specific DTH response at day 30 compared to control mice (Figure 1D, $p < 0.05$). Such an effect was not observed with non-mannosylated peptide. This suggests that treatment with M-$PLP_{139-151}$ interferes with effector functions of peptide-specific T cells in the periphery.

To study the efficacy of treatment on established disease, a single peptide dose was administered to mice that showed clinical symptoms for at least 2 days. The mice were monitored for 4 weeks after treatment and as depicted in Figure 1E, treatment with M-$PLP_{139-151}$ resulted in limited disease progression in 5 out of 7 mice. However, due to two non-responding animals, this observation did not reach significance ($p = 0.11$ compared to controls).

Collectively, these data indicate that treatment with soluble M-$PLP_{139-151}$ can mediate long-lasting modulation of an autoimmune response, well after priming of self-reactive T cells.

M-$PLP_{139-151}$ treatment inhibits disease in an adoptive transfer model of EAE

To exclude that treatment with M-$PLP_{139-151}$ only interfered with the expansion of antigen-specific T cells after active immunization, we studied the effect of peptide treatment in an adoptive transfer model of EAE. For this purpose encephalitogenic T cells were induced in donor mice and transferred into naive recipient mice.

Three adoptive transfer experiments were performed in which triple subcutaneous treatment was applied together with the transfer of encephalitogenic T cells (combined in Table 2). Due to severity of clinical symptoms in this model, prolonged monitoring until day 28 was feasible in only one experiment and these data were used to evaluate the effect of peptide treatment on the cumulative EAE score. Transfer of encephalitogenic cells into recipients that were treated with PBS only, resulted in development of clinical symptoms around day 7 in all animals (Figure 2A and Table 2). Treatment with M-$PLP_{139-151}$ resulted in a significantly reduced EAE incidence compared to control mice, while treatment with $PLP_{139-151}$ did not. (Table 2, $p < 0.05$).

Analysis of the disease course in affected mice revealed that treatment with $PLP_{139-151}$ delayed the onset of EAE ($p < 0.01$), but only slightly reduced severity of clinical symptoms (Figure 2B and Table 2). In contrast, mice treated with soluble M-$PLP_{139-151}$ showed both

Table 2. M-PLP$_{139-151}$-treatment ameliorates EAE in an adoptive transfer model

Treatment [2]	EAE incidence	Day of onset [3] (Mean ± SEM)	Maximal score (Mean ± SEM)	Cumulative score[4] (Mean ± SEM)
PBS	13/13	7.0 ± 1.2	3.7 ± 1.0	56.3 ± 25.2
PLP$_{139-151}$	12/14	8.6 ± 1.2 [b]	2.8 ± 1.3 [c]	38.9 ± 3.4
M-PLP$_{139-151}$	10/15 [a]	11.7 ± 1.9 [d]	1.0 ± 0.9 [e]	13.7 ± 9.6 [f]
M-OVA$_{371-386}$	6/8	7.0 ± 0.9	4.2 ± 0.9	30.0 ± 30.2

[1] Combined data of three experiments are presented
[2] Mice were treated s.c. on days -1, 1 and 3 with PBS or 50 µg peptide
[3] Only mice with EAE
[4] Data from one experiment, n = 5 mice / group
[a] p = 0.05 compared to controls
[b] p < 0.01 compared to controls and M-OVA$_{371-386}$-treated animals
[c] p < 0.09 compared to controls
[d] p < 0.001 compared to all other groups
[e] p < 0.001 compared to controls and PLP$_{139-151}$-treated mice and p < 0.01 compared to M-$_{371-386}$-treated animals
[f] p < 0.01 compared to controls and PLP$_{139-151}$-treated mice

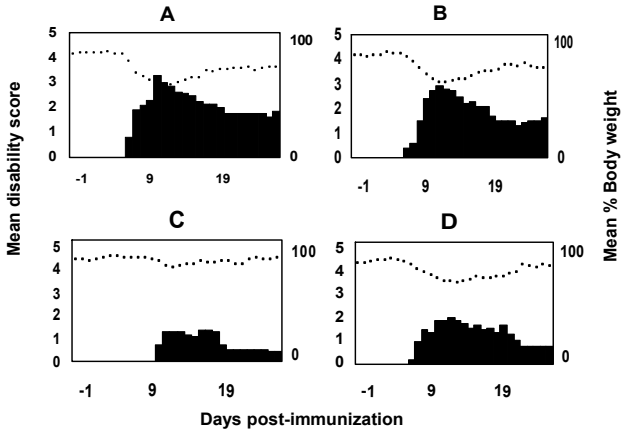

Figure 2. *M-PLP$_{139-151}$-treatment ameliorates EAE in an adoptive transfer model. Lymph node cells obtained from donor mice were cultured for three days and 3 x 10^6 T cell blasts were injected into naïve recipient mice. These animals were treated subcutaneously with PBS (A), 50 µg PLP$_{139-151}$ (B), 50 µg M-PLP$_{139-151}$ (C) or 50 µg M-OVA$_{371-386}$ (D) on days –1, 1 and 3. EAE score and body weight were assessed daily. This experiment was performed three times and mean data from a study monitored for a period of 28 days are presented. See Table 2 for combined data and statistical analysis.*

a significant delay in disease onset ($p < 0.001$) and a significantly reduced maximal EAE score compared both to controls and PLP$_{139-151}$ treated animals (Figure 2C and Table2, $p < 0.001$). Accordingly, the cumulative EAE score was significantly diminished compared to both groups ($p < 0.01$). To study the antigen specificity of mannosylated peptide treatment in this EAE model a non-relevant mannosylated OVA peptide (M-OVA$_{371-386}$) was included. Triple injection of M-OVA$_{371-386}$ into recipient mice showed no significant effect on the course

of EAE (Table 2), although two animals included in the long-term experiment presented in Figure 2D did not develop EAE.

Frequently, IL-12 is included in T cell cultures for EAE transfer, to enhance the encephalitogenic potential of T_h1 cells (23;24). In our hands culturing of T cells in the presence of IL-12 indeed resulted in severe EAE upon transfer, resulting in 40% mortality in control recipients. Even under these conditions a single dose of M-$PLP_{139-151}$ injected before disease onset resulted in ameliorated disease, while no such effect was induced by non-mannosylated $PLP_{139-151}$ (unpublished data).

Additionally, peptide was injected twice a week into recipient mice that showed clinical symptoms for at least two days. The animals were monitored for 10 days and as depicted in Figure 3A only treatment with M-$PLP_{139-151}$ ameliorated the course of disease compared to controls ($p = 0.055$). Mice were sacrificed after this treatment period and splenocytes were cultured for four days in the presence of $PLP_{139-151}$. In all treatment groups comparable peptide-specific proliferation was detected (Figure 3B).

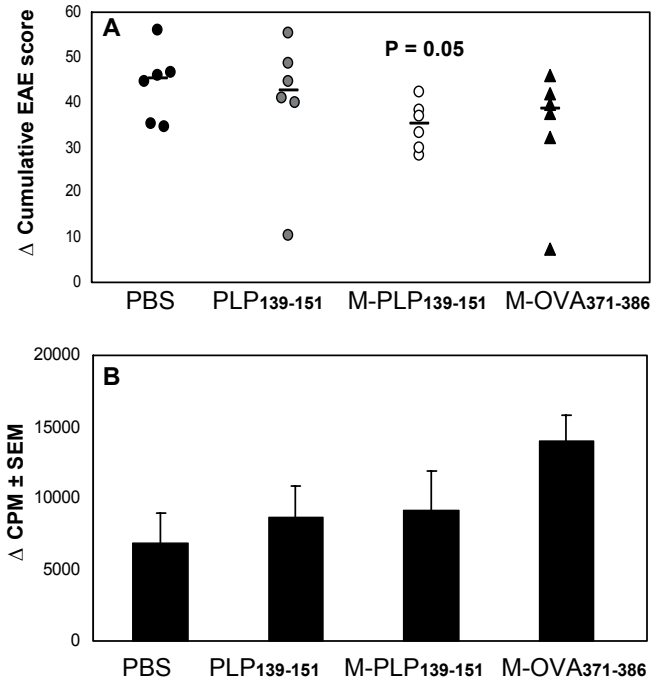

Figure 3. *Treatment of established disease with soluble M-$PLP_{139-151}$. Recipient mice of encephalitogenic T cells with apparent clinical symptoms were randomized (cumulative EAE scores ranged between 1,5 and 9,5). Subcutaneous treatment was applied twice a week with PBS , 50 µg $PLP_{139-151}$, 50 µg M-$PLP_{139-151}$ or 50 µg M-$OVA_{371-386}$ and EAE score and body weight were assessed daily. Disease progression is expressed by the cumulative EAE score during the treatment period and medians are indicated (A). Only treatment with M-$PLP_{139-151}$ resulted in a trend towards decreased disease progression as compared to controls ($p = 0.055$). Splenocytes were isolated after sacrifice and cultured with $PLP_{139-151}$ for 4 days. Proliferation revealed no differences between the groups (B).*

M-PLP$_{139-151}$ treatment is associated with reduced CNS inflammation

The effect of peptide treatment together with the transfer of autoreactive T cells on CNS inflammation was evaluated by immunohistochemistry. Haematoxylin staining revealed that on day 16 after transfer inflammatory regions were located mainly in the spinal cord (Figure 4, printed on the cover) and to some extent in the cerebellum. In control recipients (left panels), these infiltrates comprised large numbers of $CD3^+$ T cells and $CD11b^+$ cells, representing infiltrating macrophages and resident microglia. Moreover, abundant expression of MHC class II and IFN-γ was observed. The majority of infiltrates also contained IL-23 positive cells. Treatment of recipient mice with $PLP_{139-151}$ did not influence the number and composition of these infiltrates (middle panels).
In line with the decreased clinical scores, inflammation of the CNS was less severe in mice that were treated with M-$PLP_{139-151}$. This was substantiated by lower numbers of $CD11b^+$ cells and less MHC class II expression. CD3 staining revealed decreased numbers of T cells and only little IFN-γ and IL-23 expression (right panels). Besides inflammation, demyelination is a well-known neuropathological feature in MS and also in several EAE models. However, in the EAE models we employed no detectable demyelination was observed. These data indicate that treatment with M-$PLP_{139-151}$ reduced inflammation in the CNS.

Treatment with M-PLP$_{139-151}$ interferes with peripheral T cell reactivity

To study whether peptide treatment together with the transfer of autoreactive T cells interfered with T cell effector functions in the periphery, we analyzed DTH responses three to four days after transfer of $PLP_{139-151}$-specific T cells. Intradermal injection of 25 µg $PLP_{139-151}$ into the ear of control recipients resulted in a mean antigen-specific ear-swelling of 56 ± 27% (Figure 5). Treatment with M-$OVA_{371-386}$ resulted in similar DTH responses as control mice (58 ± 33%). Although not significant, $PLP_{139-151}$ treated recipients showed a slightly decreased DTH response (41 ± 19%). In contrast, a significantly decreased DTH response was detected (13 ± 11%) after treatment with M-$PLP_{139-151}$. Therefore, it can be concluded that treatment with M-$PLP_{139-151}$ affected T cell effector functions in the periphery in an antigen-specific way.
Additionally, $PLP_{139-151}$-specific antibody titers were determined in sera of recipient mice as described elsewhere (7). IgG levels in controls and M-$PLP_{139-151}$-treated mice were similar. Treatment with non-mannosylated peptide induced increased total IgG titers compared to controls, involving increased levels of IgG1 and IgG2b. In all adoptive transfer recipients IgG2a levels were below the detection limit (unpublished observations).

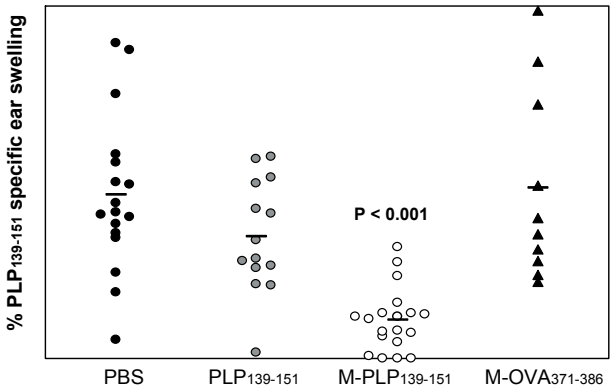

Figure 5. *Abolished DTH responses after treatment with M-PLP$_{139-151}$. Three to four days after transfer of encephalitogenic T cells, 25 μg PLP$_{139-151}$ was injected into the right ears of recipient mice; as a control saline was injected into the left ears. Peptide-specific ear swelling was determined 24 hours after challenge. Combined data of three different experiments are shown.*

Discussion

In this report we describe that the encephalitogenic potential of autoreactive T cells during EAE in SJL mice can be inhibited by treatment with soluble mannosylated myelin peptide. To demonstrate this, both active immunization and adoptive transfer EAE models were used. The fact that peptide treatment can be studied in the absence of complete adjuvant is an additional advantage of EAE transfer models. Significant reduction of EAE incidence and clinical symptoms was obtained when mannosylated peptide was administered before the onset of clinical symptoms. Treatment of established disease induced less pronounced effects, suggesting that peptide treatment is particularly effective during disease stadia involving (re)activation of autoreactive T cells. Both intravenous injection, a common way to induce peptide-specific tolerance in mice, and subcutaneous injection, more suitable for clinical application, were applied. The therapeutic effect of mannosylated myelin peptide was associated with reduced inflammation in CNS tissue and impaired T cell effector functions in the periphery as determined by DTH responses.

Irrelevant mannosylated peptide did not affect DTH responses against PLP$_{139-151}$ and only slightly modulated the course of EAE. We have infrequently observed such modest non-specific effects also during previous studies (7) and therefore we conclude that treatment effects of M-PLP$_{139-151}$ were largely antigen-specific.

Additional adjuvantia, such as Pertussis toxin, are indispensable for EAE induction in most mouse strains (25). These adjuvantia break tolerance induced by mannosylated peptides and

therefore our EAE studies have been limited to SJL mice, in which Pertussis toxin is not required for EAE induction. Besides EAE development, also DTH responses are affected by treatment with mannosylated peptide. We have used this DTH model to demonstrate that immune modulation was achieved in other mouse strains using a range of mannosylated antigens (manuscript in preparation), implicating that a broad application of mannosylated peptides for immune modulation is feasible.

Treatment with non-mannosylated $PLP_{139-151}$ showed no significant effect on DTH responses in both EAE models. Additionally, the development of disease in the adoptive transfer model was not affected. After active immunization, injection of non-mannosylated peptide significantly delayed disease onset, but did not decrease severity of clinical symptoms. Intravenous injection of soluble antigens has been described as a mechanism for tolerance induction, via clonal deletion of antigen-specific T cells (26). Although we detected slight effects, the applied dosage of $PLP_{139-151}$ was too low to mediate substantial tolerance. Therefore, we consider the tolerizing effect of mannosylated peptide dependent on a mechanism mediated by mannose-binding structures.

T_h1 effector cells play a major role in both DTH responses and inflammation during EAE (27). Because our studies show that M-$PLP_{139-151}$ modulates both inflammatory processes under conditions where autoreactive T cells are already present, it is highly likely that peptide treatment interferes with the effector functions of these T_h1 cells. Several candidate mechanisms involved in tolerance induction after injection of soluble antigens, have been described in literature, such as clonal deletion, anergy or induction of regulatory T cells (28-30).

Antigen-specific proliferation of splenocytes isolated from adoptive transfer recipient mice that were treated with mannosylated peptide was not affected. Also previous studies showed normal *in vitro* proliferation of T cells with a T_h1 phenotype after immunization with mannosylated myelin peptide (7) and preliminary data using a TCR transgenic mouse model indicate that mannosylated peptide induces normal expansion of antigen-specific T cells *in vivo* (manuscript in preparation). Together, these data are not in favor of T cell deletion or anergy induction after treatment with M-$PLP_{139-151}$. Additional analysis of $PLP_{139-151}$-specific IgG antibody levels in sera of recipient mice revealed that mannosylated myelin peptide induced no alterations in IgG antibody production. This may be considered an additional indication that mannosylated antigens do not induce a T_h2 response or a regulatory response.

Both DTH responses and EAE are highly dependent on migration of effector T cells to local tissue (6;31). Because only few T cells and little IFN-γ was detected in spinal cord tissue of recipient mice it cannot be ruled out that treatment with mannosylated M-$PLP_{139-151}$ interferes

with migration of effector cells, resulting in decreased local inflammation in tissues. Studies to elucidate the effect of mannosylated peptide on the migration capacities of T cells are ongoing.

C-type lectin family-members that recognize mannose structures, such as the mannose receptor and DEC-205, have been described to be expressed by APC in the periphery (32;33). Therefore, the routes of administration applied in our studies may result in loading of M-$PLP_{139-151}$ on peripheral APC. Flugel et al reported that encephalitogenic T cells transferred into rats reside in peripheral lymphoid organs, before they migrate towards the CNS (34). Therefore, it is very likely that injected $PLP_{139-151}$-specific T cells encounter peripheral APC loaded with mannosylated $PLP_{139-151}$. The impaired DTH response in mice treated with mannosylated peptide likely reflects a tolerizing mechanism mediated by peripheral APC.

Besides peripheral effects, mannosylated peptides may target APC in the CNS and mediate local effects that may also contribute to diminished EAE pathology. Several studies showed that local reactivation of myelin specific T cells by microglia in the CNS, for example by production of IL-23, is pivotal for initiation of EAE (24;35-37). Mannose receptor expression on CNS APC has been described in the literature (38;39). Preliminary data indicate that the mannose receptor (CD206) was indeed expressed in the CNS tissue of recipient mice during our studies, but initial experiments aimed to monitor biodistribution of peptides did not reveal selective targeting or accumulation of mannosylated myelin peptide towards the CNS or other organs.

At present, it is unclear how interactions between APC and T cells are modulated via binding of mannosylated peptides to C-type lectins. Downstream signaling of C-type lectins is still largely a black box, although a couple of clarifying studies have been published. Chieppa et al showed that cross-linking of the mannose receptor on human DC *in vitro* induces an anti-inflammatory program, including down-regulation of IL-12 production (40). This idea is further supported by Pathak et al who described that triggering of the mannose receptor inhibits NFκ-B-driven inflammatory signaling pathways (41).

In conclusion, we have shown that mannosylated $PLP_{139-151}$ is a valuable tool to inhibit the encephalitogenic potential of T cells during EAE, most likely by modulation of autoreactive T cells in the periphery. The observation that DTH responses remained suppressed for more than 4 weeks after treatment demonstrates the long-lasting effect of mannosylated peptide treatment. The ongoing search for (auto)antigens involved in (auto)immune diseases, will enhance the feasibility of selective antigen-specific treatment of patients. Mixtures of mannosylated peptides might be effective in more complex diseases, such as Multiple Sclerosis, and opens new avenues for treatment in the future.

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8

Discussion

During the last decades we have learned much about the degenerative and inflammatory processes that occur in the CNS of MS patients and result in the typical symptoms of paralysis and sensory impairment. The etiology of this disease is still unknown, although the incidence of MS is associated with living in regions with improved sanitation (1). The hygiene hypothesis proposes that the high incidence of allergy and autoimmunity in the industrialized countries results from a suboptimal development of immune regulatory mechanisms due to a lack of intense infections (2). In line with this there are some indications that parasitic infections can interfere with MS development (1).

At present no cure for MS is available and patients are treated with non-specific immune suppressive drugs that entail many side effects. This incites the search for more specific treatment strategies. Antigen-specific immune therapy may offer the prospect of specific reduction of pathological autoimmunity, without disturbing normal immune function (3). In this thesis the application of mannosylated peptides to suppress the immune system in an antigen-specific manner was studied.

An EAE model in SJL mice was used to show that immunization with mannosylated self-peptide in adjuvant does not induce EAE development, but results in antigen-specific tolerance instead **(Chapter 2)**. The *in vitro* peptide-stimulation experiments described in **Chapters 2, 5 and 6** demonstrate that immune modulation by mannosylated peptide does not result from poor recognition of these antigens by the immune system.

As described in the introduction, a variety of EAE models in rodents has been developed over time. However, the majority of these models requires pertussis toxin for disease development and as shown in **Chapter 6** this abrogates tolerance induction by mannosylated self-peptide. Fortunately, we studied EAE development as well as the ability to mount a DTH response after immunization with mannosylated self-peptide. Both in EAE and DTH the inflammatory response is driven by $CD4^+$ T cells, involving the accumulation of macrophages (4-6). We observed that tolerized mice neither developed EAE, nor mounted considerable DTH responses. Based on this correlation between DTH and EAE, other mouse strains were used to evaluate immune modulation by mannosylated peptides **(Chapter 5 and 6)**. Indeed, it was confirmed that DTH responses were reduced with the different peptides under investigation and therefore mannosylated peptides can be considered a general tool to modulate $CD4^+$ T cell-mediated inflammatory responses. It would be interesting to study whether mannosylated peptides also modulate T_h2-driven immune responses, for example in allergy.

8.1 *Modulation of antigen presenting cells*

Previous in vitro studies with human DC revealed that uptake of mannosylated antigens is mediated by the mannose receptor, resulting in enhanced antigen presentation (7-9). Within the framework of vaccine development, these were promising results. Although the studies described in this thesis indeed show that immunization with mannosylated peptide enhances antigen presentation in vivo, no inflammatory immune response developed **(Chapter 5)**. Currently, the identity of the APC and the CLR targeted by mannosylated peptide in mice is unknown, although the in vitro experiments in **Chapter 6** suggest that B cells and macrophages are not involved in the presentation of mannosylated peptides, while $CD11c^+$ DC are.

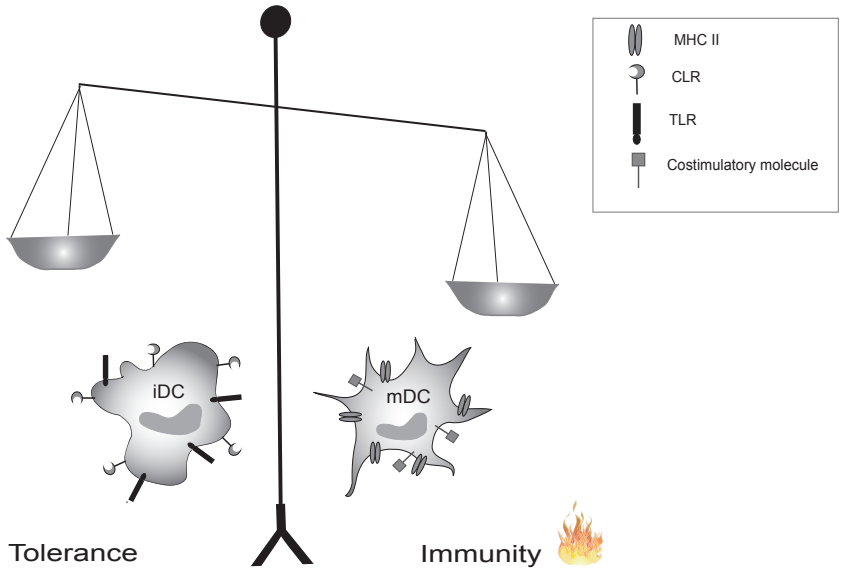

Figure 1. *The activation status of DC controls the balance between tolerance and immunity. iDC: immature DC, mDC : mature DC*

The activation status of DC controls the balance between tolerance and immunity and the expression pattern of pathogen recognition receptors contributes to these functional differences (10). Immature DC are well-equipped to endocytose large amounts of antigen, but they are poor immune stimulators. Upon DC maturation, antigen uptake decreases, while the presentation of antigens and the expression of costimulatory molecules is increased (Figure 1). DC maturation can be induced by triggering of pathogen recognition receptors or by a feedback signal of activated T cells through CD40 ligand (11-13). It is known that binding of pathogen structures to TLR induces DC maturation and results in powerful immunity.

The intracellular signaling pathways of TLR involve MyD88-dependent phosphorylation of IRAK, which in turn associates with TRAF6 and finally results in activation and translocation of NFκB to the nucleus for the transcription of proinflammatory cytokine and chemokine genes (14;15).

The role of CLR in the development of an immune response remains the subject of thorough investigation. The dual role of CLR function in the immune system is stressed by studies demonstrating immune stimulation via CLR on one hand and experiments showing immune suppression mediated by CLR on the other hand. For example, expression of the CLR family member dectin-1 is indispensable to resolve fungal infections and contributes to the induction of strong helper T cell responses (16;17). However, several microbes interact with the CLR family member DC-SIGN to suppress immune responses, that would result in clearance of the pathogen (18-20). In line with this, Shimada et al. have shown that signaling of pathogens via the mannose receptor can inhibit the fusion of phagosomes and lysosomes and prevents the production of proinflammatory cytokines (21;22).

The unraveling of the downstream signaling pathways of CLR was only recently started. Many, but not all, CLR contain activating or inhibitory motifs in their cytoplasmatic tail, which bind to adaptor proteins that induce intracellular signaling. CLR that lack such regions themselves may associate with adaptor proteins that contain such signaling motifs (23). Importantly, several studies have now demonstrated that the intracellular signaling pathways of CLR can interfere with proinflammatory TLR signaling routes. Triggering of DC-SIGN can induce T_h2-related pathways, which include the phosphorylation of ERK. In this way LPS-induced maturation of DC can be modulated, resulting in the production of IL-10 (24). In a similar way, the Man-LAM cap of M. Tuberculosis can suppress LPS-induced IL-12 production by macrophages and DC via binding to the mannose receptor or DC-SIGN. In macrophages, the ligation of CLR results in the expression of IRAK-M, which is an inhibitor of TLR pathways, because TRAF6-IRAK interactions become disturbed (25;26). In figure 2 the cross talk between intracellular signaling routes of TLR and CLR is visualized.

The fact that mannosylated antigens are able to induce tolerance despite the presence of Complete Freund's adjuvant fits well within this theory, because the TLR signaling elicited by the presence of adjuvant (27) may become overruled by the CLR signals provided by mannosylated peptides. The fact that pertussis toxin abrogates tolerance induction, while adjuvant does not, may be explained by the fact that pertussis toxin induces DC maturation via both TLR-dependent and TLR-independent signaling routes (28;29).

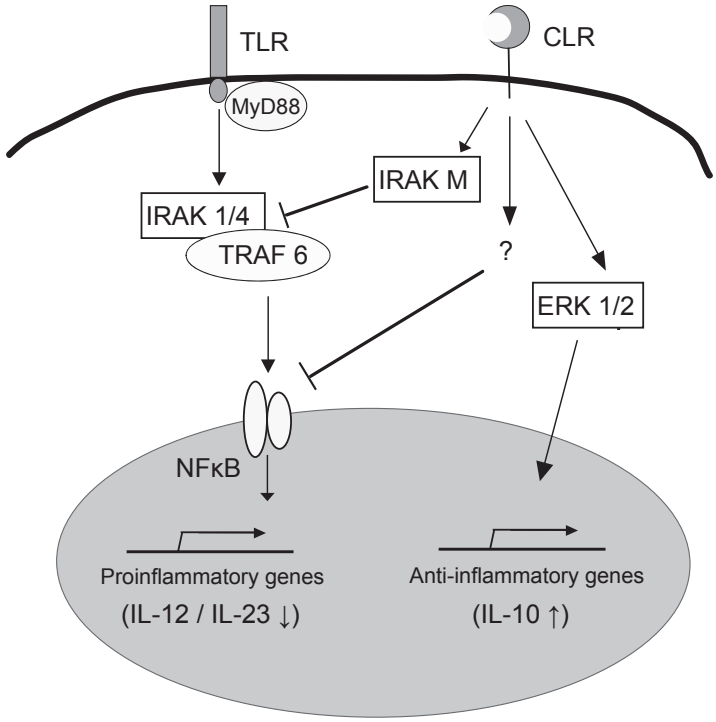

Figure 2. *Cross talk between intracellular signaling pathways of CLR and TLR*

8.2 The fate of tolerized T cells

Several strategies were applied to study the development and the fate of $CD4^+$ T cells in response to immunization with mannosylated peptides. Combining all the data described in this thesis, it can be concluded that tolerance results from a lack of local inflammation due to the development of non-functional T cells. From the literature we know that several mechanisms can contribute to the induction of tolerance, such as deletion of T cells, induction of regulatory T cells, skewing of the immune response towards a T_h2 phenotype or induction of T cell anergy (30;31). The cytokine profile of T cells as described in **Chapter 2 and 6** reveals that mannosylated peptide does not promote the development of T_h2 cells, because no IL-4 or IL-5 production was observed. Instead, high levels of IFN/γ and IL-17 were detectable in vitro, although the results described in **Chapter 5** suggest that the *in vivo* production of IFN/γ may become somewhat reduced. The fact that immunization with mannosylated peptide prevents EAE development when administered in a mixture with

the normal peptide is suggestive for active suppression of EAE development **(Chapter 2)**. However, the results in **Chapter 4** demonstrate that tolerance induction is independent of IL-10 and therefore cannot be the result of suppression via IL-10 producing regulatory T cells. Immune suppression by other mechanisms, for example via the production of TGF-β, cannot be excluded (32).

As described in **Chapter 5 and 6**, immunization with mannosylated peptide induces normal or even increased expansion of peptide-specific T cells that are non-functional. Therefore, it is unlikely that the absence of DTH responses is the result of massive clonal deletion of T cells, although specific apoptosis of subpopulations of effector T cells may occur. Importantly, the decreased antigen-specific proliferation of lymph node cells at late time points **(Chapter 2)** indicates that the formation of T cell memory might be disturbed. Memory T cells can be divided into effector and central memory T cells, based on their expression of CCR7 and CD62L. Central memory T cells remain in secondary lymphoid organs, while effector memory T cells migrate into inflamed tissue and can display immediate effector functions (33;34). The maintenance of memory T cells highly depends on the production of IL-7 and IL-15 and it would be helpful to study whether mannosylated antigens negatively affect the production of these cytokines, resulting in poor T cell memory formation.

T cell anergy is classically defined as an inactive state of T cells that can be overcome by stimulation with IL-2. Anergic T cells are long-lived and can persist *in vivo* as unresponsive cells (35). The fact that antigen-specific T cells remain present in mice after tolerance induction with mannosylated peptide, implicates that the T cells might achieve an anergic state. On the other hand, for EAE transfer experiments T cells were stimulated with IL-2 *in vitro* and these cells did not recover effector functions **(Chapter 6)**. The fact that administration of pertussis toxin abrogates tolerance induction supports several of the mechanisms described above. It might well be that pertussis toxin prevents the induction of T cell anergy (36) or inhibits the development or function of regulatory T cells (37;38).

It is most likely, that immunization with mannosylated peptide results in expansion of T cells that are incompletely differentiated. This may be reflected by the reduced blast formation of T cells **(Chapter 5 and 6)**, although proliferation and cytokine production seemed largely unaffected. Incomplete differentiation of tolerized T cells may result in impaired migration (39;40). The expression of adhesion molecules, such as VLA-4 and LFA-1, is important for migration of leukocytes across the blood brain barrier. These molecules interact with VCAM-1 and ICAM-1 expressed by endothelial cells (41-43). Possibly, mannosylated antigens alter the expression of adhesion molecules on leukocytes, contributing to the reduced accumulation of inflammatory cells. Moreover, studies in genetically deficient mice have

revealed that chemokine receptor expression is required for EAE development. While the expression of CCR2 is primarily important for macrophage migration, CCR1 is responsible for the accumulation of T cells (44-46). Expression of CCR6 by $CD4^+$ T cells is crucial in DTH responses (47), and in situ hybridization studies have revealed that T cells express CCR7, CXCR5 and CXCR3 to enter secondary lymphoid organs, the skin or CNS tissue (48;49). Besides, the effector functions of T cells are directly associated with the expression of certain chemokine receptors (50). Dedicated arrays to study the chemokine receptor profile of T cells after immunization with mannosylated peptide reveal no expression of CCR3 and CCR4, again emphasizing the absence of T_h2 cells (51). These preliminary data also suggest that immunization with mannosylated peptide alters the chemokine receptor expression of T cells, which for example results in reduced expression of CCR6 and increased levels of CCR7. To further address this issue, the surface expression of chemokine receptors on individual T cells *in vivo* should be studied.
So far, it is clear that immunization with a mannosylated peptide induces large numbers of T cells, but the question arises for what purpose these T cells are expanded, because their actual function remains unclear.

8.3 The application of mannosylated antigens in autoimmunity

Patients that suffer from an autoimmune disease visit the clinic for treatment when immune responses against self-components are ongoing and therefore the effect of mannosylated self-peptide was also evaluated during established EAE. As described in **Chapter 7**, EAE symptoms are delayed and less severe when soluble mannosylated self-peptide is administered after immunization. Moreover, repetitive peptide treatment also ameliorated established disease in an adoptive transfer EAE model. Accordingly, mannosylated peptide inhibits pre-existing T cell effector functions in a DTH model **(Chapter 5)**. Nevertheless, immune modulation by mannosylated self-peptide seems most pronounced when administered before the onset of clinical disease, suggesting that treatment during periods of T cell (re)activation is most effective. From this it can be predicted that particularly MS patients suffering from relapses would benefit from mannosylated peptide treatment, whereas such a treatment will be less effective during the more chronic phases of MS. The Biozzi EAE model, involving an initial relapse-remitting phase, followed by chronic disease, may be a suitable tool to test this hypothesis (52).
Treatment with soluble mannosylated self-peptide resulted in poor DTH responses in EAE mice, indicating that at least part of the immune modulating effect occurs in the periphery.

Indeed, it has been reported that encephalitogenic T cells reside in the periphery for several days after intravenous transfer (53), which increases the chance to encounter the injected mannosylated peptide in peripheral organs. Once encephalitogenic T cells have entered the CNS, presentation of autoantigens by endogenous APC is required for the development of full T cell effector functions that drive disease progression (54-56). Especially microglia can mediate this local reactivation of encephalitogenic T cells, because they can respond to danger signals by the upregulation of class II and costimulatory molecules and by the production of IL-23 (57-59). It cannot be excluded that the injected mannosylated self-peptide ends up in the CNS, although overt accumulation in CNS tissue was not observed **(Chapter 3)**. Uptake of mannosylated self-peptide by APC in the CNS may result in the modulation of local T cell reactivation. CLR-mediated endocytosis might occur, because during EAE macrophages and DC in the perivascular space express the mannose receptor and DEC-205 (60;61). Some studies suggest that the mannose receptor is also expressed on glial cells (62) and therefore immune modulation by mannosylated self-peptide might be extended to APC populations in the CNS parenchyma.

As described in **Chapter 2 and 7** the suppressive effect of mannosylated self-peptide is largely antigen-specific, which implies that the physiological immune functions remain intact. The moderate non-specific effects observed might be the result of interference of mannosylated peptide with CLR functions independent from antigen uptake, such as the contribution of the mannose receptor to lymphocyte migration (63). In theory, glycosylated peptides containing other sugar moieties than mannose may also induce immune modulation. This possibility has not been tested so far, but it may provide additional information for the further application of glycosylated peptides to modulate the immune system.

The success of antigen-specific immune therapy highly depends on the selection of epitopes used for treatment (64;65). The heterogeneity in MS suggests that disease progression involves multiple autoantigens and therefore a mixture of multiple mannosylated myelin peptides may be most suitable for MS treatment. To circumvent the problem of choosing appropriate autoantigens, whole mannosylated self-proteins that can be endogenously processed and presented may be a good opportunity. Moreover, whole proteins are less sensitive for degradation by proteases and due to the presence of multiple mannose clusters may result in cross-linking of CLR to increase the tolerizing signal. However, it should be mentioned that clinical trials in which oral administration of whole myelin protein was applied to MS patients, showed minimal efficacy (65).

To study the general safety of mannosylated peptides in humans, initial experiments should comprise non-pathogenic antigens, for example derived from the commensal gut flora.

Matsuzaki et al. has recently reviewed how oral administration of non-pathogenic lactobacilli can ameliorate disease in different animals models for autoimmunity, probably due to the induction of T_h2-driven immunity (66). These probiotic bacteria represent a continuous source of glycosylated molecules in the gut and the involvement of CLR in immune modulation by probiotics has been suggested, because oral administration of lactobacilli induces the expression of the mannose receptor by innate immune cells in the gut (67). Moreover, it has been shown that several lactobacilli strains can induce IL-10-producing regulatory T cells by signaling via DC-SIGN (68).

Both CLR and TLR are widely expressed among vertebrates and therefore the expression of these pathogen recognition receptors is believed to have an ancient evolutionary origin (69). The complex combination of synergistic and antagonistic signals incited by these receptors orchestrate the immune response (70;71). However, it remains unclear whether CLR have actually developed as immune stimulating pathogen recognition receptors, or whether their main function is the regulation of (chronic) inflammation via glycosylation patterns of (self-)molecules. It may well be that microorganisms evoluted in such a way that they can make use of these endogenous routes for immune regulation to induce immune suppression and to escape their eradication from the host. The fact that mannosylated antigens can be used to downregulate immune responses fits within the concept that CLR play a role in fine-tuning of immunity and may therefore be considered a valuable approach to inhibit undesirable immune reactions associated with autoimmunity or allergy.

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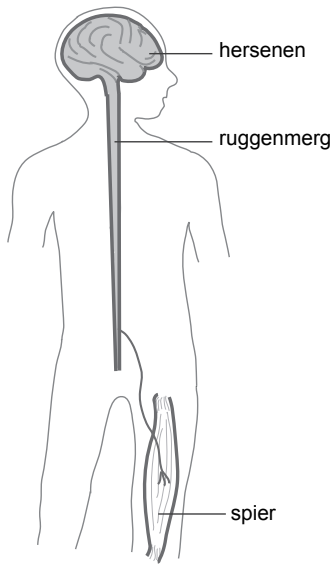
Samenvatting

1. Multiple sclerose

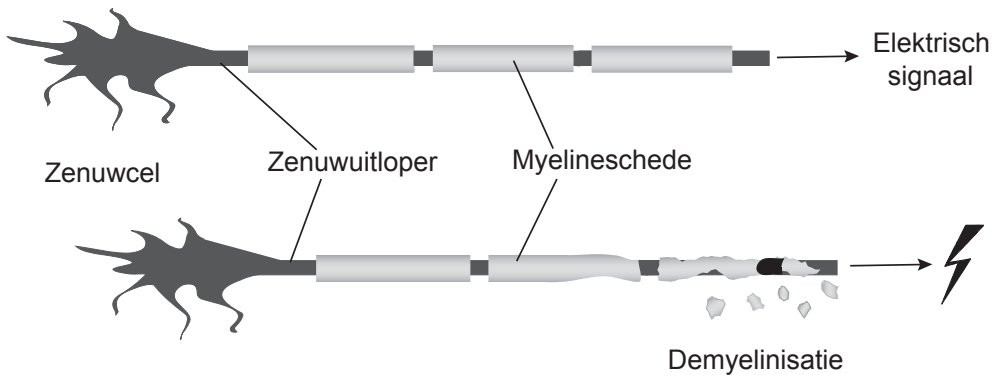
Multiple sclerose (MS) is een chronische ziekte, die meestal in de adolescentie begint. Het belangrijkste kenmerk van MS is het ontstaan van verlammingsverschijnselen, die verergeren naarmate de ziekte vordert. Daardoor zijn veel MS-patiënten aan een rolstoel gebonden. De ziekteverschijnselen worden veroorzaakt door complexe processen in de hersenen en het ruggenmerg, waardoor de aansturing (en daardoor het functioneren) van spieren wordt aangetast.



Figuur 1. *Het centrale zenuwstelsel*

De hersenen en het ruggenmerg vormen samen **het centrale zenuwstelsel (CZS)** en regelen veel functies in het lichaam (Figuur 1). Het CZS bestaat uit zenuwcellen, die met elkaar en met de rest van het lichaam communiceren via elektrische signalen. Zenuwcellen hebben uitlopers, die deze signalen transporteren door het hele lichaam. Zo kunnen vanuit het CZS bijvoorbeeld de spieren worden aangespannen en kunnen we ons bewegen. Zenuwuitlopers zijn soms behoorlijk lang: ze kunnen van de onderkant van het ruggenmerg helemaal tot in de tenen lopen. Om de geleiding van elektrische signalen via zenuwbanen te vergemakkelijken, zijn ze ingepakt in een isolatielaag. Deze laag biedt ook meteen bescherming en heet de **myelineschede**.

In het CZS van MS-patiënten raakt de myelineschede beschadigd, wat we **demyelinisatie** noemen (Figuur 2). Hierdoor verslechtert de overdracht van elektrische signalen. Soms raken de zenuwuitlopers zelf ook beschadigd. Dit alles leidt er toe dat spieren niet meer goed kunnen worden aangestuurd en er verlammingsverschijnselen optreden. Op meerdere (**'multiple'**) plaatsen in het CZS van MS-patiënten zijn zones aanwezig waar demyelinisatie plaatsvindt (zogenaamde laesies), wat uiteindelijk leidt tot de vorming van littekenweefsel (**'sclerose'**). Het is niet precies bekend waardoor demyelinisatie ontstaat, maar vanwege de verscheidenheid van symptomen in MS-patiënten wordt gedacht dat er meerdere oorzaken zijn voor het ontstaan van MS. Er zijn veel aanwijzingen dat **een verstoorde immuunrespons** bijdraagt aan het ziekteverloop in een groot deel van de MS-patiënten.



Figuur 2. Demyelinisatie van zenuwuitlopers

2. Het immuunsysteem

Het immuunsysteem verzorgt de afweer tegen potentiële ziekteverwekkers, zoals bacteriën en virussen. De eerste herkenning van ziektekiemen die het lichaam binnendringen, gebeurt door **antigeen-presenterende cellen**. Deze cellen patrouilleren door het lichaam en zijn uitgerust met allerhande **receptoren** waarmee ze hun omgeving kunnen scannen op de aanwezigheid van ziekteverwekkers.

Sommige receptoren functioneren als 'grijpers', waarmee een ziektekiem kan worden opgenomen. Binnen in de antigeen-presenterende cel worden de eiwitten van de ziektekiem in stukjes geknipt, die we **peptiden** noemen. Vervolgens kunnen al deze peptiden op het oppervlak van de cel worden gepresenteerd met behulp van eiwitten, die fungeren als **presenteerblaadjes**. Andere immuuncellen zijn in staat om de gepresenteerde peptiden waar te nemen. Een peptide die wordt herkend en een immuunreactie opwekt wordt ook wel een **antigeen** genoemd; zo komen antigeen-presenterende cellen aan hun naam.

De **T-cel** is een immuuncel, die in staat is om een peptide op het oppervlak van antigeen-presenterende cellen te herkennen. Het peptide kan alleen worden herkend als het past in de **T-cel receptor** op het oppervlak van de T-cel. Iedere T-cel heeft slechts één type T-cel receptor op zijn oppervlak, waardoor de specifieke herkenning van één antigeen mogelijk is. Om een zo groot mogelijke hoeveelheid antigenen (afkomstig van ziekteverwekkers) te kunnen herkennen, bevat het immuunsysteem dan ook enorme aantallen T-cellen, die allemaal hun eigen specificiteit hebben.
De herkenning en opname van ziektekiemen leidt tot activatie van antigeen-presenterende cellen, waardoor de presentatie van antigenen verder wordt verhoogd en de productie van signaalstoffen op gang komt. Deze signaalstoffen, in combinatie met het gepresenteerde antigeen, zijn nodig voor de optimale activatie van T-cellen, zodat de ziekteverwekker daadwerkelijk kan worden opgeruimd. Hierbij krijgt de T-cel hulp van andere immuuncellen, zoals **B cellen, granulocyten** en **macrofagen**. Dit proces, waarbij immuuncellen elkaar met behulp van signaalstoffen waarschuwen, waarna ze zich verzamelen om ziekteverwekkers op te ruimen, wordt **ontsteking** genoemd.

3. Auto-immuniteit in Multiple Sclerose

Het immuunsysteem heeft als functie een ontsteking te veroorzaken als er ziekteverwekkers aanwezig zijn, maar mag niet reageren op lichaamseigen componenten. Als het immuunsysteem niet goed in staat is om dit onderscheid te maken, kan **auto-immuniteit** ontstaan. Er ontwikkelt zich in dat geval een immuunreactie tegen lichaamseigen structuren, met vaak hele schadelijke gevolgen.
In MS-patiënten is een auto-immuunreactie tegen myeline aan de gang. Per abuis ziet het immuunsysteem myeline als een 'gevaarlijke component' en zal proberen om het op te ruimen. Dit leidt tot ontsteking in het CZS, waardoor myeline wordt afgebroken en demyelinisatie optreedt. Gealarmeerde T-cellen, macrofagen en alle ontstekingsfactoren die worden gemaakt, veroorzaken **onherstelbare schade** aan de myelineschede en de zenuwuitlopers. Hierdoor is het proces van verlamming in MS-patiënten grotendeels onomkeerbaar.
De oorzaak van auto-immuniteit is een **verkeerde communicatie** tussen antigeen-presenterende cellen en T-cellen. In het geval van MS laat een antigeen-presenterende cel ten onrechte aan de T-cel weten dat de gepresenteerde myeline-peptiden 'gevaarlijk' zijn, waardoor de T-cel een auto-immuunreactie tegen het eigen lichaam in gang zet. Veel onderzoek heeft dan ook tot doel de communicatie tussen beide celtypes beter te begrijpen. Het **corrigeren van de communicatie** tussen antigeen-presenterende cellen en T-cellen zou een waardevolle

bijdrage kunnen leveren aan de behandeling van patiënten die lijden aan een auto-immuun ziekte. Als lichaamseigen componenten niet langer als 'gevaarlijk' worden bestempeld, zal minder ontsteking optreden en zal het ziekteverloop van patiënten verbeteren.

4. Receptoren op antigeen-presenterende cellen

Ziekteverwekkers hebben specifieke structuren, waardoor het menselijk immuunsysteem ze kan herkennen als lichaamsvreemd. Tot nu toe zijn er twee soorten receptoren bekend die antigeen-presenterende cellen kunnen gebruiken om **lichaamsvreemde structuren** te onderscheiden.
Ten eerste zijn er **Toll-like receptoren**, die specifieke structuren van ziektekiemen kunnen herkennen. Binding van ziektekiemen aan Toll-like receptoren leidt tot activatie van antigeen-presenterende cellen en het in gang zetten van een immuunreactie, maar niet tot opname van ziekteverwekkers door de antigeen-presenterende cel.
Daarnaast gebruiken antigeen-presenterende cellen **C-type lectinereceptoren** om **gesuikerde structuren** waar te nemen. Veel ziektekiemen hebben gesuikerde structuren op hun oppervlak. De meeste C-type lectinereceptoren fungeren als grijpers en de opname van ziektekiemen draagt bij aan de ontwikkeling van een immuunreactie .
Er zijn echter ook **lichaamseigen moleculen die suikers bevatten**. Sommige C-type lectinereceptoren blijken juist betrokken te zijn bij het opruimen van dergelijke moleculen wanneer er te veel van aanwezig zijn. In zulke gevallen leidt opname van gesuikerde moleculen uiteraard niet tot een immuunreactie. De reactie van een antigeen-presenterende cel op signalering via de C-type lectinereceptoren is dan ook niet altijd dezelfde. Sommige onderzoeken wijzen uit dat binding van gesuikerde structuren aan C-type lectinereceptoren leidt tot activatie van de antigeen-presenterende cel, waardoor een potente immuunrespons ontstaat. Andere studies laten juist zien dat signalering via C-type lectinereceptoren leidt tot onderdrukking van het immuunsysteem en het ontstaan van immunologische **tolerantie**.

5. 'Multiple Sclerose' in muizen

Om meer inzicht te verkrijgen in de ziekteprocessen die mogelijk een rol spelen in MS zijn er modellen in proefdieren ontwikkeld. In muizen kan een MS-achtige ziekte worden opgewekt door ze te injecteren met lichaamseigen myeline-peptide, in combinatie met lichaamsvreemde structuren die kunnen binden aan Toll-like receptoren en dus door antigeen-presenterende cellen als 'gevaarlijk' worden bestempeld. Dit wordt **immuniseren** genoemd. De immunisatie

leidt tot alarmering van antigeen-presenterende cellen, terwijl ze het lichaamseigen myeline-peptide aan T-cellen presenteren. Hierdoor wordt een auto-immuun respons tegen myeline in gang gezet en ontwikkelt zich de ziekte **experimentele auto-immuun encephalomyelitis** (EAE). Op meerdere plekken in het CZS ontstaan ontstekingen waarin T-cellen en macrofagen een grote rol spelen. Door deze ontstekingen raken de muizen verlamd en doen zich dezelfde verschijnselen voor als bij MS-patiënten.
Voor het beantwoorden van de vraagstellingen in dit onderzoek was het niet in alle gevallen mogelijk of noodzakelijk om daadwerkelijk EAE op te wekken in muizen. Daarom zijn tevens zogenaamde **'vertraagd type overgevoeligheid reacties'** (*Delayed Type Hypersensitivity*, **DTH**) gemeten. Na de immunisatie (zie hierboven) wordt een kleine hoeveelheid peptide in het oor van de muis gespoten, waardoor ter plekke een ontstekingsreactie optreedt. Dit wordt een DTH-respons genoemd. Net als EAE is deze immuunreactie afhankelijk van T-cellen en macrofagen. De mate van ontsteking is gemakkelijk waar te nemen aan het oortje, dat rood en dik wordt.

6. Het promotieonderzoek

Dit proefschrift gaat over het verminderen van auto-immuniteit in EAE, door de communicatie tussen antigeen-presenterende cellen en T-cellen te veranderen. Steeds meer onderzoeken wijzen uit dat signalering via C-type lectinereceptoren op het oppervlak van antigeen-presenterende cellen kan leiden tot onderdrukking van de immuunrespons. Hoe dit gebeurt is nog grotendeels onduidelijk, maar er is wel bekend dat een veranderde communicatie van antigeen-presenterende cellen naar T-cellen een rol speelt.
In dit onderzoek is bestudeerd of gesuikerde structuren die kunnen binden aan C-type lectinereceptoren bruikbaar zijn om de auto-immuunrespons in muizen met EAE te corrigeren. Voor dit doel is gebruik gemaakt van aangepaste myeline-peptiden met twee suikergroepen, namelijk **mannoses**. Er is bestudeerd of de ontwikkeling van EAE vermindert onder invloed van **gemannosyleerde peptiden** en hoe T-cellen zich ontwikkelen na herkenning van een gemannosyleerd antigeen.

Tolerantie tegen EAE
Het immuniseren van muizen met een myeline-peptide leidt tot de ontwikkeling van EAE, maar immunisatie met gemannosyleerd myeline-peptide niet. De muizen worden daadwerkelijk **tolerant voor het myeline-peptide**, want ze zijn vervolgens beschermd tegen

EAE-ontwikkeling door her-immunisatie met het normale myeline-peptide. Deze tolerantie is **peptide-specifiek**, want het is niet mogelijk om tolerantie op te wekken met een willekeurig gemannosyleerd peptide. In de tolerante muizen is er nauwelijks ontsteking in het CZS. Ook de DTH-respons in de oren is verlaagd. Dit betekent dat het immuunsysteem na stimulatie met een gemannosyleerd myeline-peptide niet meer in staat is om een auto-immuunreactie te ontwikkelen. Hierdoor wordt het ontstaan van een ontsteking en weefselschade voorkomen.

Verspreiding van gemannosyleerd peptide

Gemannosyleerd myeline-peptide kan worden herkend door C-type lectinereceptoren, maar het normale myeline-peptide uiteraard niet. Dit leidt mogelijk tot een ongelijke distributie van beide peptiden in muizen en het zou een eerste aanwijzing zijn voor een mechanisme dat ten grondslag ligt aan tolerantie tegen EAE. Door het normale myeline-peptide en het gemannosyleerde myeline-peptide te koppelen aan **tracers** (een soort 'vlaggetjes') en vervolgens te injecteren in muizen, is bestudeerd of het gemannosyleerde myeline-peptide in een bepaald orgaan of celtype ophoopt. Hiervoor zijn geen aanwijzingen gevonden.

Tolerantie en interleukine-10

Interleukine-10 is een belangrijke signaalstof binnen het immuunsysteem om de heftigheid van immuunreacties te reguleren. In muizen die geen IL-10 kunnen maken, is dit controlemechanisme afwezig, wat de muizen extra gevoelig maakt voor het ontstaan van heftige ontstekingsreacties.

Om te bestuderen of interleukine-10 een rol speelt in het ontstaan van peptide-specifieke tolerantie tegen EAE, zijn muizen die geen interleukine-10 kunnen maken geïmmuniseerd met gemannosyleerd myeline-peptide. In muizen zonder interleukine-10 is de ontwikkeling van tolerantie tegen EAE even robuust als in normale muizen. Tevens ontwikkelt zich in deze muizen geen DTH-respons. Kortom, interleukine-10 speelt geen rol bij het onderdrukken van auto-immuniteit door gemannosyleerd myeline-peptide.

Het lot van T-cellen

Omdat T-cellen een cruciale rol spelen in de ontwikkeling van EAE en de DTH-respons, hebben we het lot van T-cellen na immunisatie met gemannosyleerd peptide bestudeerd. Daarvoor hebben we gebruik gemaakt van zogenaamde **T-cel receptor transgene T-cellen**, wat betekent dat álle T-cellen dezelfde T-cel receptor hebben en dus hetzelfde peptide herkennen. Door het grote aantal peptide-specifieke T-cellen is het heel gemakkelijk om te bestuderen wat er met deze T-cellen gebeurt.

Het is gebleken dat de T-cellen geactiveerd raken na herkenning van een gemannosyleerd peptide en dat ze normaal of zelfs iets sneller gaan delen. De T-cellen lijken goed in staat te zijn om signaalstoffen te maken en om zich te verplaatsen. Tot dusver zijn er geen duidelijke aanwijzingen dat de T-cellen verdwijnen of dat ze ongevoelig worden voor stimulatie met het gemannosyleerde peptide. Het blijft vooralsnog onduidelijk waarom grote aantallen peptide-specifieke T-cellen ontstaan, terwijl die niet in staat zijn om een ontsteking in gang te zetten.

Behandeling van bestaande ziekte

Omdat MS-patiënten al ziekteverschijnselen hebben als ze naar de kliniek komen voor behandeling, is bestudeerd of gemannosyleerd myeline-peptide geschikt is om bestaande ver lammingsverschijnselen te bestrijden. Hiervoor zijn muizen behandeld met gemannosyleerd myeline-peptide pas ná het ontstaan van EAE symptomen, wat leidt tot een duidelijk aantoonbare **vermindering van de ziekteverschijnselen**. In het CZS van de behandelde muizen werden veel minder ontstekingscellen aangetroffen, en ook de DTH-respons in deze muizen was aanzienlijk verlaagd. Dit betekent dat gemannosyleerd myeline-peptide gebruikt kan worden om een bestaande auto-immuunreactie te corrigeren.

Conclusie

De resultaten van dit promotieonderzoek laten zien dat gemannosyleerd peptide leidt tot peptide-specifieke tolerantie tegen EAE. Stimulatie met een gemannosyleerd peptide leidt tot activatie en deling van peptide-specifieke T-cellen, die echter niet in staat zijn om ontsteking en weefselschade te veroorzaken. Het is nog onbekend waarom de T-cellen niet in staat zijn om een auto-immuunreactie in gang te zetten. Met het oog op de behandeling van MS-patiënten is duidelijk geworden dat gemannosyleerde peptides bruikbaar zijn om bestaande auto-immuniteit in EAE te verminderen, zodanig dat het ziektebeeld verbetert.

List of publications

Luca, M.E., Kel, J.M., van Rijs, W., Drijfhout, J.W., Koning, F., Nagelkerken, L. Mannosylated PLP(139-151) induces peptide-specific tolerance to experimental autoimmune encephalomyelitis.
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Kel, J., Oldenampsen, J., Luca, M., Drijfhout, J.W., Koning, F., Nagelkerken, L. Soluble mannosylated myelin peptide inhibits the encephalitogenicity of autoreactive T cells during Experimental Autoimmune Encephalomyelitis.
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Junda M. Kel, Eveline D. de Geus, Marianne J. van Stipdonk, Jan W. Drijfhout, Frits Koning, Lex Nagelkerken. Immunization with mannosylated peptide induces poor T cell effector functions despite enhanced antigen presentation
International Immunology 2008 Jan; 20 (1): 117-27

Junda M. Kel, Bram Slutter, Jan Wouter Drijfhout, Frits Koning, Lex Nagelkerken. Mannosylated self-peptide inhibits the development of experimental autoimmune encephalomyelitis via expansion of non-encephalitogenic T cells
Submitted for publication

Abbreviations

APC	Antigen presenting cell
BBB	Blood brain barrier
CLR	C-type lectin receptor
CFA	Complete Freund's adjuvant
CNS	Central nervous system
DC	Dendritic cell
iDC	Immature dendritic cell
mDC	Mature dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MR	Mannose receptor
MS	Multiple Sclerosis
OVA	Ovalbumin
PLP	Proteolipid protein
PTX	Pertussis toxin
T_h	T helper cell
TLR	Toll like receptor

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Wellicht een wat luguber begin, maar ik ontkom er niet aan om de muisjes te noemen, die zo'n waardevolle bijdrage aan mijn onderzoek hebben geleverd. Lieve Inge, hyperactieve duizendpoot, je denkt ook aan alles! Dank je wel voor al je hulp en de ontelbare koppen thee (met zelfgemaakte zoetigheid). Ik ben blij dat jij tijdens de promotie naast me staat en alles in goede banen leidt.

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Gelukkig heb ik niet vier jaar in mijn eentje geploeterd en daarom prijken de namen van 'mijn studenten' boven de verschillende hoofdstukken van dit proefschrift. Ed en Willem, de verdeel-en-heers-politiek heeft duidelijk niet gewerkt…Gelukkig ben ik dankzij jullie wel altijd in evenwicht en hou ik het randjespunt nauwkeurig in de gaten! "Bram, pak een stoel." Nee grapje, dat hoeft nu niet meer. Ik hoop dat je een glansrijke academische carrière tegemoet gaat! Lianne, AIO lotgenoten zijn onmisbaar. Dank je wel voor alles. Wanneer kom je eten in Utrecht, want je hebt nog steeds een promotiecadeau van me te goed?!

Naast TNO-ers zijn er dan ook nog 'de mensen van de overkant', ofwel het LUMC, die altijd voor me klaar stonden met peptiden, muizen, werkbesprekingen, enz. Ik wil alle medewerkers bij peptidenchemie hartelijk danken voor het maken van alle peptiden, in het bijzonder natuurlijk Willemien. De tumorimmunologie club met al die M-en (Marianne, Marjolein, Marieke, Marjolein etc.) wil ik bedanken voor de OTII muizen, de gebietste FACS antilichamen en protocollen. De afdeling nierziekten was gastvrij, ik kon altijd terecht voor het homogeniseren van organen.

Oei, en dan het Utrechtse front. Waar te beginnen? Bij de AIO lotgenoten maar… Esther, chica, mijn boek is eíndelijk ook af, dus laat het feest beginnen! Bas en Sigrid, laten we proberen onze eet- en koffietraditie ondanks alle schrijfstress in ere te herstellen?! Girlies (Christine, Assia, Maartje en Rozanne), ik vind het fijn dat ik mezelf één van de TFTers mag noemen. Ook al zit ik er niet op, ik doe wel vaak mijn best om er van te zijn, dus blijf me vooral meeslepen en heropvoeden! Wouter: CTRL-ALT-W…. Wrap it baby! :-)
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Si vous dites: je vous aime, c'est déjà le language que vous vous mettez à aimer, c'est donc déjà une forme de rupture et d'infidélité.
Jean Baudrillard

Curriculum Vitae

Junda Mariska Kel is geboren op 12 juni 1978 in IJsselstein. In 1996 behaalde zij haar gymnasium diploma aan College De Klop te Utrecht. In september van datzelfde jaar begon zij aan de studie Biologie aan de Universiteit van Utrecht en in 1997 behaalde zij het propedeusediploma. Tijdens de doctoraalfase deed ze onderzoek bij de Wetenschapswinkel Biologie in samenwerking met de afdeling Milieutoxicologie onder supervisie van Drs. M. Vaal en Dr. J. Tolls en tevens bij TNO Voeding onder begeleiding van Drs. Steven Spanhaak en Dr. R.H.H. Pieters. In februari 2001 behaalde zij het doctoraaldiploma en kwam zij als assistent-projectleider in dienst van TNO Voeding in Zeist.

Van april 2002 tot oktober 2006 was zij werkzaam als assistent in opleiding (AIO) aan de Universiteit van Leiden. Het onderzoeksproject werd gesubsidieerd door Stichting Vrienden MS Research (00-432) en is uitgevoerd bij TNO Kwaliteit van Leven en in het Leids Universitair Medisch Centrum. Het in dit proefschrift beschreven onderzoek is verricht onder begeleiding van Dr. Lex Nagelkerken, Dr. Jan Wouter Drijfhout en Prof. dr. Frits Koning.

Sinds maart 2007 is Junda werkzaam als postdoc onderzoeker in the AMC, binnen de afdeling Celbiologie en Histologie in de groep van Dr. B.E. Clausen.