Recruitment of regulatory cells by repetitive antigens

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FABIOLA PUENTES CORREDOR

from Sogamoso, Colombia

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1st Reviewer: Prof. Dr. Thomas Blankestein

2nd Reviewer: Prof. Dr. Wolfgang Uckert

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por toda tu paciencia y tu bondad

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1 INTRODUCTION

For many years the studies on prevention and treatment of autoimmune diseases have been focused to the identification of antigens responsible for the triggering of the disease and intended to the regulation of undesired immune responses. Autoimmune diseases are mediated by sustained adaptive immune response specific for auto-antigens through unknown mechanisms and they result from a breakdown in self-tolerance [1].

There are multiple factors that influence the disease process such as environmental factors and genetic susceptibility. It has been determined that one of the major causes of numerous human autoimmune diseases is the abnormal activation of self-reactive T cells and susceptibility to them is very often associated with MHC genes. There is evidence that certain MHC class II allotypes are strongly correlated with specific autoimmune diseases: HLA DR2 is positively correlated with Systemic Lupus erythematosus (SLE) and Multiple sclerosis (MS) [2, 3] and negatively correlated with Diabetes mellitus (DM) Type 1; HLA DR3 is strongly correlated with Sjögren's syndrome, myasthenia gravis, SLE and DM Type 1 [4]; HLA DR4 is correlated with Rheumatoid arthritis (RA) and DM Type 1 [5, 6].

In essence, the breakdown of T cell tolerance may arise from different mechanisms: potential peripheral occurrence of T cells that escape thymic deletion, the presentation of antigens that are normally silenced, activation by a foreign antigen or the breakdown in suppressor mechanisms among others [7, 8].

1.1 The immune system and immunologic tolerance

The immune system is a biological defense mechanism essential for the survival of multicellular organisms, which discriminates non-self antigens from self-tissues [9]. An example for this recognition is provided by toll-like receptors on leukocytes, which induce the synthesis of molecules that initiate innate and adaptive immune responses [10]. In this way, the diversity of the T cell receptor (TCR) and antibodies uses plenty of specific mechanisms for the recognition of non-self pathogens [11]. This process is controlled by major histocompatibility complex (MHC) class I and class II molecules, as each MHC molecule that displays an antigen is recognized by a matching or compatible T-cell receptor [12].

Therefore, diverse regulatory mechanisms function both to control responses to foreign infectious and non-infectious antigens [13], balancing the immune system after the antigen has been cleared, and to maintain unresponsiveness to self-antigens [14]. In this aspect the clonal selection theory postulates the existence of a mechanism for the acquisition of a state of self-tolerance [15]. Based on this concept, tolerance has been defined as a state of antigen-specific unresponsiveness induced by prior exposure to the antigen [16].

Self tolerance mechanisms that mediate the prevention of the development of autoimmune diseases have been extensively studied and some specific characteristics of central and peripheral tolerance have been defined.

1.1.1 Central and peripheral tolerance

Central tolerance is a set of mechanisms that ensure the absence of self-reactivity during lymphocyte development while peripheral tolerance occurs after lymphocytes leave the primary organs. Differentiation of precursor cells into the thymus is characterized by the coordinated expression of cell surface proteins on the thymocyte, including the TCRβ rearrangements [17]. Next, rearrangement of TCRα takes place and these cells, expressing α/β heterodimeric T cell receptors depending the ligand specificity, are subjected either to positive or negative selection [18, 19].

The major mechanism of T-cell central tolerance is clonal deletion, which eliminates T-cell progenitors that have high affinity for self-antigens. Important central tolerance mechanisms are mediated by medullary thymic epithelial cells (mTECs) that express peripheral-tissue antigens and present them on the context of major histocompatibility complex (MHC) class I and II molecules, allowing deletion of self-reactive thymocytes [20].

Although central-tolerance mechanisms are efficient, they cannot eliminate all self-reactive lymphocytes. Then, peripheral tolerance mechanisms either ensure the elimination of circulating lymphocytes at first encounter of their cognate self-antigen or render them tolerant by modifying the function of self-reactive T cells. Under non-inflammatory conditions, antigen presenting cells (APC) that carry proteins from parenchymal tissues can lead to the elimination or inactivation of self-reactive T cells. Peripheral-tissue antigens expression has been previously observed only in mTECs, dendritic cells, lymph node stromal cells and spermatocytes [7, 21].

In general, at the cellular level the mechanisms of T cell tolerance to self and exogenous antigens have common characteristics. They include thymic and peripheral T cell deletion, anergy, down-regulation of surface expression of the TCR and/or coreceptor molecules (i.e., CD8, CD4) and immune deviation mediated by cytokines [22, 23]. Likewise, active suppression by regulatory cells constitutes an essential factor in the control of autoreactivity [14].

1.1.1.1 Clonal deletion

In central tolerance, there are two stages of clonal deletion: early in cortical epithelial cells and late in medullary epithelial cells [20]. The process of clonal deletion is initiated in the

thymus by interactions between cell-surface molecules on stromal antigen-presenting cells and differentiating thymocytes [24]. Some of the processes of selection in the thymus have been explained by the TCR affinity for self-peptide-MHC ligands, determining the stage at which clonal deletion takes place. Therefore, progenitors that have no affinity or very low affinity are eliminated [25].

Positive selection occurs when the TCR has a low affinity for self-peptide-MHC, then the progenitor is likely to survive and differentiate [26]. It has been postulated that progenitors with high affinity for self-peptide-MHC can be deleted by a process of negative selection or they could differentiate into cells with a regulatory phenotype [27]. On the other hand, it is important the role of dendritic cells (DCs) recruited in the thymus, contributing actively to central tolerance by inducing clonal deletion of autoreactive thymocytes [28, 29].

1.1.1.2 Anergy

T cell anergy represents an important mechanism of peripheral tolerance, in which the lymphocyte is functionally inactivated after encounter with the antigen but remains alive in a hyporesponsive status [30, 31]. The anergic state has been very controversially characterized in regard to cell division, cell differentiation and cytokine production, since anergic cells could be present for long time and may not require the persistence of antigen [32], which can be explained by the fact that clonal anergy seems to be generated from incomplete T cell activation [30].

To explain the process by which anergy is induced, it has been proposed the two-signal model. The model establishes that T cells are completely activated if they recognize the antigen (signal 1) in conjunction with costimulatory molecules (signal 2). In the absence of costimulation, T cells become anergic [33]. In this sense, the outcome of the immune response is determined at some extent by the type and the activation state of APCs [28, 34].

1.1.1.3 Active suppression

Active suppression is normally developed in a tolerance state that is characterized by the immune deviation of the response mediated by the secretion of certain types of cytokines where regulatory T cells (Tregs) play a key role [35, 36]. This mechanism can also be correlated with the expansion of a particular subset of cells as well with the expression of surface markers [37-39].

There are number of studies focussed on the mechanisms underlying the induction of active tolerance. One of these approaches attempt to follow the course of the immune response on activated T cells when deprived of certain cytokines [40, 41]. In addition, active suppression

has been demonstrated to be induced after oral administration of low doses of antigens, as antigens presented by gut associated antigen presenting cells preferentially induce regulatory T cells that in turn, modulate the effector response [42-44].

In active suppression, the shift of the immune response towards the secretion of suppressive cytokines such as TGF-β and IL-10 has been widely described [45, 46]. At the same time, bystander suppression mechanisms may take place, affecting cells with antigen-specificities different from the antigen responsible for the induced tolerance [47]. Likewise, a critical role of APC in immune regulation of unrelated-antigen specific T effector cells has been established. A recent publication describes two possible models for the role of dendritic cells in bystander suppression. First, they mediate interactions between T cells by presenting MHC-peptide ligands to T regulatory cells, stimulating them to produce anti-inflammatory cytokines that would affect unrelated-antigen specific T effector cells. Second, dendritic cells could serve as a bridge by transmitting signals from regulatory T cells to effector T cells [48]. In addition, it has also been described that dendritic cells use the thrombospondin pathway to control TGF-β for bystander suppression [49]. Furthermore, plasmacytoid dendritic cells could produce IDO that limit T effector cells survival and promotes the TGF-β expression by $CD8^+$ T regulatory cells [50].

1.2 Regulatory/suppressor T cells

The main function of regulatory T cells is to release factors to down-regulate or suppress the immune response helping to maintain homeostatic conditions [38, 51]. It is not completely understood how a T cell is selected to acquire a regulatory cell phenotype. The origin of regulatory T cells may be explained by the selection of high-affinity self-reactive cells, most likely mediated by IL-2 [52, 53]. In this way, it has been shown that Treg cells are relatively more resistant to clonal deletion in the thymus in comparison with effector T cells. These Treg cells are possibly committed to suppress low affinity antigen-specific effectors, thus playing an important function in the control of autoimmune diseases [54]. Recent reports also show that the presence of antagonist peptides do not succeed to promote positive selection, while the presence of an agonist peptide in the thymus can result in differentiation of $CD4^+CD25^+$ regulatory T cells [55].

The repertoire of suppressor T cells can also be modulated in the periphery by the generation of Tregs from naive T cells and by selection of specific Treg cells after contact with the antigen [56]. Some cytokines and costimulatory molecules like IL-2, TGFβ and CD28 have significant roles in the maintenance and generation of Treg cells in the periphery [57].

1.2.1 $CD25^+CD4^+$ regulatory T cells

One of the features of $CD25^+CD4^+$ regulatory T cells is their high expression of CD25; these cells keep the immune system from excessive inflammation and/or autoimmunity [58]. Under optimal conditions, there must a relationship between memory and regulatory cells, however, it is not clear whether both subsets operate independently or they regulate each other [59].

The expression of Foxp3 has been demonstrated to be a crucial factor in the regulatory function of Tregs. The immune dysregulation in Foxp3 knockout mice develops a range of autoimmune disorders [60, 61] and the defective function of Foxp3 in humans is correlated with X-linked syndromes (IPEX) [62] and autoimmune destruction in humans [63, 64].

In mouse models, depletion of this subset of cells leads to abnormal proliferation of lymphocytes and induce destabilization of the immune response. Therefore, adoptive transfer and *in vivo* cell depletion approaches have been useful to study the function and relevance of CD25 in the regulation of immune responses [65, 66] such as in infection, tumors, transplants and graft versus host disease [67]. *In vitro* methodologies reveal that typically $CD4^+CD25^+$ regulatory cells are anergic upon TCR-mediated stimulation in vitro, failing to undergo proliferation and suppressing the activation and proliferation of other $CD4^+$ and $CD8^+$ T cells [68, 69]. Some molecular mechanisms for the function of suppressive $CD4^+CD25^+$ T cells point to a key role of CTLA-4 molecule, which is expressed and critical for the activation of suppression [70]. A variety of markers such as CD45RB, GITR, TLR4 and CD103 [71] as well as the differential secretion of anti-inflammatory cytokines IL-10 and TGF-β have been used to define $CD4^+CD25^+$ regulatory cells [72, 73].

1.2.2 Tr1 and Th3 regulatory cells

Tr1 and Th3 cells are populations of regulatory T cells that produce high amounts of IL-10 and/or TGF-β and whose suppressive activity is mainly mediated by these immunosuppressive cytokines [46, 74]. While Tr1 cells have been characterized by the particular production of IL-10 [75]. Th3 $CD4^+$ regulatory cells mainly secrete TGF-β. Th3 cells were primarily identified during the process of investigating mechanisms associated with oral tolerance [76] and these regulatory cells are related with the induction of protective mucosal immunity [77] and with the induction of Foxp3 [78].

1.2.3 Regulatory B cells

For long time MS has been considered an autoimmune disease mediated principally by T cells, but recently the functional role of B cells in the pathogenesis of the disease has gained more attention [79]. B cells associated with MS severity produce pathogenic antibodies

against myelin and neural antigens, which help to the recruitment of cells that express Fc receptors and at the same time, activate complement signalling factors [80, 81]. It has been demonstrated the presence of proliferating B cells in lymphoid follicles in the meninges of patients with MS [82].

However, a number of studies have demonstrated that B cells are also important in the suppression of autoimmune responses. One of the early observations concerning the anti-inflammatory role for B cells was achieved by Flood, et al., who showed that B cell depletion might interfere with the function of suppressor T cells [83]. Afterwards, characterization of regulatory B cells showed a particular phenotype of IL-10 producing $CD1^{high}CD5^{+}$ B cell subset that suppresses progression of intestinal inflammation [84]. Moreover, transfer of B cells that produce IL-10 is correlated with modulation and prevention of arthritis [85]. An important study by Fillatreau et al. shows that B cells that produce Il-10 are correlated with recovery of EAE. These B cells produce antigen-specific IL-10, and remarkably, are able to transfer suppression to EAE induced in bone marrow chimeric mice, in which IL-10 deficiency was restricted to B cells [86]. More recently, a subset of B suppressor cells was characterized as transitional 2-marginal zone precursor B cells and this phenotype was associated with remission of arthritis and IL-10 secretion [87].

Despite the correlation of B cells with MS pathogenesis, the regulatory function of B cells is also important. MS patients present a decreased production of IL-10 by B cells in comparison with healthy individuals [88] and depletion of these cells is associated with worsening of autoimmune symptoms [89].

Consequently, therapies to treat autoimmune diseases might exploit the fact that B cells exhibit both pathologic and regulatory roles.

1.3 Cytokine-mediated suppression

During the activation process, upon the engagement of the TCR, the fate of T cells depends largely on the presence of cytokines. Particular sensitivity to some cytokines may determine the development of T effectors or T regulatory cells as well as the induction of cell death [90]. IL-10 and TGFβ are pleotropic cytokines known for their inhibitory activity on multiple immune functions and have suppressive properties in both Th1 and Th2 cells [72, 91, 92]. IL-10 and TGF-β also exert anti-inflammatory effects on macrophages and dendritic cells by suppressing the production of pro-inflammatory cytokines like IFN-γ, TNF-α, IL-1 and IL-6 [93, 94]. IL-10 and TGF-β can also suppress the ability of antigen presenting cells to initiate adaptive immune responses through inhibition of cell surface expression of stimulatory molecules like MHC class II, CD40, and B7 [95]. In tolerance studies, the shift of different

pattern of cytokines helps to understand the course of the immune response and is somehow determiner for the evaluation of therapeutic agents aimed to the control of autoimmune diseases [35].

1.3.1 Suppression by IL-10

IL-10 is an important anti-inflammatoty mediator required for the proper function of regulatory T cell [96-98], modulates the production of pro-inflammatory cytokines in APCs [93] and is crucial in the control of many autoimmune diseases as it has been demonstrated with the neutralization of cytokines [99, 100].

The down-regulatory and immunomodulatory effects of IL-10 are somehow controversial because of its pleiotropic effect since the inflammatory response might be exacerbated depending of the dose and of the immune system status [101].

One of the clearest proposed mechanisms for the function of IL-10 is the inhibition of the anti-CD28-stimulated proliferation in T cells [102, 103]. IL-10 seems to inhibit proliferation of T cells in which low numbers of TCRs have been triggered and in addition need CD28 costimulation. These results by Akdis, et al. demonstrate a relationship between IL-10R and CD28 in which CD28 stimulation induce IL-10R expression, consequently these cells are more susceptible to IL-10- mediated suppression.

One of the early events after binding of IL-10 to its receptor is the activation of the signaling via the Janus kinase-STAT. Experimental data reveal its essential role, since the inactivation of STAT3 in myeloid cells results in abrogated IL-10 responses correlated with the development of autoimmune disorders [104].

1.3.2 Suppression by TGF-β.

The transforming growth factor b (TGF-β) mediates a variety of cellular processes including development and function of the immune system. TGF-β was firstly identified as a growth factor and subsequently its importance in immunoregulatory processes, including inhibition of B and T cells, has been widely demonstrated [105, 106]. Many studies suggest that TGF-β may be the key cytokine that mediates active suppression [78], with a broad range of effects both in *in vitro* systems and *in vivo*. Modulation of cell migration, inflammatory cytokines and the extracellular matrix are some of the central functions of TGF-β [107].

This suppressive cytokine is secreted as a latent molecule, which is converted into an active form to become functional, able to interact with the high-affinity TGF-β receptors [49, 108].

The latent complex can be associated with additional binding proteins like LAP. The LAP present in the small latent complex is usually the target of different latent TGF-β activators

such as proteases, integrins and thrombospondin-1 (TSP-1) [109-111]. Recent studies focussed on the complex mechanism of TGF-β activation involve the accurate detection of this cytokine by tracking the associated proteins [106, 112].

Since active suppression when developed in a tolerance state is characterized by immune deviation, the analysis of a defined profile of cytokines and approaches such as the *in vivo* neutralization of cytokines, *in vivo* depletion of $CD25^+$ cells and adoptive transfer experiments represent useful tools to identify the immunoregulatory activities during tolerance induction. Some of these methodologies have been applied in the present study to determine the mechanisms involved in the suppression induced by repetitive sequences in the EAE model for multiple sclerosis.

1.3.3 EAE as a model for human multiple sclerosis

Multiple sclerosis (MS) is an inflammatory neurodegenerative disease that is primarily mediated by T cells with self-reactivity directed against myelin-derived antigens, producing damage of the central nervous system (CNS) [113]. It is unclear what triggers the process in which potentially autoreactive $CD4^+$ T cells are activated in the periphery [114]. Genetic factors or viral infections have been mainly correlated with susceptibility to develop autoimmune reactions. Once autoreactive T cells are activated, they are able to transmigrate through the blood brain barrier via adhesion molecules such as LFA-1 and VLA-4 [115, 116]. Subsequently, proinflamatory cytokines and chemokines activate resident cells such as microglia and astrocytes that induce the recruitment of other immune cells from the peripheral blood and induce the formation of the inflammatory lesion [117]. The result of inflammation induce oligodendrocytes to undergo apoptosis leading to substantial axonal damage that concludes in neurological disability [118].

Diagnostic tests used to determine a clinical diagnosis of MS include brain and spinal cord magnetic resonance imaging (MRI), sensorial analysis of evoked potentials and analysis of cerebrospinal fluid used to detect increased amounts of immunoglobulin or oligoclonal bands [119, 120].

Some immunoregulators have been approved for clinical use: several recombinant beta interferons, natalizumab and the polypeptide Copoloymer 1 [121-123].

The first pharmacon with proved efficacy for the treatment of patients with the RRMS or RPMS form of MS was interferon-β1b (IFN-β1b) [122, 124]. IFN-β has immunomodulatory activities that include the upregulation of adhesion molecules, induction of IL-10 and

neurotrophic factors, blocking of BBB opening via inhibition of MMP-2 and MMP-9 [124, 125].

More recently Natalizumab, an antibody that binds to the integrin VLA-4 preventing migration of immune cells into the brain, has shown to be an effective agent for the treatment of MS relapses [123].

Glatiramer-acetate (GA), known also as Copolymer-1 (Cop-1) or Copaxone, is another approved therapy for RRMS [126]. GA is a random copolymer composed of the aminoacids L-alanine, L-lysine, L-glutamic acid and L-tyrosine [127] and is cross-reactive with MBP [128, 129]. Its suppressive effect has been explained in terms of this cross-reactivity. The mechanism of activity of GA seems to be based on the binding of GA to a range of MHC class II molecules. Other effects after GA treatment include polyclonal T cell stimulation, Th2 activation, [130], immunomodulatory effect on antigen presenting cells and upregulation of suppressive cytokines like IL-10 and TGF-β.

A typical animal model of experimental autoimmune encephalomyelitis (EAE) has been very useful for the study of MS, given that in both diseases autoimmune cells home to the CNS to attack myelin components. The use of the EAE model has made possible the identification of drugs currently used to treat MS and has helped to define some of the mechanisms controlling the development of this disease [131]. This experimental model has provided important information concerning the immune cell types involved in the pathogenesis of the demyelinating disease.

In the same way, as in MS, in the EAE model the disease is characterized by progressive ascending paralysis resulting from CNS mononuclear cell infiltration [132, 133]. EAE can be induced in genetically susceptible animals by active immunization with myelin antigens or by adoptive transfer of myelin-reactive $CD4^+$ T cells and there is evidence for the involvement of DCs in initiating EAE [134]. The most relevant myelin antigens in the pathogenesis of MS and EAE are the Myelin Basic Protein (MBP), the Myelin Oligodendrocyte Glycoprotein (MOG) and the Proteolipid Protein (PLP).

MBP is the second most abundant myelin protein and was the first used in EAE. It is present at the intracellular surface of myelin membranes and is involved in maintaining the structure of myelin. MBP is found in high concentrations in both central and peripheral myelin and its transcripts have also been demonstrated in peripheral lymphoid organs [135]. EAE can be induced with MBP in several mouse and rat strains, guinea pigs and nonhumans primates [136, 137].

MOG is a transmembrane glycoprotein much less abundant than the major myelin proteins and located on the outer surface of the olygodendrocyte membrane. Therefore, MOG is directly accessible to antibodies and significantly induce a strong cellular and humoral immune response in MS [138]. Its immunodominant epitopes are located predominantly in the Ig-like extracellular domain. MOG-induced EAE is well characterized in C57/BL6 mice, in which MOG(35-55) peptide induces a chronic monophasic nonrelapsing EAE [139].

PLP is the most abundant CNS myelin protein highly hydrophobic and evolutionarily conserved across species. PLP is primarily expressed in brain and spinal cord prior to myelination and in peripheral lymphoid organs. It has been shown that PLP is a stronger encephalitogen than MBP and the PLP(139-151) immunodominant epitope is commonly used for EAE induction in SJL/J mice [140].

Recently, it has been proposed that at least two subpopulations with encephalitogenic properties are differentiated once the antigen triggers naive $CD4^+$ T cells. These subpopulations have been characterized into Th1 and Th17. Th1 cells produce principally IL-2, IL-6, TNFα, and IFNγ, important in the activation of macrophages, and induce DTH responses in eae [141, 142]. On the other hand, Th17 produce IL-17, which has been identified to be highly pro-inflammatory and is correlated with the induction of severe autoimmunity [143, 144]. This cytokine has an important role in the pathogenesis of EAE as recent studies indicate that it is the major mediator of tissue inflammation [144]. However, the diversity of experimental models has shown that the pathogenic role of IL-17 found in EAE depends, at some extent, on the mouse strain and the antigen used for the disease induction [145]. Likewise, the correlation of IL-17 with severity of MS is still controversial [146].

After activation, encephalitogenic cells are able to traverse all tissue compartments, to get into the CNS and to interact with the cerebrovascular endothelium via VLA-4 and VCAM-1 [115]. Chemokines have a crucial role in these events; they induce and activate leukocyte adhesion molecules and mediate the recruitment of leukocytes in the CNS [147].

During the EAE progression, the inflammatory process leads to a substantial recruitment of macrophages and antigen-specific and non-specific T cells to the CNS. Eventually the demyelination process and axonal damage induce paralysis [148].

The resolution of EAE involves the participation of regulatory T cells that suppress effector cells through the production of IL-10 and TGF-β principally. It has been shown that recovery from EAE in mice and rats is associated with accumulation of Th2 and Th3 cells in the CNS [149].

1.3.3.1 Prevention and treatment of EAE

Given that there is strong association between certain autoimmune diseases and defined alleles of the MHC complex in humans, therapies of EAE have been mainly focused to the function of these molecules [150]. Many studies have proposed that blockade of the binding site of MHC or the TCR by high affinity antagonist peptides represent a method for immunotherapeutic intervention. [151]. As a result, it was suggested that a kind of competition could take place between the response to an antigen *in vivo* with another competing peptide [152]. It has been shown that analogues of encephalitogenic peptides are able to protect animals from the induction of EAE [153].

Basically, analogue peptides work as TCR antagonists by blocking the activity of encepahlitogenic Th1 cells in vitro. Moreover, inhibition of EAE can result after coimmunization with a mixture of self peptides and TCR antagonist peptides. Different strategies have been studied with the aim of controlling the EAE development: repetitive antigen stimulation, injection of high or very low doses of the antigen, treatment and vaccination with repetitive peptides, modulation of costimulatory molecules such as B7/CD28, CD40/CD154 and treatment with monoclonal antibodies against the TCR among others [45, 154-159].

1.3.4 Role of the multivalent antigens in the control of autoimmune diseases

Tolerant effects based on the persistence of antigen are achieved after injection of high-dose antigen or repeated injections of low dose soluble antigens. T cell deletion, anergy mechanisms and a change in the balance of transcription factors have been characterized to be important for the maintenance of *in vivo* tolerance induced by these means [154, 160].

Studies have also shown that Copolymer 1, Glatiramer Acetate (GA), a molecule containing repetitive sequences, is effective for MS treatment [161]. Experimental data demonstrate that copolymers bind promiscuously to MHC molecules, co-localizing on the surface of antigen presenting cells [162]. In the treatment of EAE, it has been observed how copolymers displace myelin antigens for its binding to MHC molecules, limiting the proliferation of autoreactive cells. However, the major role in the mechanism of activity of GA is the immunomodulatory effect, in which an induction of specific T suppressor cells can cross the blood brain barrier, accumulate in the brain and induce bystander suppression [163]. These T suppressor cells are characterized as Th2/Th3 type cells secreting anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β in response to myelin proteins [164]. In addition, adoptive transfer of copolymer-specific Th2 cell lines that secrete IL-4 and IL-10 induces resistance to EAE [165, 166].

Another class of repetitive tolerogenic antigens are epitope oligomers. Epitope oligomers are polypeptide constructs containing multiple copies of defined T cell epitopes. They have proved to be effective in inducing strong T cell proliferation. Linear epitope oligomers show increased antigenicity because of their ability to crosslink efficiently MHC class II molecules to trigger signalling through the TCR [167]. Likewise, these oligomers induce activation in antigen presenting cells, enhancing the expression of MHC class II molecules and intercellular adhesion molecules such as ICAM-1 [168].

Similar to the high dose suppression, repetitive sequences used at low concentrations also produce tolerizing effects; this is reflected in the hyporesponsiveness induced in T cell lines due to a downregulation of the TCR and to the induction of apoptosis [169].

Vaccination trials in experimental autoimmune neuritis (EAN) using a polypeptide oligomer (16-mer) of the neuritogenic epitope of myelin P2 protein have shown to prevent EAN induction and to have a long lasting effect, even after re-induction of EAN [156]

When oligomerized peptides are administrated *in vivo* under certain conditions, such as concomitant injection with the corresponding monomer, they are able to induce a tolerogenic effect. It was successfully shown how the vaccination and treatment with multivalent antigens control the development or severity of autoimmune diseases. A single injection of oligomers, consisting of 16 repeats of an encephalitogenic T cell epitope from myelin antigens, controlled the development of EAE in mice [155]. In addition, immunohistochemical analysis of the CNS after the treatment with multimerized peptides showed minor infiltration and no demyelination. As well, a similar approach, using multimerized self epitopes in the type I diabetes model induced protection and demonstrated that autoreactive T cells do not respond after *in vitro* stimulation with the oligomerized peptide [170]. It was also observed that the induction of cells with regulatory properties contributes to the tolerant phenotype provoked after immunotherapy with multimerized self-peptides. This report suggests that the suppression achieved is due to induction of active tolerance, which constitutes an alternative approach for the use of oligomers in the treatment of autoimmune diseases.

1.4 Natural repetitive structures within parasite proteins

Tandem repeated domains occur within protein antigens of many parasitic taxa, including *Plasmodium*, *Leishmania*, *Trypanosoma* and *Toxoplasma* [171]. Down regulation of proliferative immune responses is a feature of different parasitic infections and most likely related with mechanisms of parasite survival. In parasites, and predominantly in *Plasmodium*, repeats are common among several proteins and are present in different developmental stages.

Some allelic variants of a protein often have unique repeats and more than one variety of repeat can be present within some proteins [172].

1.4.1 Repetitive domains within the protein antigens of *Plasmodium*

Different repetitive antigens are expressed during the multiple phases of the *Plasmodium* parasite's life cycle and are target by stage-specific immunity [172] [173]. Repetitive domains are present in proteins including soluble antigens released during schizogony (S-Antigens), proteins associated with the infected red blood cells (RESA), rhoptry organelle proteins and surface antigens of sporozoites (CS protein), the antigen containing repeats of acidic and basic residues (ABRA) and merozoites (MSP) proteins [174] [175] [176]. Unlike single or non-repetitive epitopes, tandemly repeated structures crosslink surface immunoglobulin on B cells with a thymus independent activation signal [177, 178]. This feature seems to be crucial in the general mechanism of evasion used by the parasite since there is evidence that antibodies generated to repetitive sequences after exposure to parasites are eventually non-protective [172, 173, 179]. Moreover, the fact that T cell independent response is normally weak for B-cell memory induction together with the incomplete activation or cooperation of T cells**,** may be a parasite's evasion mechanism to persist into the host. On the other hand, the repeats are suggested to present a broad arrangement of crossreactive epitopes that ultimately avoid the affinity maturation of the response to immunogenic epitopes and consequently help the parasite to evade immunity [180].

Theories about the function of repeats propose that each repeat unit acts as an individual ligand for host structures such as red blood cells and hepatocytes. The repetitiveness allows a multimeric high-avidity interaction between parasite and receptor. Another property of repetitive sequences is that cross-reactivity can occur among domains of different proteins. Such is the case of CS and CSP-2 protein of *P. falciparum* and *P. Berghei*, which is serologically crossreactive even when these parasites exist in different hosts [172, 181].

The repeats are particularly constrained to certain amino acids that allow the domains to be efficient B-cell epitopes, favouring hydrophilicity and an appropriate conformation.

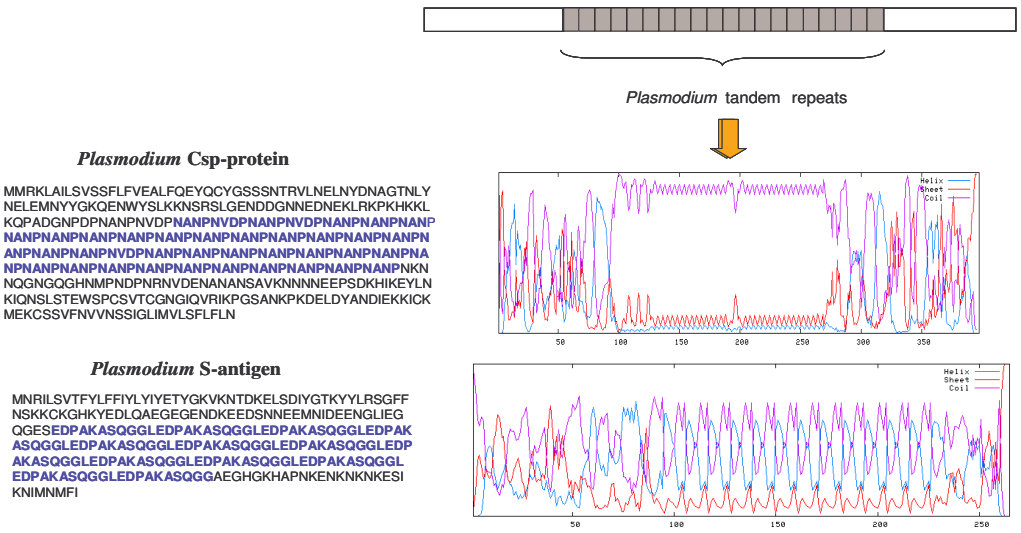


Figure 1. **The general structure of repetitiveness**
Repeat sequences within the Csp protein and S-antigen of *P. falciparum* (isolate NF7). The left side shows the protein sequence, the right side a theoretical plot of the secondary structure. It corresponds to the GOR IV secondary structure prediction method [182].

Analysis of various genomes revealed that tandem repeats of amino acid sequences are particularly frequent among parasites. A striking example of tandem repeats of amino acid sequences is the S-antigen of *Plasmodium.* These repeats cover nearly two thirds of the protein which is secreted in large amounts during the erythrocyte stage and function as a target for the immune system. In *Plasmodium,* different variants of the S-Ag are found as they are determined by the high polymorphism of the repeated regions [174].

1.4.2 Immunosuppressive response during malaria infection

Immunosuppression is a known feature of acute malaria in humans and it is associated with increased susceptibility to concomitant infections and reduced humoral immune responses to vaccination [183]. The ability of *P. falciparum* derived antigens to lower cellular immune responses in mice and humans has been demonstrated both *in vivo* and *in vitro* [184, 185].

In general, the immune response during malaria infections is related with increased frequency of $CD8^+$ and $CD4^+$ T suppressor cells, defective activation of T helper cells and lymphocytotoxic antibody formation among others [186, 187]. Recently, attention has been focused in the role of regulatory T cells, which significantly alter cellular immune responses to various protozoan infections.

In malaria, regulation of Th1 effectors by IL-10 and TGF-β is essential for limiting immunopathology. It has also been shown that depletion of $CD25^+$ cells provokes a Th1 response and delays early parasite replication, facilitating the resolution of the infection [184]. Recent results reveal that in human *P. falciparum* malaria infection there is a production of

systemic TGF-β early on the first detection of blood stage infection. This early secretion of TGF-β activity is strongly associated with suppression of proinflammatory cytokines, parasite growth and induction of regulatory T cells [186]. In this case, TGF-β is produced by monocytes-macrophages, in line with many reports about the importance of antigen presenting cells in the production of modulatory cytokines that control T cell responses during malaria and other parasitic infections [188, 189].

Experimental infection with *Plasmodium yoelli* shows that IDO inhibition, which mediates the catabolism of tryptophan in dendritic cells, suppresses to some extent parasite density and enhances proliferation and IFN-γ production by $CD4^+$ T cells in response to malaria parasites [190].

There are several reports about the relevance of suppressor mechanisms in other parasitic diseases. In *Leishmania* infections, an imbalance in the number of antigen-specific regulatory and effector cells is important for the outcome of the disease, including the reactivation of latent infections. It is clear that naturally occurring regulatory T cells can facilitate persistence of *Leishmania major* in healed lesions. Moreover, the mRNA expression for both IL-10 and Foxp3 is highly upregulated in non-healing lesions and IL-$10^{-/-}$ mice or mice treated with anti-CD25 antibody are able to resolve the infection [191-193].

Likewise in *Trypanosoma cruzi* infections, there exists induction of a strong Th1 type response as well as immunosuppression during the acute phase of the disease [194]. For example, macrophages from *T. cruzi* infected mice produce large amounts of nitric oxide that seems to be the main effector molecule that cause suppression of lymphoproliferative response to parasite antigens. Regulatory cytokines such as IL-10 and TGF-β has also an important role in the suppression observed during the Chagas' disease [195].

One more representative example is the evasion mechanism of both B and Th cell recognition by african trypanosomes, which use the switching of the immunodominant variant surface glycoprotein (VSG), a repetitive protein, to keep away from immune elimination [196].

Correlation with the outcome of autoimmune diseases has been shown during concomitant parasitic infections. This is the case observed after coinfection with *T. cruzi,* which interferes with the EAE development, likely due to a modulatory effect of IL-10 on Th1 expansion and also to induced apoptotic molecules [197]. Similarly, protection from diabetes and inflammatory bowel disease has also been shown during helminth infection. These parasites have the ability to alter the host response towards a Th2 phenotype, characterized by increased IL-4, IL-5 and IL-13 cytokine production [198].

These findings about the ability of parasites to modify the host response, particularly towards a Th2 and regulatory T cell phenotype, are very important and likely constitute the mechanism responsible for the induced protection against autoimmune and diseases.

2 OBJECTIVES

Purpose of the study was the characterization of the tolerogenic capacity of repeat antigens to control the development of EAE.

Primary objectives were:

1. Determination of the therapeutic effect of oligomers for the EAE treatment.
2. Characterization of the principles of suppression underlying the oligomer induced protection of EAE.
3. Identification of the cellular mechanisms involved in the tolerogenic effect induced by oligomer treatment.
4. Determination and characterization of the suppressive capacity of parasite-derived repetitive sequences containing self epitopes using the EAE model.

3 EXPERIMENTAL PROCEDURE

3.1 Materials

3.1.1 Mice

All mouse strains were maintained in the animal facility at the Max Delbrück Center (MDC). Females 10 to 12 weeks old were used throughout the experiments. SJL/J mice were purchased from Taconic Laboratories. C57BL/6 mice were purchased from Charles River Laboratories.

3.1.2 Peptides and T cell oligomers

Proteolipid protein derived PLP139–151 (C140S) ((HSLGKWLGHPDKF) later referred as PLP139-151 and myelin oligodendrocyte glycoprotein MOG35-55 (MEVGWYRSPFSRVV HLYRNGK) peptides (Research Genetics EMC), were synthesized by using standard solid phase F-moc chemistry. All peptides were purified on a C4 -HPLC column (Vydac).

Oligomerized T cell epitopes were produced in *Escherichia coli* bacteria using recombinant techniques as described [168]. In brief, double-stranded oligonucleotide units encoding the T cell epitopes of the PLP139–151(C140S) oligomers were generated by annealing two complementary strands of synthetic oligonucleotides (PLP139–151 (C140S), + strand: 5'-TCACTCTCTGGGTAAATGGCTGGGTCACCCGGATAAATTCGG, and - strand: 5'-GAATTT-ATCCGGGTGACCCAGCCATTTACCCAGAGAGTGACC). They were linked to the nucleotide sequence of the S3 spacer GGPGGGPGGGPGG by cloning the oligonucleotides into theBsrDI site of a modified pCITE vector (Novagen), which contained the DNA encoding the S3 spacer as described [155, 168]. The HA107-119 4-mer consists of four covalently linked linear HA107–119 peptides (SVSSFERFEIFPK) each linked by the S3 spacer. All oligomers were produced in *E coli* using recombinant techniques as previously described [168]. Endotoxin was removed from the polypeptide oligomers by separation on a reversed-phase C4-HPLC column (Vydac) and tested for endotoxin with the colorimetric limulus test (Charles River).

3.1.3 Fusion proteins based in repetitive sequences from parasites

The design of the epitope fusion-proteins with repeat sequences of the S-antigen (S-Ag) of *Plasmodium falciparum* was carried out as follows: The repeat unit, consisting of 24 repeats of a 8-mer unit derived from the NF7 isolate (A(L/R)KSDEAE), was linked by S3 spacer to N-terminus of $CD4^+$ T cell epitopes such as MOG38-51 (S-AgMOG 38-51), PLP139-151 (C140S) (S-AgPLP 139-151) and OVA323-339 (S-AgOVA 323-339). Since the natural units

contained either L or R in their repeats, building blocks in which the two amino acids alternate in tandem repeats were used.

The generation and production was carried out by recombinant techniques in *E. coli* as described [168]. The constructs were isolated using a His-tag located at the C-terminal site of the construct and purified by RP-HPLC to remove endotoxins. Testing for endotoxin levels was performed using the limulus test.

3.1.4 Antibodies used for *in vivo* depletion or neutralization

Following antibodies were produced from hybridoma cell lines and purified at the Max Delbrück Center (MDC): 1B1.3a (anti-mIL-10R) [199], JES5-2A5 (anti-mIL-10) [200], 1D1 (anti-mTGFβ) [201], PC61 (anti-mCD25) [202] and rat IgG1 as isotype control. Antibodies were harvested from culture supernatants and after ammonium sulphate precipitation purified over Protein G columns, followed by dialysis against PBS. The protein concentration has been determined by Bradford test and tested for endotoxin with the limulus test. Endotoxin levels were < 1 EU/mg protein.

The list of antibodies used for FACS analysis and for ELISA detection is specified in Table 1 and the reagents (solutions and buffers) and laboratory supplies are described in Table 2.

3.2 Methods

3.2.1 EAE Induction

10- week-old female SJL/J mice were immunized subcutaneously at two sites (base of neck and tail), with 50 µg of PLP $_{139\text{-}151}$ (C140S) peptide in incomplete Freund's adjuvant, containing 400 µg of *Mycobacterium tuberculosis* H37Ra. One day after priming, mice were injected intravenously with 200 ng of *Pertussis* toxin and scored daily for clinical signs of EAE, on a scale from 1 to 5 according to the severity of the disease as follows: 0, no clinical sign; 1, flaccid tail; 2, flaccid tail and impaired righting reflex (defined as the ability to get on their feet after being placed on their back); 3, partial hind limb paralysis; 4, hind and forelimb paralysis; 5. Moribund. It is according to a standard scale to monitor the EAE course [203].

EAE induction in C57BL/6 mice was performed as previously described, but using 50 µg of MOG $_{35\text{-}55}$ peptide in incomplete Freund's adjuvant containing 400 µg of *Mycobacterium tuberculosis*. Next day, mice were injected intravenously with 500 ng of *Pertussis* toxin.

For challenging**,** mice were injected for a second time at different periods after the first immunization. Injections were made subcutaneously, with 50 µg of PLP $_{139\text{-}151}$ (C140S)

peptide in incomplete Freund's adjuvant. Next day, 200 ng of *Pertussis* toxin was administered intravenously.

3.2.2 Vaccination and treatment of EAE

Animals were treated by injection in the tail vein with 50 μg of PLP $_{139\text{-}151}$- 16-mer peptide (oligomer containing 16 repeats of the encephalitogenic epitope) on day 7 after immunization. For vaccination experiments, the injection with the oligomer was performed 7 days before EAE induction.

For treatment and vaccination with repetitive sequences derived from parasites, the same protocol described for oligomers was used. Concentrations of 70 μg of the S-AgMOG $_{38\text{-}51}$ and 50 μg of the S-Ag PLP $_{139\text{-}151}$ were injected at day 7 into C57BL/6 and SJL/J mice respectively.

3.2.3 General protocol for isolation of cells from lymphoid tissues and bone marrow

Spleen and lymph node cells were isolated at different time points after induction of EAE. Cell suspensions were prepared by mincing tissue fragments in warm medium. Large fragments were removed by passing the cell suspension through a nylon mesh. Erythrocytes were lysed with a hypotonic ammonium chloride solution (NH_4Cl 0.83%) for 3 minutes at 37°C. Cells were counted in a Neubauer chamber, based on the trypan blue exclusion, for viability discrimination.

Bone marrow was obtained by flushing the femurs and tibias with RPMI medium containing 2% foetal calf serum. Erythrocytes were lysed with NH_4Cl as described above. The remaining cells were filtered through nylon mesh and then resuspended in MACS buffer for FACS analysis.

3.2.4 Isolation of CNS-infiltrating mononuclear cells

Infiltrating cells in the CNS of animals with EAE were analysed at the peak phase of the disease. Mice were anesthetized (by intraperitoneal injection with Ketamine: 50 mg/kg body weight and Xylazine: 10mg/Kg body weight) and perfused intracardially through the left ventricle with 50 ml of ice-cold PBS until the effluent was free of erythrocytes. Cells in the CNS were isolated and purified by a Percoll density gradient. Briefly, the brain was dissociated and digested with 0.5 mg/ml of Collagenase type VIII at 37° C for 20 min. The suspension was washed and centrifuged for 5 minutes at 1300 rpm. A 60% Percoll solution was overlayed with the 30% Percoll solution containing the brain suspension. The gradients were centrifuged at 1600 rpm for 20 min.; mononuclear infiltrating cells were collected from

the 30% / 60% interface after first removing the myelin layer at the top. Cells were washed 2x in medium and resuspended in RPMI 5% FCS for FACS analysis. Numbers of infiltrating cells were counted and plotted for comparison between protected and diseased mice.

3.2.5 Proliferation assay

Draining lymph node and spleen cells from immunized and/or oligomer treated mice were isolated at different time points (on days 2, 4 and 6 after oligomer treatment) and cultured in complete RPMI-1640 5% FCS at a density of $3x10^5$ cell/well in 96-well plates. The cultures were stimulated without antigenic peptide or with the designated peptide concentration (titrated amounts from 20µg/ml of PLP 139-151 peptide). Cells were pulsed with 1 µCi (1 Ci = 37 GBq) of [^{3}H] thymidine after 72 hours of culture and then, harvested for scintillation counting in a Microplate Counter.

3.2.6 Determination of antibody response

EAE was induced in SJL/J mice by subcutaneous injection of PLP 139-151 peptide in complete adjuvant. Five groups of mice were tested as follows: (1). Control group: EAE induction without treatment; (2). EAE induction with PLP 139-151 16-mer oligomer; (3). EAE induction and treatment with PLP 139-151 16-mer oligomer on day 7 after immunization; (4). Intravenous injection of PLP 139-151 16-mer oligomer, without previous priming and (5). Naive mice were used as a control of the experiment. Peripheral blood obtained by eye bleeding was collected and centrifugated at 10.000 rpm at 4°C, serum was collected and stored at -20°C until the analysis was performed. The immunoglobulin class of the antibodies (IgM, IgG1, IgG2a, IgG2b and IgG3) was determined in serum samples (Table 1). The antibody titer was made from a 150-fold prediluted serum and was then titrated in a 4-fold dilution. Antibody titers were determined on days 4, 7, 9, 11, 15 and 23 after EAE induction.

3.2.7 $PLP_{139-151}$-specific antibody determination by ELISA

Nunc-Immuno 96-well ELISA plates were coated overnight with 5 µg/ml of $PLP_{139-151}$ 16-mer peptide in PBS. The plates were washed and blocked in a PBS/BSA 2% solution before the addition of serum at the indicated dilution in duplicate. The samples were incubated for 2 hours at room temperature and the plates were washed before the addition of the first antibody (isotype-specific rat anti-mouse antibody: IgM, IgG1, IgG2a, IgG2b or IgG3) [204] diluted in PBS/BSA 2%. The plates were incubated for 1 h at room temperature and then the secondary antibody was added (horseradish peroxidase (HRP)-labeled anti-rat, previously absorbed in mouse serum) and incubated for an additional 1 h. Between incubations, plates were washed four times with PBS/Tween-20 0.05%. After the final wash, a peroxidase substrate TMB

(Tetramethylbenzidine/ H_2O_2) was added and the reaction was stopped by the addition of 1M H_3PO_4. OD was measured at 450 nm in an ELISA reader. Wells coated with an irrelevant peptide (HA-16-mer peptide) were used as a negative control.

3.2.8 B cell characterization

Spleen and bone marrow cells were isolated on day 13 after EAE induction. Cells from PLP 139-151 16-mer treated and untreated mice were analyzed phenotypically for expression of B220, IgM, IgD, CD21, CD23 and CD24. Analysis of different subpopulations was performed by FACS.

3.2.9 *In vitro* restimulation and IL-10 determination

Inguinal and axillary lymph nodes and/or spleens were isolated from PLP 139-151 16-mer treated and untreated mice on day 24 after EAE induction. $4x10^5$ cells were cultured at 37°C in complete RPMI-1640 10% FCS. Cells were stimulated with PLP 139-151 peptide at 10 µg/ml; 5 days later, the medium was replaced and irradiated antigen presenting cells were added. At that time, cultures were restimulated with 5 µg/ml of PLP 139-151 16-mer oligomer. Different peptide combinations were tested for stimulation (see Table 3). Cells were seeded in duplicated plates. After a total of 8-10 days in culture, supernatants were collected for determining IL-10 levels. Measurement of the cytokine was performed by ELISA using cytokine-specific capture and detection antibodies (Table 1).

3.2.10 ELISA for Cytokine determination

The OptEIA ™ set for mouse IL-10 quantification was used. Plates were coated overnight at 4°C with the capture antibody (anti-mouse IL-10) in the corresponding coating buffer. After washing, plates were blocked with a PBS/10% FCS solution (assay diluent) at room temperature for 1 hour. After the adding of the detection antibody (biotinylated anti-mouse IL-10), avidin-horseradish peroxidase conjugate was added. Following 1 hour incubation, plates were washed and the substrate solution (TMB and Hydrogen Peroxide) was added; the reaction was stopped with 1M H_3PO_4.

IL-10 levels in culture supernatants were determined by interpolation from the IL-10 standard curve. All solutions are described in the reagent table (Table 2). Reactions were measured at 450 nm absorbance in an ELISA reader.

3.2.11 Cell-ELISA for Cytokine determination

Cell-ELISA method, which combines cultured cells and ELISA for increasing sensitivity of cytokine detection, was used [205]. This method allows the determination of relative low levels of cytokines and it has been particularly used for the IL-10 detection in B cells [86].

$4x10^6$ purified B cells (negatively selected using the CD43 antibody) (Figure 3) were resuspended in complete RPMI-1640 5% FCS and seeded in 96-well plates previously coated with 15 µg/ml of PLP $_{139-151}$ 16-mer peptide. Stimulation trough CD40 ligation was performed by adding 5 µg/ml of purified anti-CD40. Cultures were incubated at 37°C in an atmosphere of 5% CO_2 during 4 days. At this time, cells were transferred to an ELISA plate precoated with anti-IL-10 (2 µg/ml) and incubated for additional 24 hours before the IL-10 ELISA was carried out as described above in the protocol for cytokine determination.

3.2.12 Adoptive transfer experiments

3.2.12.1 Transfer of total lymph node cells

Total lymph node cells from oligomer treated and non treated mice were collected 13 days after EAE induction. $5x10^6$ cells were transferred intravenously in mice in which EAE had been induced 4 days before. Animals were daily scored during at least 30 days after the onset of the disease.

3.2.12.2 Purification of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells and *in vivo* adoptive transfer

15 days after EAE induction, lymph node cells together with spleen cells were collected from oligomer treated and not treated mice. Subpopulations were separated by immunomagnetic cell sorting (Figure 2). Negative and positive selection was used to isolate $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. Purity of cells used in this study was greater than 95% as determined by flow cytometry. $5x10^6$ cells from each population were intravenously transferred in mice, one day before the EAE induction. In some experiments, cell transfer was carried out 4 days after disease induction with the aim to establish possible differences or any effect depending on the time of transfer. The method used for isolation of different subpopulations is depicted in Figure 2.

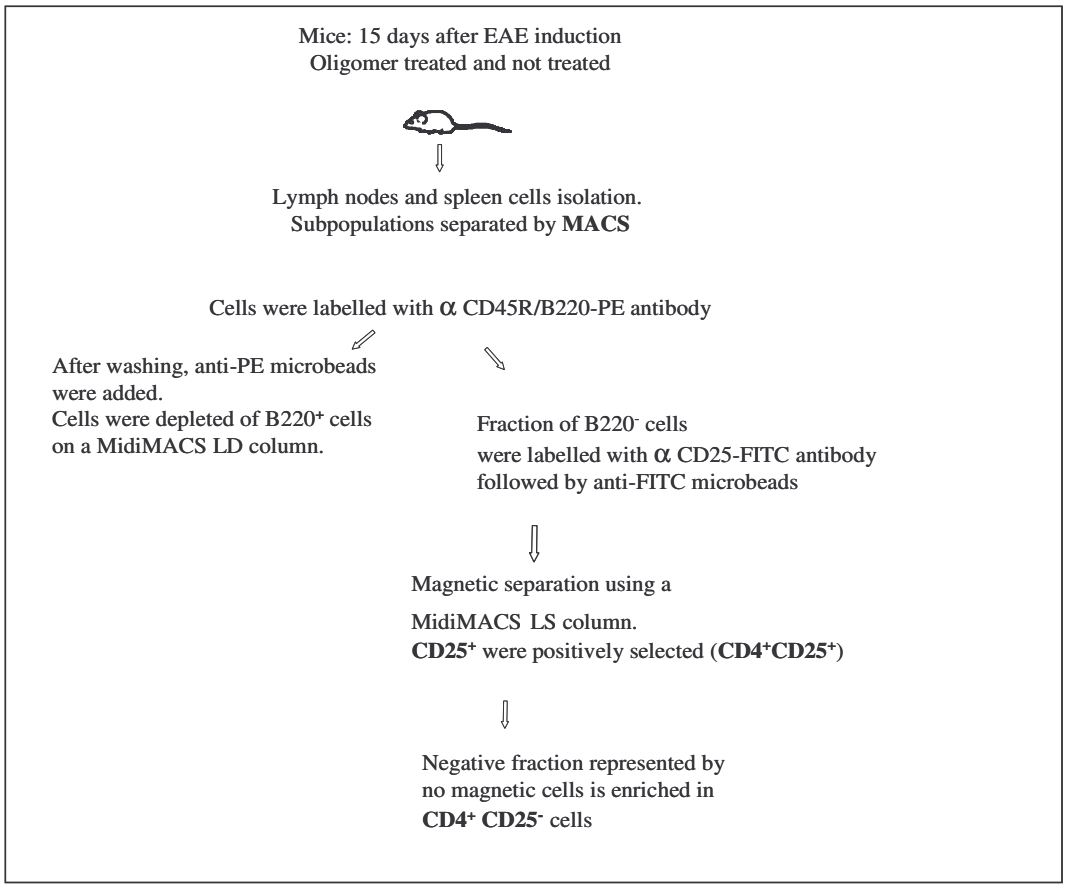


Figure 2. Purification of $CD4^+CD25^+$ and $CD4^+CD25^-$
Cells from lymph nodes and spleen cells were isolated on day 15 after disease induction. Subpopulation of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were separated by Magnetic cell sorting (MACS). Cells were washed and resuspended in 100µl of MACS buffer/$1x10^7$ cells. Corresponding antibodies were added at a final dilution of 1:100 and incubated for 20 min at 4°C. After washing, cells were resuspended in 100µl of MACS buffer and 10µl of microbeads were added per $1x10^7$ total cells, gently mixed and incubated for 15 min at 4°C. Cells were washed and then centrifuged at 1000 rpm for 10 min. Magnetic separation was performed; the magnetically labelled cells are separated over a MACS Column placed in a MACS Separator. They are retained on the LS column, while unlabeled cells pass through. These cells can be collected as the unlabeled fraction ($CD4^+CD25^-$). The retained cells are eluted from the MACS column after removal from the magnet ($CD4^+CD25^+$).

3.2.12.3 B cell isolation and adoptive transfer

A negative selection procedure was chosen for purification of splenic B cells (Figure 3). By using CD43 microbeads, all CD43-expressing cells were magnetically labelled. Since CD43 is not expressed on conventional peripheral B cells [206], this subset stays untouched and it was collected in the column effluent. The enriched cell fraction was tested for the expression of the B220 marker. B cell isolation was performed at different times after EAE induction (at days 18 and 23). $4x10^6$ purified B cells from oligomer treated and untreated mice were transferred intravenously in recipients one day prior to the disease induction.

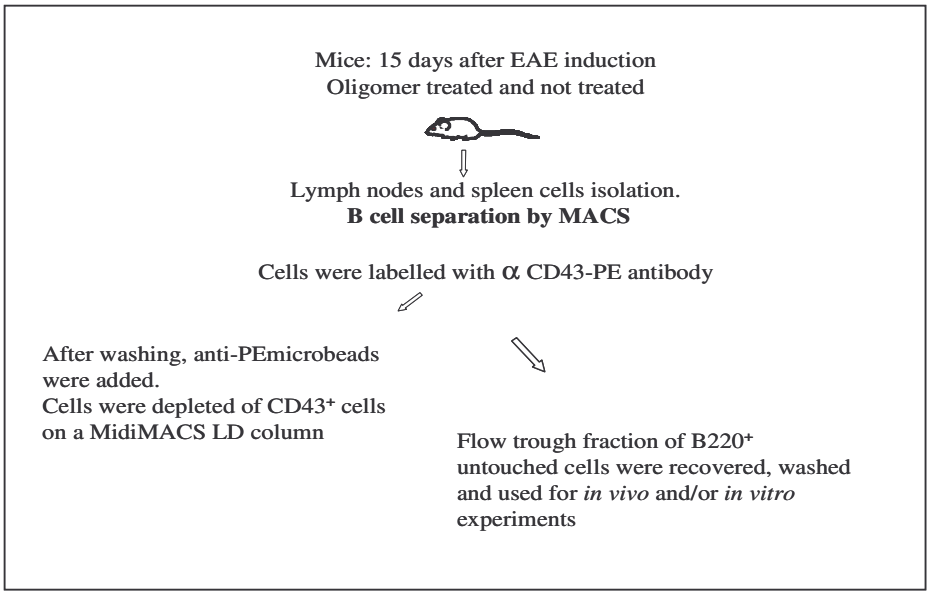


Figure 3. Purification of B cells
Cells from lymph nodes and spleen cells were isolated on day 15 after disease induction. Cells were washed and resuspended in 100µl of MACS buffer/1x10^7 cells. 20 µl of anti-CD43PE was added per 1x10^7 total cells and incubated for 20 min at 4°C. After washing, cells were resuspended in 100µl of MACS buffer and 10µl of anti-PE microbeads was added per 1x10^7 total cells; this was gently mixed and incubated for 15 min at 4°C. Cells were washed and then centrifuged at 1000 rpm for 10 min. Magnetic separation was performed; untouched B cells are collected as column effluent using a MidiMACS LD column.

3.2.13 CD25 *in vivo* depletion

$CD25^+$ cell depletion was performed with 200 µg of anti-CD25 PC61 mAb injected intraperitoneally 15 days before EAE induction. Depletion was evaluated by using anti-CD25 7 D4 mAb. The effect of depletion of $CD25^+$ cells was tested in oligomer-vaccinated and not vaccinated mice. Vaccination was carried out 7 days before disease induction. As a control of the experiment, mice received the same concentration of rat IgG1.

3.2.14 *In vivo* neutralization of cytokines

Purified rat anti-mIL-10, anti-mIL-10R, anti-mTGF-β and IgG1 isotype control, all containing less than 1 IU of endotoxin/ml of antibody, were used for *in vivo* neutralization. Specifications are described in the antibody table (Table 1). PLP $_{139-151}$ 16-mer, S-AgMOG $_{38-51}$ or S-AgPLP $_{139-151}$ treated mice were injected intraperitoneally on days 7, 9, 11 and 13 after disease induction with 0.5 mg of the corresponding antibody. The effect on the clinical score after neutralization was monitored daily. The clinical score of five mice per group was analysed.

Table 1. **Antibodies used for FACS staining, ELISA and for *in vivo* Neutralization**

Anti-mouse antibodies

Anti-CD antibodies						
Antibody	**Label**	**Synonym**	**Clone**	**Isotype**	**Staining conc.**	**Manufacturer**
CD4	PerCP-Cy5.5	L3T4	RM4-5	rat IgG2a, k	1 µg/ml	PharMingen
CD8a	PE-Cy7	Ly-2	53-6.7	rat IgG2a, k	0,5 µg/ml	PharMingen
CD11c	APC	Integrin αX chain	N418	hamster IgG	2 µg/ml	Caltag
CD11c	PE	Integrin αX chain	N418	hamster IgG	2 µg/ml	Miltenyi
CD21	FITC		B-ly4	rat IgG2a	2 µg/ml	PharMingen
CD23	PE		B3B4	rat IgG2a	3 µg/ml	PharMingen
CD24	biotin	HSA	30-F1	rat IgG2c, k	0,2µg/ml	PharMingen
CD25	APC	IL-2R α chain p55	PC61 5.3	rat IgG1	0,5 µg/ml	Caltag
CD25	PE-Cy7	IL-2R α chain p55	PC61	rat IgG1, l	0,2 µg/ml	PharMingen
CD40	purified		HM40-3	hamster IgM, k	1 µg/ml	PharMingen
CD40	FITC		HM40-3	hamster IgM, k	1 µg/ml	PharMingen
CD43	PE		L11	rat IgG2a	1 µg/ml	Miltenyi
CD45R/B220	PE		RA3-6B2	rat IgG2a	0,5 µg/ml	PharMingen
CD45RB	FITC		16A	rat IgG2a	0,5 µg/ml	PharMingen
CD49d	Biotin	Integrin α4 chain	R1-2	rat IgG2b, k	0,2 µg/ml	PharMingen
CD54	FITC	ICAM-1	3E2	hamster IgG1, k	1 µg/ml	PharMingen
CD69	PE		H1.2F3	hamster IgG	1 µg/ml	PharMingen
CD80	PE	B7-1	RMMP-1	rat IgG2a	1 µg/ml	Caltag
CD83	PE		Michel17	rat IgG1	1 µg/ml	PharMingen
CD86	FITC	B7-2	GL1	rat IgG2a	1 µg/ml	PharMingen
CD95	FITC	Fas	Jo2	hamster IgG	1 µg/ml	PharMingen
CD103	Biotin	Integrin αIEL chain	M290	rat IgG2a, k	0,2 µg/ml	PharMingen
CD103	FITC	Integrin αIEL chain	M290	rat IgG2a, k	1 µg/ml	PharMingen
CD152	PE	CTLA 4	UC10 4F	hamster IgG1, k	0,2 µg/ml	PharMingen
CD162	PE	PSGL-1	KPL-1	ratIgG2a	0,2 µg/ml	PharMingen
CXCR3	Biotin		1C6	ratIgG2a	0,2 µg/ml	PharMingen
CCR5	Biotin		2D7	rat IgG2a	0,2 µg/ml	PharMingen
GITR	Biotin	TNFRSF18	polyclonal	goat IgG	0,2 µg/ml	R&D
Pan-NKcells	PE		DX5	rat IgG2a	1 µg/ml	PharMingen
Foxp3	PE		FJK-16s	rat IgM, k	1 µg/ml	Biosciences
anti-IgM	FITC		R6-60.2	ratIgG1	1 µg/ml	PharMingen
anti-IgD	FITC		11-26c.2a	ratIgG1	1 µg/ml	PharMingen

Table 1 (Continued)

Antibodies used for FACS staining, ELISA and for *in vivo* Neutralization

Anti-mouse antibodies

Antibody	Label	Synonym	Clone	Isotype	Staining conc.	Manufacturer
Secondary antibodies						
Streptavidin	APC				0,5 µg/ml	Caltag
Streptavidin	FITC				0,5 µg/ml	Caltag
Streptavidin	PE				0,2 µg/ml	Caltag
anti-rat IgG	FITC			goat-IgG	0,5 µg/ml	PharMingen
anti-rat Ig	FITC		polyclonal	rat-Ig	0,5 µg/ml	PharMingen
ELISA Detection						
IL-10	OptEIA	Mouse IL-10 Set				PharMingen
Isotype antibody	purified		A85-1	rat-IgG1	0,5 µg/ml	PharMingen
Isotype antibody	purified		R19-15	rat-IgG2a	0,5 µg/ml	PharMingen
Isotype antibody	purified		RMG2b-1	rat-IgG2b	0,5 µg/ml	PharMingen
Isotype antibody	purified		R40-82	rat-IgG3	0,5 µg/ml	PharMingen
Isotype antibody	purified		R6-60.2	rat-IgM	0,5 µg/ml	PharMingen
Neutralizing Antibodies						
anti-IL-10	purified		JES-A25			MDC*
anti-IL10R	purified		1B1 3.9			MDC*
anti-TGF–β	purified		1D1			MDC*
Isotype Control	purified		Gl 113	rat-IgG1		Sigma
Antibodies for in vitro stimulation						
CD40	purified		HM40-3	hamster IgM, k	1-5 µg/ml	PharMingen

*These antibodies were produced and purified at Max Delbrück Center (MDC).
Antibodies are referenced in material and methods.

Table 2. Chemicals and laboratory supplies

Chemicals and laboratory supplies	Manufacturer
RPMI-1640 medium	Invitrogen (Karlsruhe, D)
FCS (fetal calf serum)	Invitrogen
Penicillin/Streptomycin	Cambrex Bio Science (Verviers, B)
L-glutamine	Invitrogen
Hepes	Invitrogen
Sodium piruvate	Invitrogen
β-Mercaptoethanol	Invitrogen
NEA (non essential aminoacids)	Invitrogen
PBS-D	Invitrogen
Trypan blue	Invitrogen
Cytofix-Perm solution	BD Biosciences
Saponin	Calbiochem
BSA (Bovine serum albumin)	Sigma
EDTA (Ethilendiamin-tetra-acetic acid)	Sigma
Collagenase type VIII	Sigma
Percoll	Amersham (Uppsala, S)
Tween20	Sigma
Ketaminhydrochlorid (Exalgon 1000)	Merial (Hallbergmoos, D)
Xylazinhydrochlorid (Rompun)	Bayer AG (Leverkusen, D)
[^{3}H] thymidine	Amersham, Freiburg, Germany
Limulus test	Charles River
TMB (Tetramethylbenzidine/ H_2O_2)	KPL Gaithersburg, Maryland USA.
Horseradish peroxidase (HRP)	Sigma
Complete Freund's adjuvant	Sigma
Incomplete Freund's adjuvant	Sigma
Pertussis toxin	List Biological Laboratories, Inc UK
Mycobacterium tuberculosis H37Ra	Difco Laboratories (Detroit, USA)
MACS columns (LD, LS)	Miltenyi
Immunomagnetic cell sorting	Miltenyi
Cell strainer	Falcon, BD Bioscience
Nunc-Immuno 96-well ELISA plates (maxisorp)	Nunc

Table 2 (Continued)

Chemicals and laboratory supplies

Culture medium and Buffer's composition	
Complete RPMI-1640 medium	Supplemented with: 100 µg/ml Penicillin, 100U/ml Streptomycin, 2 mM L-glutamine, Hepes, 5µM Sodium pyruvate, 5mM β-Mercaptoethanol, 1mM NEA
MACS buffer	PBS, 0.5mM EDTA, 0.5% BSA
PBS (Phosphate buffered salt solution)	4.3 mM Na2H2PO4, 137mM NaCl, 1.4mM KHPO4, 2.7mM KCl, PH 7.4
Saponin buffer	PBS, 0.5% saponin, 5% FCS, 0.1% NaN3
Lysis solution for erythrocytes	0.87% NH4Cl, 10mM Tris/HCl
Staining buffer	PBS, 2% FCS

Instruments	
96 well plate ELISA washer	TECAN (Männedorf, Switzerland)
ELISA reader -VictoR 3V TM	Perkin Elmer (Germany)
LSR II cytometer	BD Bioscience (San Jose, USA)
FACSCalibur	BD Bioscience (San Jose, USA)
Megafuge 3.0R	Heraeus-Sephatech
HEPA Filtered, IR Incubator	Labor-Technik (Göttingen)
Microscope	OLYMPUS (Japan)
96 well plate Harvester	Tomtec (New Heaven, USA)
β-Scintillation Counter	Wallac (Turku, FIN)

4 RESULTS

In previous studies, it was shown that repeated antigens have been able to suppress a number of autoimmune diseases. This was demonstrated in various experimental models, e.g. in type 1 diabetes, autoimmune neuritis and MS models [155, 156, 170]. In order to establish a system that allows evaluating the function of the repetitive sequences at the MDC, EAE was induced in SJL/J mice to determine the effect of the treatment with the repetitive peptide. The PLP oligomer construct (PLP 139-151 16-mer) used for the EAE treatment consist of linear polypeptide chains containing 16 repeats of the encephalitogenic T cell epitope derived from the proteolipid protein (PLP), separated by a spacer (S3). Analysis of the oligomer purification is shown in Figure 4. The disease is induced by a subcutaneous injection of the PLP 139-151 peptide in incomplete adjuvant (IFA) containing 400µg/ml mycobacteria followed by an intravenous injection of *Pertussis* toxin. At these conditions, the SJL/J mice used in this model develop mild EAE with an incidence of 80% to 100%.

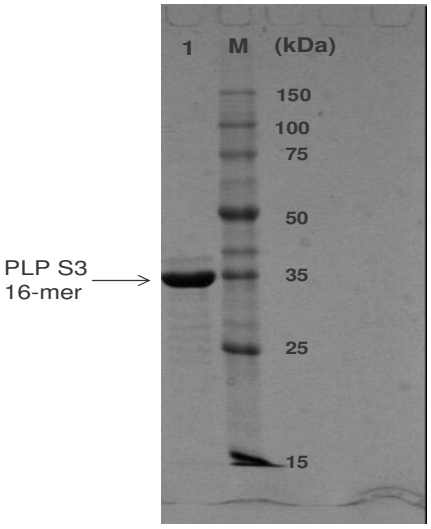


Figure 4. SDS-PAGE analysis of PLP 139-151 16-mer.
Oligomers were produced in *E. coli* using recombinant techniques as described [168]. Purification of PLP 139-151 16-mer: lane 1, a single band with a molecular weight of 39 kDa; lane M, molecular weight markers.

4.1 Protection of EAE induced by PLP 139-151 16-mer

4.1.1 Treatment of EAE by intravenous application of PLP 139-151 16-mer

The effect on the suppression of EAE by the administration of the epitope oligomer was evaluated based on the severity of the clinical signs. EAE development was tested after treatment with PLP 139-151 16-mer. Interestingly, as seen in Figure 5 only one injection of the

T cell epitope-oligomer 7 days after induction inhibits the paralysis associated with EAE almost completely.

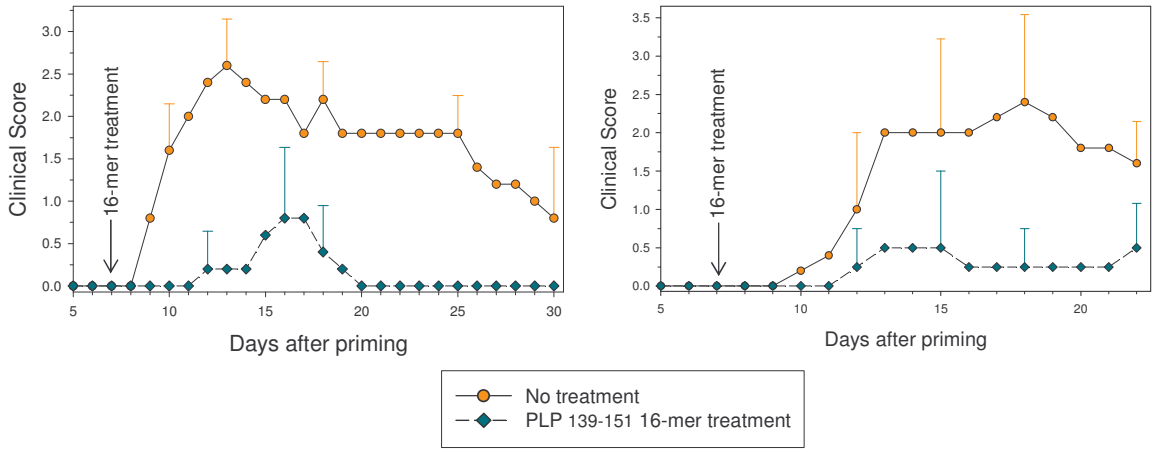


Figure 5. Protection against the EAE development after treatment with PLP 139-151 16-mer.
EAE was induced in SJL mice with the self epitope PLP 139-151. A group of 5 mice was treated intravenously with 50 µg of the PLP 139-151 16-mer on day 7 after disease induction. **(A)** and **(B)** are two representative experiments along this study. The clinical score was performed based on the scale described in methods.

Only few of the treated mice developed very weak symptoms (mean maximal clinical score of 0.8) at the peak of the disease and rapidly they recover on the next days, in contrast to untreated animals, which had a maximal score of 2.2 (Figure 5). Along this study, most of the oligomer-treated mice never developed any symptoms of EAE at any time. This is in line with previous results, which show the effectiveness of the PLP 139-151 16-mer and MBP 86-101 16-mer in the treatment of EAE [155]. Recent data also demonstrate that the protection of experimental autoimmune diabetes was provided by low dosage of the HA 107-119 4-mer peptide [170]. These results suggest that antigen specific oligomer-therapy may be useful in treating human autoimmune diseases.

4.1.2 Intradermal or intravenous PLP 139-151 16-mer treatment renders mice tolerant to EAE

It is possible that each route of administration of the tolerising agent follows a different mechanism to reach protection. These mechanisms can differ by the type of antigen presenting cell encountered and the duration of antigen delivery during the injection. It is known that systemic distribution during intravenous injection of antigens leads to a rapid response, whereas antigen delivery intradermally is mainly taken by langerhans cells, which migrate to lymph nodes presenting the antigen during long periods of time [207].

In order to compare whether the route of injection could influence the effect induced by the repetitive epitope, mice were treated either intravenous or intradermally with the same concentration of the oligomer.

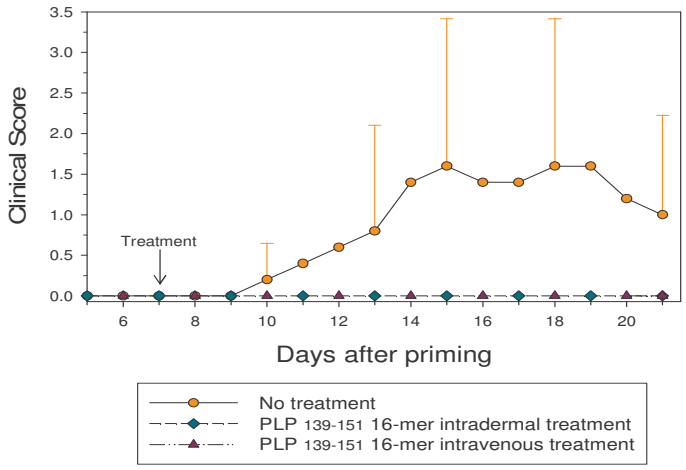


Figure 6. Comparison between intradermal and intravenous treatment with PLP 139-151 16-mer.
EAE was induced in SJL mice using PLP 139-151 peptide. Mice were treated intravenously or intradermally with 50 µg of the PLP-16mer peptide on day 7 after EAE induction. Animals were observed daily and the mean of the clinical score of five mice per group was plotted.

In each case, after intravenous or intradermal injection with the multimerized peptide, mice were completely protected. Results in Figure 6 show that in this experiment none of the treated mice developed any symptoms of EAE, while untreated mice developed mild disease with a maximal mean score of 1.6.

4.1.3 Vaccination with the PLP 139-151 16-mer peptide for EAE prevention

The previous experiments showed that the PLP 16-mer was effective when employed after the induction of disease. In order to determine whether the application of the PLP 16-mer before the disease induction could also prevent the EAE development; vaccination experiments were performed. Figure 7 shows that epitope-oligomer vaccination of either subcutaneous or intravenous injection routs prevents the EAE development. There was a significant decrease in the severity of the disease with a maximal mean clinical score of 0.6 in both vaccinated groups compared with a score of 2.8 in the control group. These results are consistent with previous reports that demonstrate the PLP 139-151 16-mer ability to block EAE when given as a treatment or prior to the induction of the disease [155, 156].

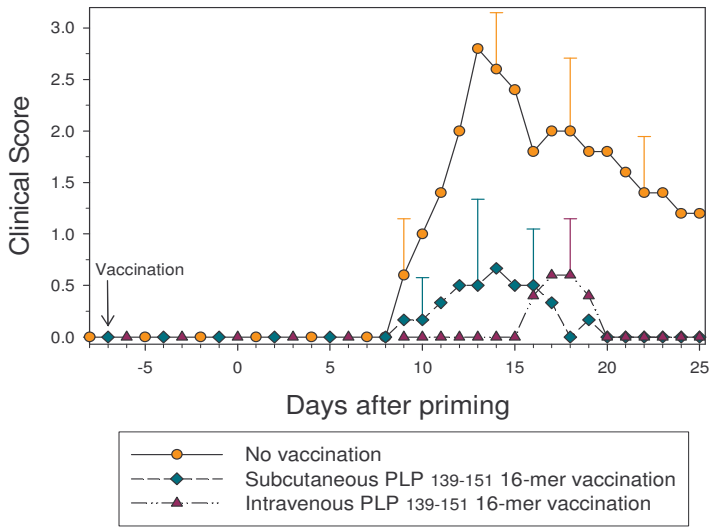


Figure 7. Vaccination with PLP 139-151 16-mer.
Mice were vaccinated subcutaneously or intravenously with 50 µg of PLP 139-151 16-mer. Vaccination was performed 7 days before EAE induction. Subcutaneous injection was given in incomplete adjuvant and for the intravenous injection, the oligomer was dissolved in PBS. Mean clinical score for each group was plotted from day 0 to day 25. One representative experiment of two performed is shown.

Intravenous vaccination in all experiments was in fact slightly more effective, which might be due to the faster delivery to the immune system. In all experiments, intravenous injection route was therefore used in this study.

4.1.4 Treatment with the PLP 139-151 16-mer after the onset of EAE

The oligomer-vaccination seems to be an effective approach to prevent the appearance of autoimmune diseases and therapeutic treatments for EAE represent an important approach for the future treatment of human multiple sclerosis [208]. In order to determine the therapeutic effect of the oligomer in suppression of EAE, it was given very close to the onset of EAE (Figure 8). In the EAE model described in the present study, the normal kinetic of the disease shows that the first clinical signs of EAE are manifested between days 10 and 12. Therefore, a group of mice received a single intravenous injection of the PLP 139-151 16-mer on day 12; at this time, 25% of mice had already developed the first symptoms.

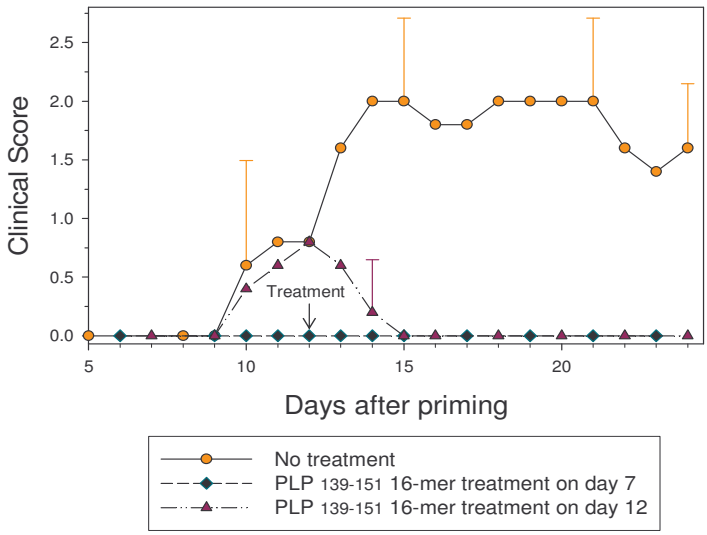
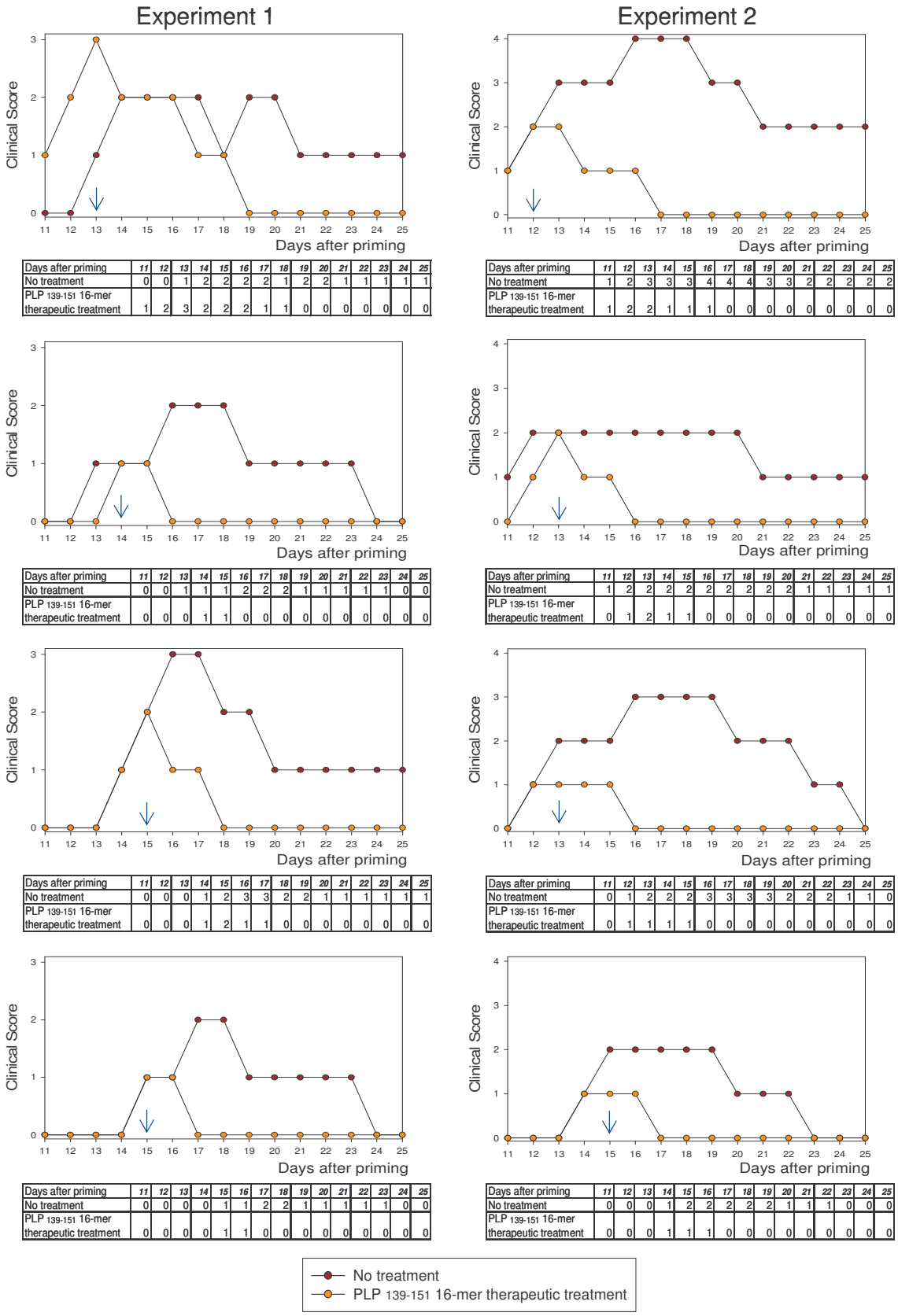


Figure 8. Treatment with PLP 139-151 16-mer close to the onset of EAE.
Mice were given the epitope-oligomer at a time close to the onset of EAE. A single dose with 50 µg of the epitope-oligomer was administrated intravenously on day 12 after disease induction. A group of mice treated on day 7 was included as a control of the experiment. Values are specified as a mean clinical score in groups of five mice each.

The experiment revealed that the administration of the oligomer at a late time point (day 12) prevents the development of EAE (mean maximal score of 0.8, incidence of 40%). Figure 8 illustrates that all mice that do not receive any treatment start to develop EAE symptoms on days 11-14 (maximal mean clinical score of 2, incidence of 100%). Nevertheless, once mice were given the epitope-oligomer, an inhibition of the disease development was observed in 100 % of the mice from this group. Thus, the process to get this suppressive status is very rapid and seems to last for more than 10 days. To determine whether the effect of the epitope oligomer can be used as a real therapy for EAE, mice were given the oligomer not at a fixed time point but instead only after the appearance of the first clinical symptoms.



Experiment 1	Days after priming	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
	No treatment	0	0	1	2	2	2	2	1	2	2	1	1	1	1	1
	PLP 139-151 16-mer therapeutic treatment	1	2	3	2	2	2	1	1	0	0	0	0	0	0	0
	No treatment	0	0	1	1	1	2	2	2	1	1	1	1	1	0	0
	PLP 139-151 16-mer therapeutic treatment	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
	No treatment	0	0	0	1	2	3	3	2	2	1	1	1	1	1	1
	PLP 139-151 16-mer therapeutic treatment	0	0	0	1	2	1	1	0	0	0	0	0	0	0	0
	No treatment	0	0	0	0	1	1	2	2	1	1	1	1	1	0	0
	PLP 139-151 16-mer therapeutic treatment	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0

Experiment 2	Days after priming	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
	No treatment	1	2	3	3	3	4	4	4	3	3	2	2	2	2	2
	PLP 139-151 16-mer therapeutic treatment	1	2	2	1	1	1	0	0	0	0	0	0	0	0	0
	No treatment	1	2	2	2	2	2	2	2	2	2	1	1	1	1	1
	PLP 139-151 16-mer therapeutic treatment	0	1	2	1	1	0	0	0	0	0	0	0	0	0	0
	No treatment	1	2	2	2	2	3	3	3	3	2	2	2	1	1	0
	PLP 139-151 16-mer therapeutic treatment	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0
	No treatment	0	0	0	1	2	2	2	2	2	1	1	1	0	0	0
	PLP 139-151 16-mer therapeutic treatment	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0

Figure 9. **Treatment with the epitope-oligomer after the onset of EAE controls the ongoing disease.**
Mice were treated individually once the first clinical symptoms appeared. For the treatment, 50 µg of the PLP 139-151 16-mer was given intravenously in diseased mice. Score of 4 mice from two independent experiments are shown. The arrow in each figure indicates the day of the treatment.

As seen in Figure 9, even when the treatment was delayed until the first disease symptoms were established, the contact with the epitope-oligomer clearly prevents the progression of the disease. In general, mice were treated between days 12-15, once the score 1-2 was apparent. It is important to note that just few days after the injection with the oligomer, mice started to recover, inhibiting completely the development of EAE.

The therapeutic use of oligomers therefore seems to be a promising approach in the control of autoimmune diseases.

4.1.5 Suppressive effect induced by the epitope-oligomer is antigen specific

To ensure that the tolerogenic effect induced by oligomer-treatment in the EAE model is antigen specific, the effect on the suppression of EAE was tested by the use of oligomers (4-mers) of an epitope derived from the influenza virus hemagglutinin protein (HA 107-119), which has been shown to have a suppressive effect in the RIP-HA model of type 1 diabetes as described recently [170]. HA 4-mer also contains the same spacer sequence linked to the NH2-terminal side (S3 spacer) as the oligomerized encephalitogenic antigen PLP 139-151 16-mer.

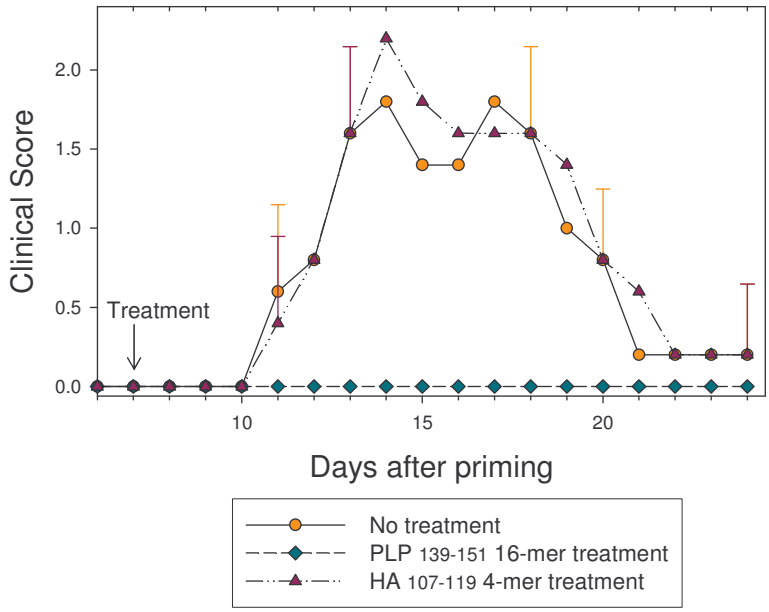


Figure 10. Antigen specific effect of the PLP 139-151 16-mer.
EAE was induced in SJL mice with the T cell encephalitogenic peptide PLP 139-151 in incomplete Freund's adjuvant containing *Mycobacterium tuberculosis*. The oligomer (4-mer) of an epitope derived of the influenza virus hemagglutinin protein HA 107-119 was administrated intravenously in mice on day 7 after disease induction. Normal treatment using the PLP 139-151 16-mer was carried out as a control of the experiment (day 7). Mice were monitored daily and the average of the clinical score of five mice per group was plotted.

The results show that no protection was obtained in mice treated with the HA 107-119 4-mer which had a comparable mean maximal clinical score in relation with the untreated ones: 2.2 and 1.8 respectively (100% of incidence in both cases), while mice treated with PLP 139-151 16-mer were completely protected against the disease development. The tolerogenic function during the oligomer treatment seems to require antigen-specific triggering that, in some extent, could control effector cells when given the cognate antigen in the oligomerized form. The causative role of non-antigen specific repeats, e.g. linker structures, in inducing suppression of EAE was ruled out based on the result in this experiment.

4.2 Cellular characterization of oligomer mediated protection of EAE

As indicated above, the effect of oligomers in the control of EAE as treatment and preventive measure was evident. Even more important, they were equally effective in the context of the therapeutic settings. Therefore, it is of particular interest to find out on which cellular mechanisms the suppression it is based on.

4.2.1 In vitro proliferation in response to the specific antigen

For a long time, self peptides have been used in approaches to treat autoimmune encephalomyelitis [209, 210]. The mechanism to inhibit the disease is very often correlated with the lack of antigen specific T cell proliferation. This phenomenon has been explained either by activation induced cell death (AICD) or by anergy. Although, several reports also demonstrate different mechanisms that question the specific T cell anergy [211].

The absence of the disease observed in oligomer treated mice could lead to think that also in this case, effector cells are modulated somehow through a direct induction of anergy or apoptosis. To determine whether $CD4^+$ T cells from oligomer treated mice were affected by the treatment, their *ex vivo* capacity to respond against the PLP encephalitogenic epitope was tested. For this purpose, spleen cells were isolated at different times after the treatment and were challenged *in vitro* with the corresponding peptide.

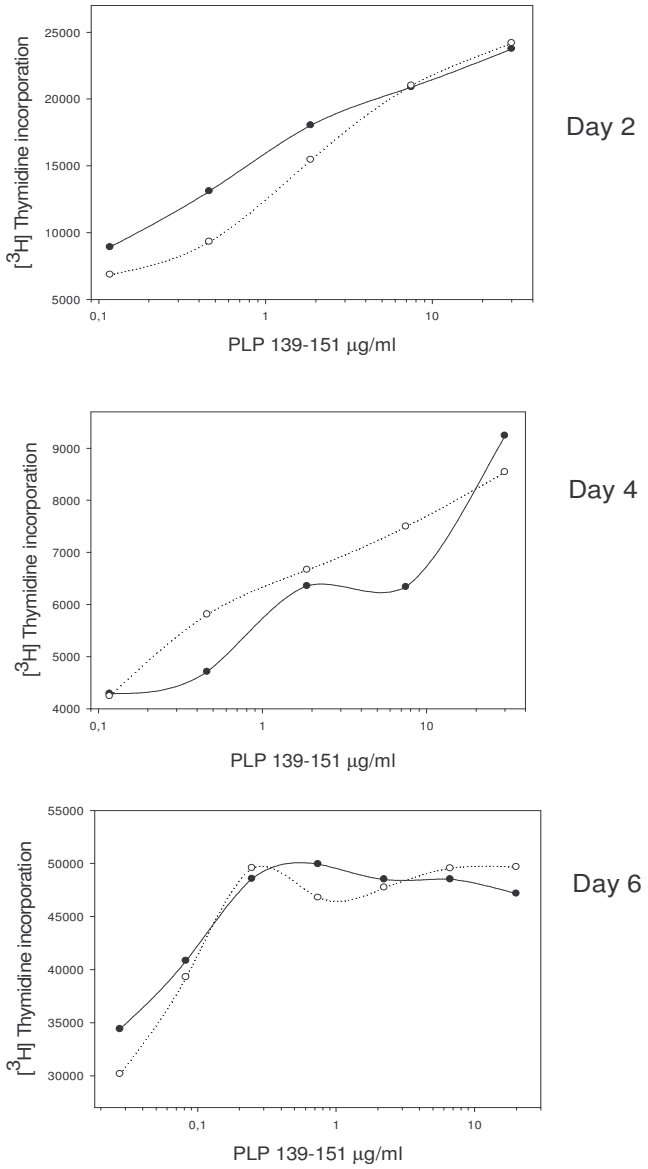


Figure 11. **Comparison on the proliferative capacity of splenocyte cells derived from untreated and oligomer treated mice.**
Mice were treated with PLP 139-151 16-mer on day 7 after EAE induction. Cells were isolated: on days 2, 4, and 6 after treatment with the repetitive epitope. Proliferation experiments were performed in absence or presence of titrated amounts of PLP 139-151 peptide, $3x10^5$ cells were plated during 72 hours and the proliferative response was measured by [^{3}H] thymidine incorporation. Results are expressed as cpm.

Since oligomer effect is evident soon after its injection, the *ex vivo* response to the encephalitogenic epitope was monitored during the first week after treatment. Proliferation assays were performed on days 2, 4 and 6 after treatment with PLP 139-151 16-mer. Surprisingly however, the results showed that cells from both oligomer treated and untreated mice respond equally to stimulation at different dosages of PLP 139-151 peptide (Figure 11). Proliferation data were also similar when lymph node cells were tested (not shown). The results observed in the present study suggest that the suppressive effect induced during the

oligomer-treatment is not based on tolerance mechanisms that involve anergy or apoptosis. Even when treated mice do not show clinical symptoms of the disease, the isolated spleen cells preserve the capacity to respond against the self antigen. This suggests the existence of other indirect mechanisms that somehow silence the autoreactive cells.

4.2.2 Phenotypic characterization of lymphocytes from treated mice

Measurement of expression of cell surface markers has been useful to study immune responses at the cellular level and to identify mechanisms of immune system modulation. Several global markers were therefore analysed with the purpose of establishing phenotypic differences between PLP $_{139\text{-}151}$ 16-mer treated and untreated animals.

Markers of activation and costimulation (CD25, CD69, CD80, CD83, CD40, ICAM1, MHC-II), markers associated with regulatory functions (CTLA-4, GITR, Foxp3, CD45RB, CD103) and migration markers (VLA-4 (CD49d), PSGL-1 (CD162), CXCR3, CCR5) were analyzed. The expression of common lymphocyte markers was examined by flow cytometry.

A comparison of the expression of different markers, however, showed no significant differences between treated and untreated mice. Some representative examples are shown in Figure 12.

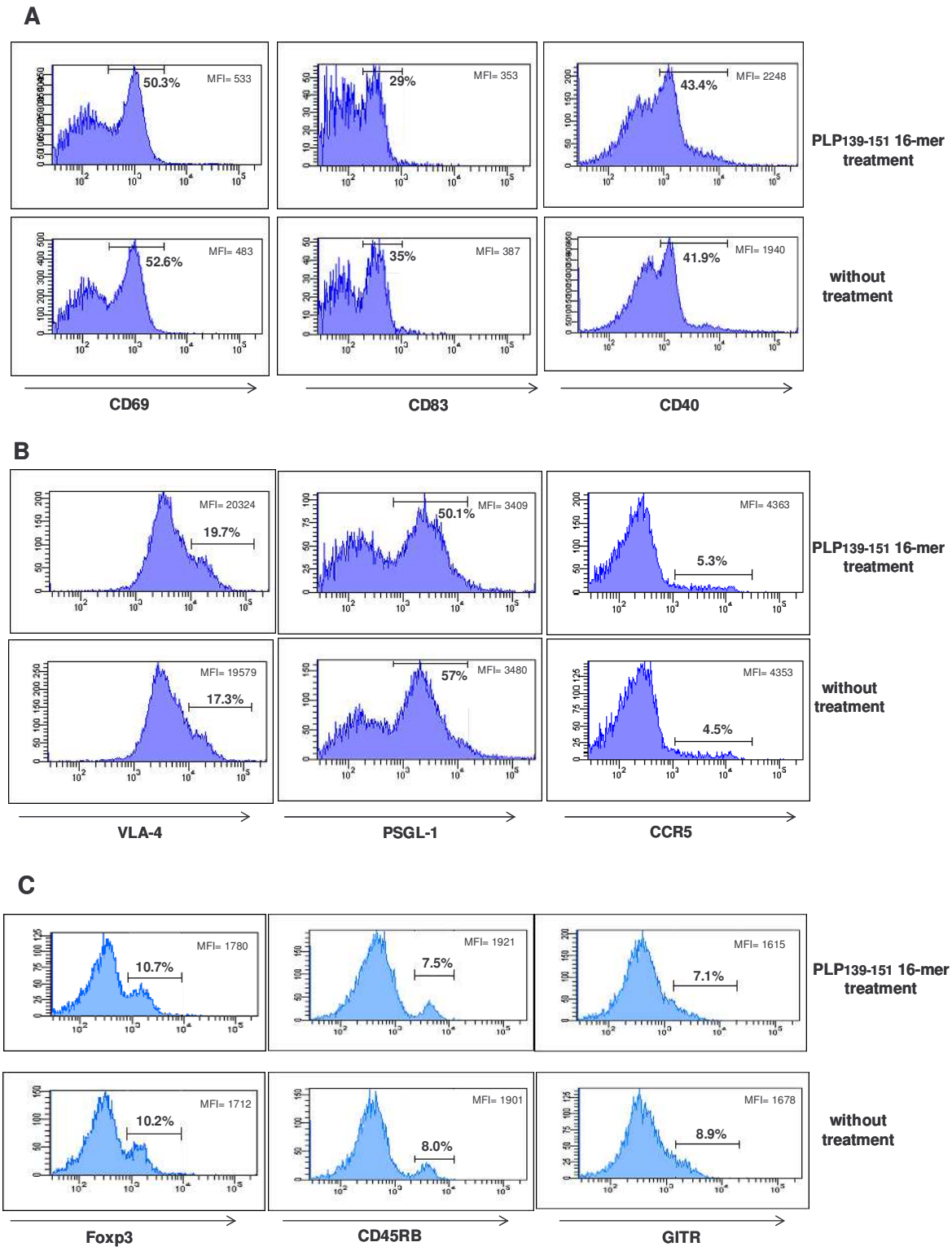


Figure 12. Phenotypic characterization of lymphocytes derived from PLP 139-151 16-mer treated and untreated mice.

Total lymph node cells were isolated on day 13 after EAE induction and tested for the expression of different markers. Phenotypic profile of cells by flow cytometry analysis is shown. **(A).** Expression of activation and costimulatory markers: CD69, CD83 and CD40. **(B).** Expression of migration markers: VLA-4, CD162 and CCR5. **(C).** Expression of markers associated with regulatory functions: Foxp3 intracellular staining, CD45RB and GITR surface staining. Analysis was performed on gated $CD4^+$ with an exception for CD83 and CD40, in which the analysis was done in total lymph node cells. Percentage of cells and the mean fluorescence intensity (MFI) is indicated in each histogram. A representative staining of four mice per group is shown.

One of the important mechanisms of immunosuppression is the down-modulation of co-stimulatory and activation molecules. The expression of the T-cell early-activation marker CD69 and activation/costimulatory molecules such as CD83 and CD40 was similar in PLP 139-151 16-mer treated and untreated mice, both showing an activated T cell phenotype (Figure 12A).

The adhesion molecules VLA-4 and PSGL-1, important in leukocyte-endothelial interactions and in the migration of leukocytes to sites of inflammation [212, 213], were expressed similarly in oligomer treated and untreated mice. Likewise, low levels of CCR5 were observed in both groups of mice (Figure 12B). Certain chemokine receptors are commonly associated with MS pathogenesis, in particular CCR5 and CXCR3 may play an important role in the migratory process of cells to the brain [214].

The expression of markers relevant for regulatory functions was also tested; among them, a key marker for regulatory T cells Foxp3, the CD45RB molecule that is down-regulated on antigen experienced/memory T cells and GITR, which is expressed at high levels on resting $CD4^+CD25^+$. Analysis of the expression of all these markers, however, showed no differences between oligomer treated and untreated mice (Figure 12C). Phenotypic analysis showed that the relative expression of several markers is very similar in mice treated with PLP 139-151 16-mer and mice that did not receive treatment. Nevertheless, the protective status of mice that received the oligomer was correlated with higher levels of CD103 (the α_E chain of $\alpha_E\beta_7$) integrin. Figure 13 illustrates one representative experiment.

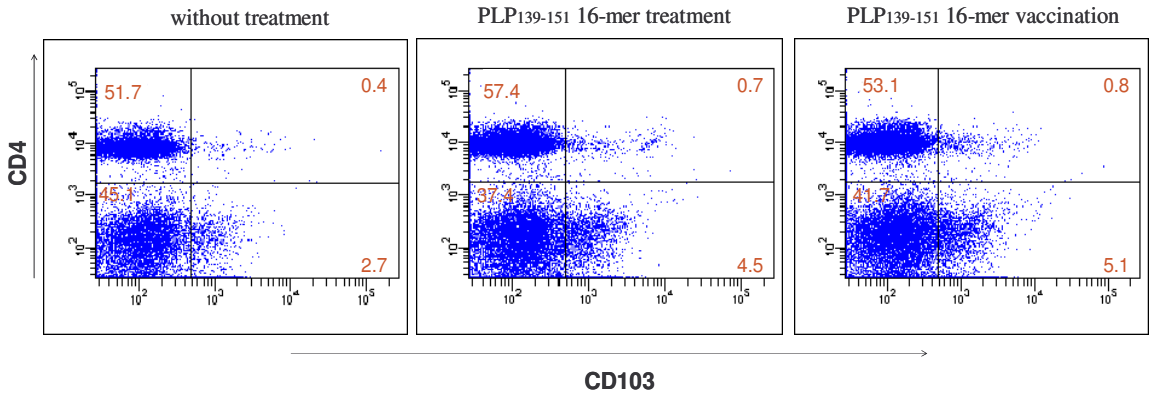


Figure 13. Accumulation of $CD103^+$ cells in lymph nodes of PLP 139-151 16-mer vaccinated and treated mice.

7 days before EAE induction, oligomer vaccination was carried out in a group of 5 mice. Treatment was performed 7 days after disease induction in a second group of 5 mice. For vaccination and treatment, 50 µg of the PLP 139-151 16-mer was injected intravenously. Untreated mice were included as a control of the experiment. Recent isolated cells from lymph nodes were recovered 15 days after EAE induction and tested for the CD103 expression. FACS plots display one representative staining pattern in individual mice out of three animals per group. Numbers in the quadrants show the percentages of the cell populations. SD in $CD4^-CD103^+$ cells n=3 without treatment 2.7%±1.5, PLP 139-151 16-mer treatment 4.9%±0.4, PLP 139-151 16-mer vaccination 5.5%±1.1.

Both oligomer treated and vaccinated mice had a marked increase in the numbers of $CD4^+CD103^+$ cells (0.7 % and 0.8% respectively) in comparison with untreated mice (0.4%). Similar differences were also observed in the CD4 negative population. These corresponded to 4.5% and 5.1% of $CD4^-$ cells expressing CD103 in treated and vaccinated mice respectively, in comparison to 2.7 % in non-treated mice (Figure 13).

Since $CD103^+$ cells were accumulated particularly in the non-CD4 cell fraction, it was of interest to determine the nature of these cells. Some surface markers including CD4, CD8, CD11c, B220, CD25 and DX5 were further tested. It was observed that $CD103^+$ cells co-stain mainly with $CD8^+$ cells. The analysis of the relative percentage of different subpopulations that express the CD103 molecule showed a profile of: 13.5% $CD4^+$; 83% $CD8^+$, 7.5% $CD11c^+$, 3.5% $B220^+$, 10.2% $CD25^+$ and 3.5% NK cells.

$CD103^+$ regulatory cells are considered peripherally generated and potent suppressors with specific migratory patterns. It has been demonstrated that $CD103^+CD4^+$ cells provide protection from EAE [215] and play a role in the control of homeostasis [216] and $CD103^+CD8^+$ cells have important regulatory functions in the balance of alloreactive and Th1 type responses [217, 218].

4.3 CNS infiltration

4.3.1 Epitope-oligomer treatment decreases the inflammatory cell infiltration into the CNS

In order to test whether the oligomer treatment has an influence in the inflammatory process caused by infiltration of cells to the CNS, different groups of mice were perfused for analysis of the cells present into the brain and spinal cord. Significant differences in the number of $CD4^+$ infiltrating cells between treated and non-treated mice were found (Figure 14).

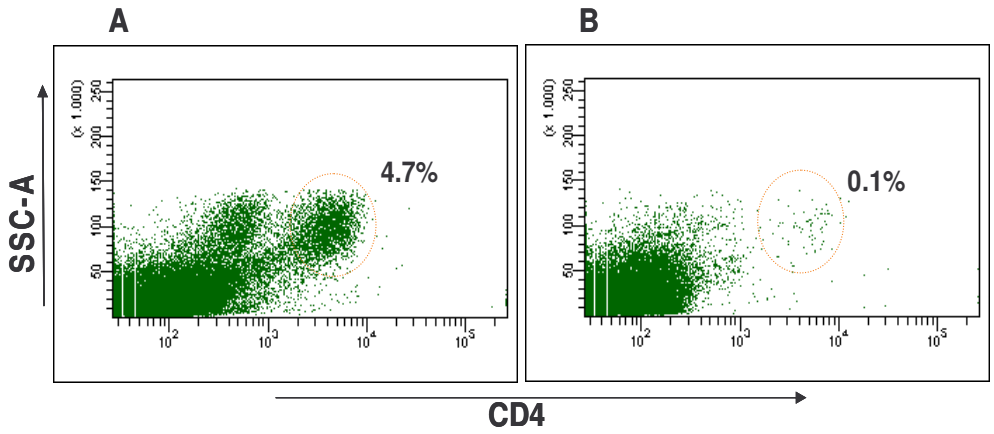


Figure 14. FACS analysis of cells recovered from CNS.
CNS cells were isolated on day 16 after EAE induction. Mice were perfused and cells from the brain and spinal cord were recovered using a percoll gradient. Percentage of $CD4^+$ cells in recent isolated CNS cells is shown: **(A)** PLP 139-151 16mer treated group (clinical score= 0) and **(B)** Non-treated group (clinical score= 3). Plots are representative of 4 experiments. SD n=4 non-treated mice 4.6%±1.1 and PLP 139-151 16-mer treated mice 0.25%± 0.19.

Oligomer treated mice had reduced leukocyte infiltration in the CNS. Cells were recovered from mice at the peak of clinical EAE. The FACS analysis showed a reduction of $CD4^+$ T cell infiltration into the CNS of oligomer treated animals, which correlates with the inhibitory effect on disease development. In contrast, abundant T cell infiltration was seen in the CNS of non treated mice (Figure 14). It is worthy to mention that the opposite situation was found in lymph nodes, where the total number of cells was notably decreased in sick animals. Percentages of $CD4^+$ CNS infiltrating cells correspond to 4.7% in non treated mice, versus 0.1% in mice treated with the oligomer. It indicates that PLP 139-151 16-mer treatment seems to interfere with the process of migration and infiltration of mononuclear cells across the blood–brain barrier. Thus, the CD103 up-regulation previously observed in mice receiving the oligomer could suggest a relevant role of this integrin in the control of migration of pathogenic cells to the CNS. Due to the very low number of cells present in the CNS of treated animals, the analysis and detection of cytokines and surface markers in these experiments were difficult to carry out and no conclusive results were obtained.

4.4 Adoptive cell transfer experiments

Since the oligomer treatment appears to control the CNS infiltration, the actual suppression may be based on active tolerance. Active suppression is one of the major mechanisms of peripheral tolerance generated to prevent autoimmune diseases [35]. As it is mediated by suppressor cells, adoptive transfer experiments were carried out to determine whether they play a role in oligomer-mediated suppression.

4.4.1 Tolerance of oligomer treated mice is transferable

First, the influence of the timing of adoptive transfer was tested by transferring the cells before and after the EAE induction. Cells were isolated from different groups of mice and were adoptively transferred in EAE induced mice. Initially, cells of total lymph nodes from oligomer treated and untreated mice were transferred 4 days after EAE induction.

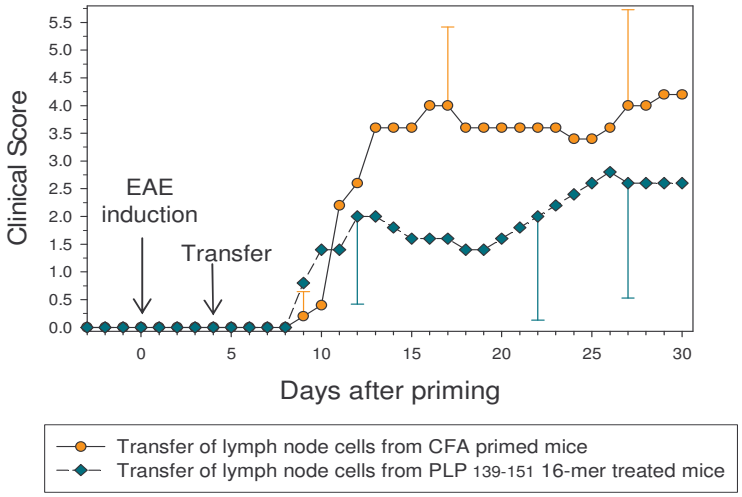


Figure 15. Adoptive transfer of total lymph node cells from oligomer treated mice.
Lymph node cells were obtained from PLP 139-151 16-mer treated mice on day 13 after EAE induction. Cells from CFA primed mice were used as a control of the experiment. $5x10^6$ cells were transferred intravenously into recipient mice in which EAE had been induced 4 days prior to the cell transfer. Animals were daily scored during at least 30 days after the onset of the disease. Results are expressed as mean disease score of a group of five mice and are representative of several experiments.

A modulation of the course of EAE was observed in mice injected with cells of PLP 139-151 16-mer treated mice. Mice had decreased clinical signs of disease, displaying a mean maximal score of 2. In contrast, mice that received CFA primed cells developed a normal form of EAE (mean maximal score of 4) with a relapsing phase at the later time point of the disease (Figure 15).

Thus, protection of EAE was transferred by cells from PLP 139-151 16-mer treated mice. This observation indicates that oligomer induced protection of EAE may in fact be caused by induction of active tolerance. Next, it was significant to investigate whether a particular subset of cells is committed in the suppression induced during the oligomer treatment.

4.4.2 Adoptive transfer of $CD4^+CD25^+$ cells

The transfer of antigen specific $CD4^+CD25^+$ regulatory cells have been widely demonstrated to mediate the balance between tolerance and pro-inflammatory immunity [219]. It was studied whether oligomer treatment could lead to the induction of regulatory $CD25^+$ cells,

which may attenuate the severity of EAE after *in vivo* transfer. The difficulty to have a system to test antigen-specificity, i.e. by the use of tetramers, made difficult the identification of a special subset of cells involve in the oligomer induced suppression. Therefore, different populations were transferred independently of their antigen specificity.

4.4.3 Adoptive transfer of $CD4^+CD25^+$ *vs* $CD4^+CD25^-$ cells from PLP 139-151 16-mer treated mice

Some reports have shown that CD25 depleted $CD4^+$ T cells are able to protect from spontaneous EAE in transgenic mice [220], suggesting that both $CD4^+CD25^+$ and $CD4^+CD25^-$ regulatory cells may work in most situations. In order to compare the protective capacity between different cell subsets, purified sub-populations of $CD4^+CD25^+$ and $CD4^+CD25^-$ from 16-mer treated mice were transferred into recipient mice in which EAE was induced one day after the transfer (Figure 16).

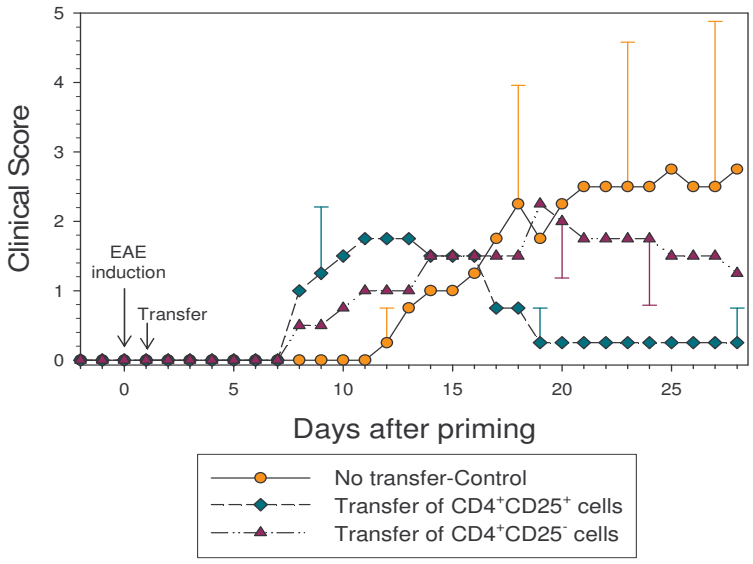


Figure 16. Transfer of purified $CD25^+$ and $CD25^-$ $CD4^+$ cells from PLP 139-151 16-mer treated mice.
Lymph node cells were isolated from 16-mer treated mice 15 days after immunization. $CD25^+$ and $CD25^-$ $CD4^+$ cells were purified by MACS bead separation as described in Figure 2. One day after EAE induction $5x10^6$ cells were intravenously injected and the mice were daily scored. Results are expressed as mean disease score of a group of five mice.

The transfer of $CD4^+CD25^+$ cells showed significantly decreased maximal EAE signs with a mean clinical score of 1.7 in comparison with the control mice that did not receive transferred cells; they had a mean maximal score of 2.7.

It indicates that although mice transferred with $CD4^+CD25^+$ cells had an earlier onset of EAE, they manifested a reduced severity of paralysis at the initial phase of the disease and most

importantly, around one week after, these mice were almost completely recovered. No striking effect was observed when $CD4^+CD25^-$ cells were transferred. The disease was just slightly less severe in the second phase (around day 20) and mice entered in remission faster in comparison with mice that did not receive transferred cells.

4.5 Role of $CD25^+$ cells in the PLP $^{139-151}$ 16-mer induced protection against EAE

One of the best characterized cell subsets having suppressive functions is defined by a constitutive expression of CD25. These $CD4^+CD25^+$ regulatory T cells play a central role in the maintenance of peripheral tolerance and the depletion of these cells leads to various autoimmune diseases [58]. *In vivo* depletion of $CD25^+$ cells is a method to monitor their significance in experimental systems. This approach seems appropriate even when CD25 expression is also characteristic of stimulated effector cells, as several *in vivo* studies have effectively associated regulatory activity to this cellular subset and most of the $CD25^+$ cells in a normal mouse express Foxp3 [74].

Initially, some experiments were carried out to ensure a substantial depletion of $CD4^+CD25^+$ cells. The depletion showed an efficient removing of these cells which was verified by FACS analysis of CD25 expression in peripheral blood. This effect remained for at least 4 weeks (data not shown).

In previous experiments, it was also observed that $CD25^+$ depletion performed at a close time point to EAE induction induced amelioration of the disease. Since depletion of $CD25^+$ cells depletes also activated $CD25^+$ effector cells, it was important to choose an appropriate time point to avoid unwanted depletion of effector cells. Therefore, the approach by vaccination with the oligomer was carried out to determine the effect of $CD4^+CD25^+$ T regulatory cells.

To determine the functional involvement of $CD25^+$ cells in the suppressive effect induced by the oligomer, depletion of this cell population was performed 15 days prior to the disease induction (day -15) and one week after (day -7) mice were vaccinated with PLP $^{139-151}$ 16-mer (see Figure 17).

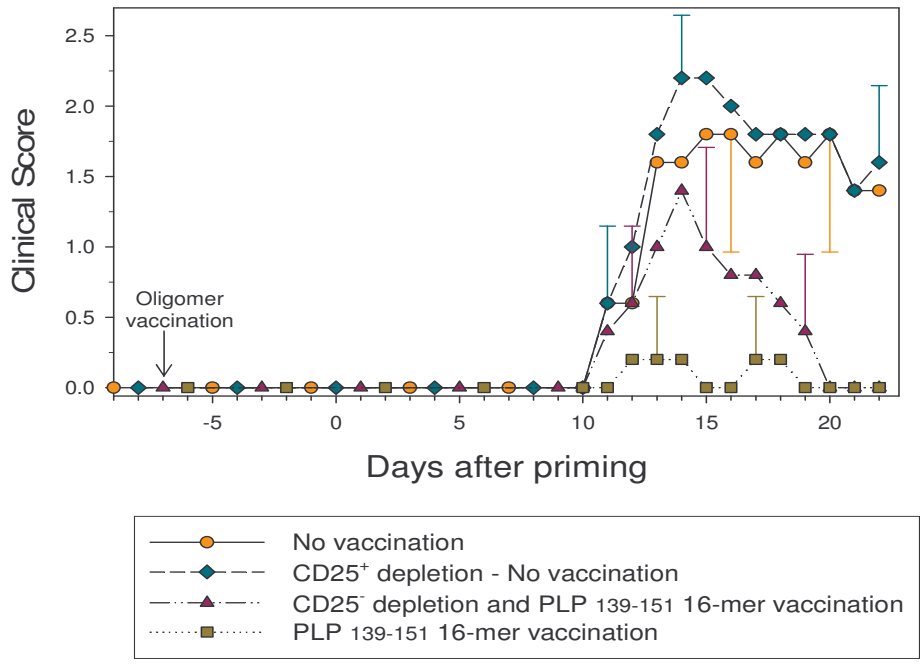


Figure 17. **Effect of *in vivo* CD25$^+$ cell depletion in oligomer treated mice**
15 days previous the EAE induction, mice were injected intraperitoneally with a depleting anti-CD25 antibody. 7 days prior disease induction mice were vaccinated by giving 50 µg of PLP 139-151 16-mer intravenously. A control group without CD25 depletion was included to monitor the normal course of EAE. Each point represents the mean clinical score of five mice.

Depletion of CD25$^+$ cells in oligomer vaccinated mice resulted in a partial abrogation of tolerance as indicated by the appearance of weak signs of EAE (Figure 17).

The results show that a transient effect is only achieved in CD25$^+$ depleted 16-mer treated mice. At the peak of the disease (day 14) and for a few days, mice revealed symptoms of EAE (mean maximal score of 1.4) and afterwards they recover rapidly. In contrast, oligomer vaccinated mice that were not depleted of CD25$^+$ cells were almost completely protected (mean maximal score of 0.2), whereas non-vaccinated mice developed EAE with a mean maximal score of 1.6, comparable to the 2.2 score seen in CD25$^+$ depleted non-vaccinated mice (Figure 17). Nevertheless, a partial contribution of CD25$^+$ cells in the protective effect induced during the 16-mer vaccination was evident according to the significance of the general clinical score during the experiment; in which all mice belonging to the group (CD25$^+$ depleted 16-mer vaccinated) developed mild symptoms of EAE at the same time and showed remission within a few days.

Next experiments were focussed to study the role of other subsets of cells, such as B cells, that might have relevance in the tolerance induced after the oligomer treatment. Antibody production, adoptive transfer experiments and phenotypic characterization of B cells were carried out.

4.6 Characterization of different subclasses of antigen specific immunoglobulins

It is widely described that the differentiation of B cells is very efficiently stimulated when an antigen is presented in a repetitive form and consequently, a strong immunoglobulin secretory response is induced [178]. In the first part of these experiments, it was examined whether an antibody response was generated during the oligomer treatment. The occurrence and characterization of different subclasses of antigen specific immunoglobulins was tested in different groups of oligomer treated and untreated mice.

The analysis of the humoral response in this study reveals that soon after oligomer treatment, mice were able to induce high levels of antibodies, in contrast with untreated mice that only produce low amounts of the different isotypes (Figure 18).

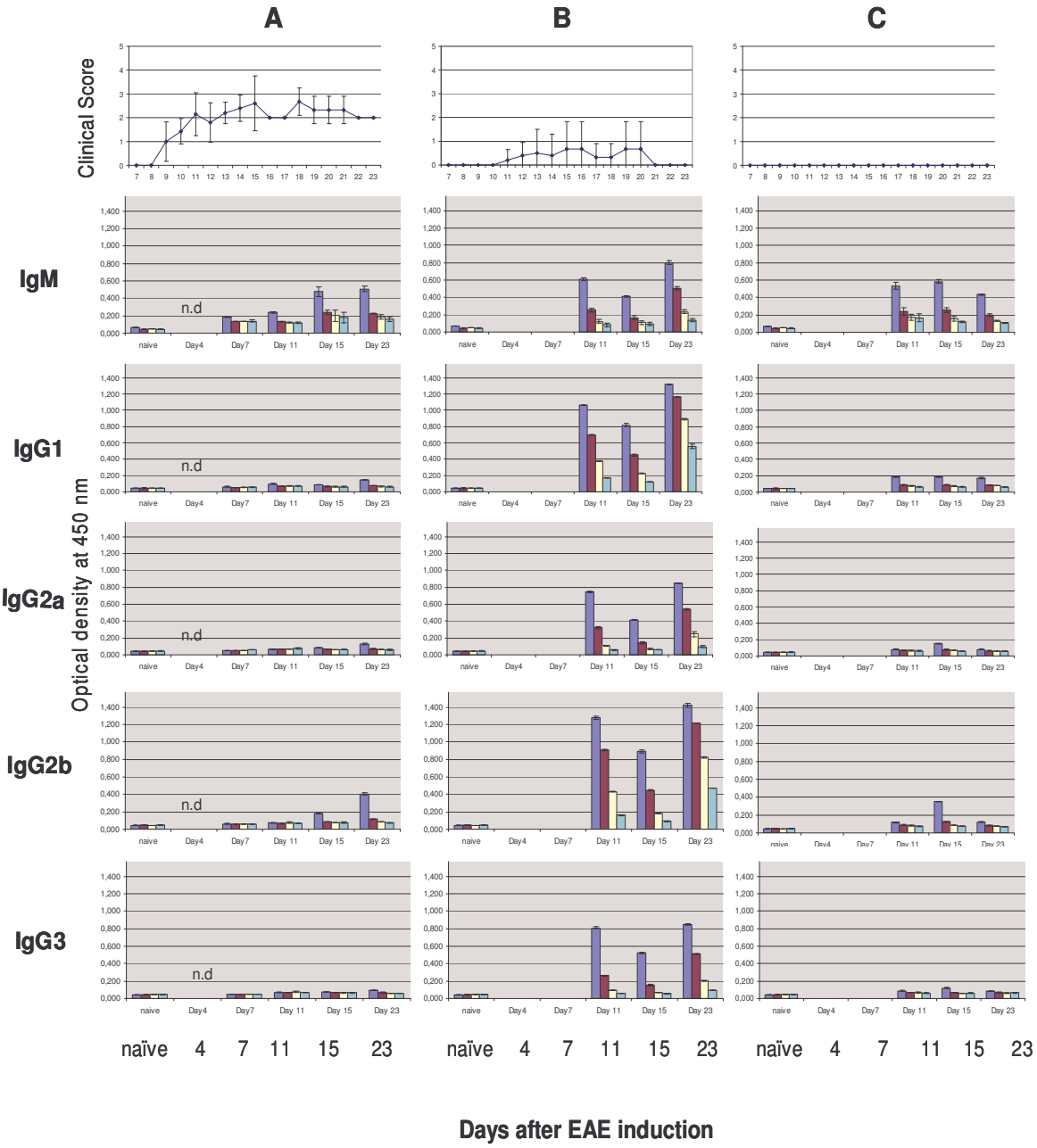


Figure 18. Characterization of different subclasses of antigen specific immunoglobulins
Production of IgG isotypes in serum of **(A)** disease induced mice without treatment; **(B)** disease induced mice treated with the PLP 139-151 16-mer (50 µg) and **(C)** unprimed mice but treated with the PLP 139-151 16-mer (50 µg). The data belonging to day 7 in **B** and **C** were not determined due to the oligomer treatment. Serum was collected at different time points after disease induction. The bars represent the anti-PLP139-151 antibody serum titer: 1:150; 1:600; 1:2400; 1:9600. All anti-antibody titers are indicated from 150- fold prediluted serum titrated 1:4 over 4 dilution steps; n.d, not determined. Upper panel represent the daily mean clinical score between different groups of mice.

It is generally accepted that increased levels of antigen specific IgG1 and IgG2b antibodies reflects a Th2 immune response to the antigen, whereas a higher amount of IgG2a results from amplification of a Th1 immune response [221]. In this sense, no conclusive results could be achieved from these assays. However, analysis of the clinical score shows that in treated

mice there was association between the antibody titers to the PLP 139-151 epitope and the suppression of the clinical disease, as observed in the upper panel of Figure 18.
Antigen-specificity reaction in these experiments was verified by the use of the oligomer HA 107-119 4-mer, which also contains the same spacer sequence than the PLP 139-151 16-mer. No reactivity of the immunoglobulins was found when the HA oligomer was used for coating (data not shown). Mice that were only intravenously with the oligomer but were not primed did not show any significant antibody production, comparable with non-treated mice. It is probable that an antigen-specific inflammatory reaction together with the oligomer, which harbours a cognate antigen, is needed for the activation of the humoral immune response.

For some time, cross-linking of repetitive antigens has been identified as a strong signal that induces differentiation and activation of B cells modulating the strength of the immune response [177]. A possible role of B cells during the PLP 139-151 16-mer treatment was studied in the next experiments. Their potential suppressive functions were tested in adoptive transfer experiments and their phenotypic characterization was carried out.

4.7 Adoptive transfer and characterization of B cells

To test whether B cells from epitope-oligomer treated mice could transfer protection against EAE, *in vivo* transfer experiments were performed. B cells were collected from the mice at different time intervals after the treatment (days 10 and 15) (see Figure 3); at these periods untreated mice usually display a peak of relapsing, whereas oligomer treated animals remain completely healthy. In addition, it was of interest to check the effect of B cells from treated mice through time after the oligomer administration.

4.7.1 Amelioration of EAE by transfer of B cells from oligomer treated animals

Donor B cells from PLP 139-151 16-mer treated and untreated mice were isolated at different time points and $4x10^6$ purified B cells were intravenously injected in recipients to monitor the course of EAE in comparison to the control mice which did not receive transferred cells.

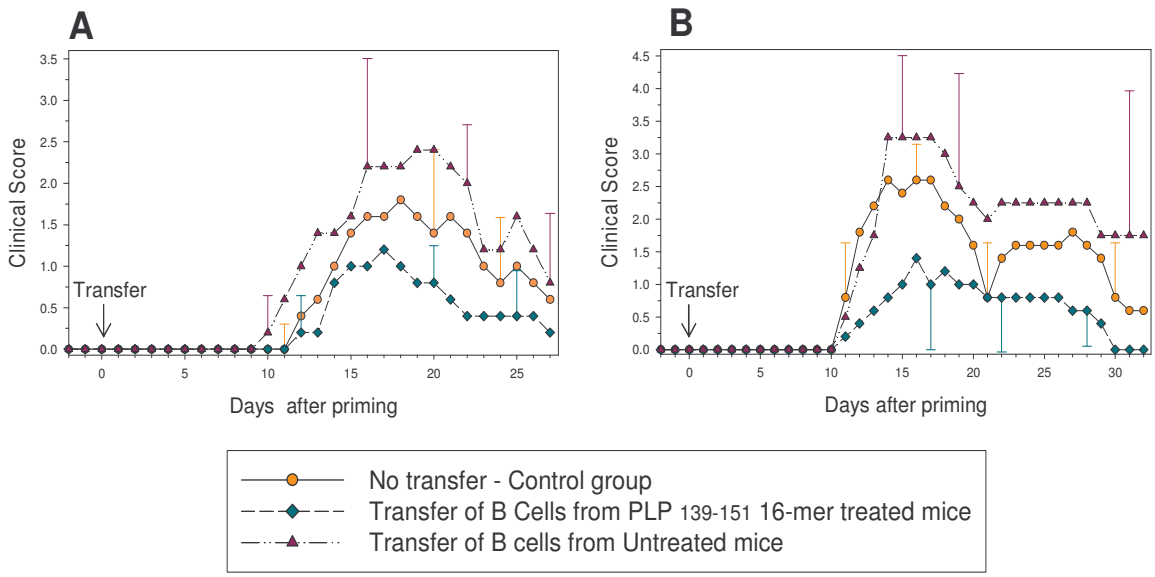


Figure 19. **Suppression of EAE upon adoptive transfer of B cells from oligomer treated mice.**
Spleen and lymph node cells were recovered from mice at different times**: (A)** 10 days and **(B)** 15 days after oligomer-treatment. Suspension cells were incubated with 20µl of anti-CD43 PE antibody per $1x10^7$ total cells; after washing, 10µl of anti-PE microbeads was added per $1x10^7$ total cells. After sorting, the negative fraction was recovered and assessed for B220 expression (see Figure 3). The purity was more than 95%. $4x10^6$ purified B cells were transferred intravenously into naïve mice. EAE was induced one day after the transfer.

Mice transferred with B cells derived from untreated mice showed stronger disease symptoms than those transferred with B cells from treated mice, which were able to control the severity the disease. Figure 19 shows two representative experiments that demonstrate the role of B cells from oligomer treated mice in induction of suppression. B cells isolated at different periods after the treatment had the capacity to adoptively transfer suppression of EAE in recipient mice. In this case (transfer of B cells recovered on days 10 and 15 after the treatment), a delay in the onset of the disease was observed. Mice receiving B cells from oligomer treated mice showed in general significantly decreased maximal EAE signs (mean score of 1.4 with an incidence of 60%) in comparison with mice which received B cells from untreated animals (mean maximal clinical score of 2.6 with an incidence of 100%).

B cells from untreated mice failed to transfer suppression. Moreover, in some experiments they had an earlier onset of disease, reaching a maximal mean score of 2.4 with an incidence of 100% in comparison with mice that did not receive transferred B cells, which showed a maximal score of 1.8 and an incidence of 80% (Figure 19).

Therefore, these results indicate a potential role for B cells in the oligomer induced suppression of EAE**.** With the aim to characterize the nature of B cells from treated mice with potential role to transfer suppression, different B cell surface markers were analyzed comparatively with untreated and naive mice.

4.7.2 Phenotypic characterization of B cells

During the B-cell development, every step is tightly controlled by the expression and function of the B-cell receptor (BCR) and by the ability to interact with the microenvironment. The coordination between proliferative expansion and differentiation of bone marrow (BM) precursor cells is essential at all stages of hematopoiesis, which can be described by cell surface markers.

4.7.2.1 Analysis of bone marrow B cells

In the B cell development, multiple stages determine whether they will survive or die. In the bone marrow, the pre-BCR signals the rearrangement of the light chain and subsequent differentiation into immature B cells. The $B220^{+}IgM^{-}$ population characterize the early B-cell population (pro and pre-B cells). The $IgM^{+}IgD^{-}$ population defines the immature B cells and the mature B-cell population is composed of $IgM^{+}IgD^{+}$ B cells.

In adult BM, the surface markers that distinguish pro-B cells from cells at later stages of B-cell development include B220, CD43, CD24, IgM, IgD, and MHC class II (Ia) among others. CD24 has been traditionally used as a differentiation marker for B and T cell ontogeny because of its lineage-specific and developmentally regulated expression.

In order to compare the phenotype of B cells in different groups of mice, bone marrow derived cells were isolated at the peak of the EAE and the $B220^{+}$ population was analysed for surface expression of IgM, IgD and CD24.

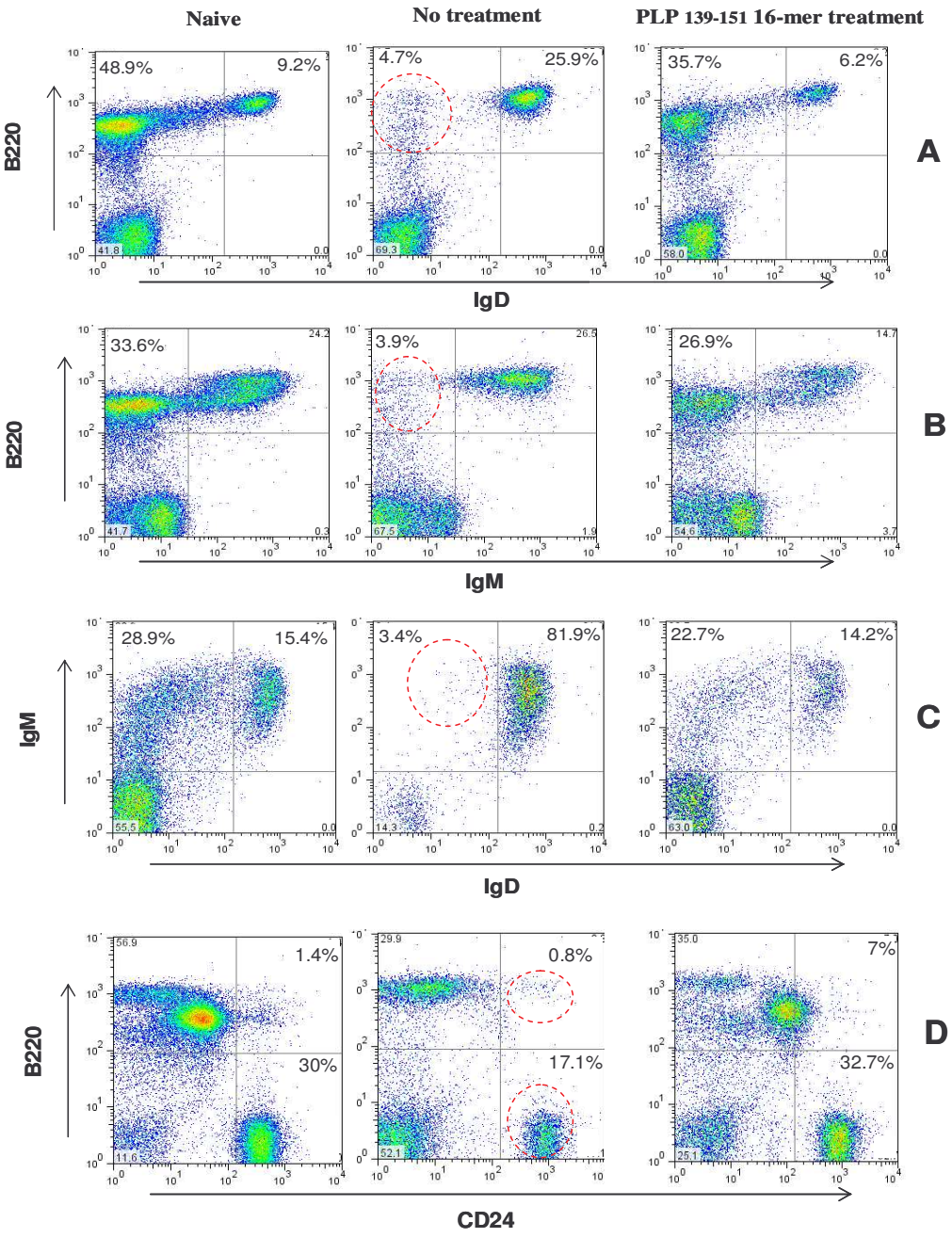


Figure 20. **Analysis of bone marrow derived B cells.**
Total bone marrow cells were isolated on day 13 after disease induction. Cells were examined for surface expression of **(A)** $B220^+$ and IgD; **(B)** $B220^+$ and IgM; **(C)** $B220^+$ gated bone marrow cells were analysed for the expression of IgM versus IgD and **(D)** analysis of bone marrow cells for the expression of $B220^+$ and CD24. The relative expression of the different markers was compared between untreated, PLP 139-151 16-mer treated and naive mice. The percentage of B cell subpopulations in each quadrant is indicated.

Figure 20A shows that the number of bone marrow $B220^+IgD^+$ cells was similar in treated (6.2%) and naïve mice (9.2%); however, a substantial increasing of these cells was observed in untreated animals (25.9%). Likewise, the relative number of $B220^+IgM^-$ cells

corresponding to pre-B cells (Figure 20B) is decreased in untreated mice (3.9%) in comparison with oligomer treated (33.6%) and naive (26.9%) mice.

Flow cytometric analysis of surface IgM versus IgD expression on $B220^+$ cells in the bone marrow (Figure 20C) indicates a decreased percentage of immature (IgM^+IgD^-) B cells in untreated mice in comparison with PLP 139-151 16-mer treated and naive mice. The majority (~85%) of bone marrow $B220^+$ IgM^+ positive cells derived from mice with active EAE (untreated) also stained positively for membrane IgD , which is present on mature B cells.

The remarkable deficiency in immature B cells is denoted with a red dotted circle in Figure 20C, which shows a similar profile in IgM^+IgD^- B cells of treated (22.7%) and naive mice (28.9%) in contrast to untreated mice in which this population was reduced by 3.4%.

It seems clear that normally, the ability of the marrow to produce and maintain lymphoid cells is severely compromised during active EAE. The lymphopenic status in EAE has been attributed to a reduction in the number of bone marrow B cell precursors or defects in B lymphocyte development, activation and survival [137, 222].

Because CD24 is a useful marker for characterizing the maturation stage of B lineages, the expression of CD24 in $B220^+$ cells was tested in different groups of mice. Figure 20D shows that in untreated mice the percentage of $CD24^+$ cells is markedly reduced in both $B220^+$ and $B220^-$ populations (0.8 and 17.1% respectively), in comparison with PLP 139-151 16-mer treated (7% $B220^+CD24^+$ and 32.7% $B220^-CD24^+$) and naive mice (1.4% $B220^+CD24^+$ and 30% $B220^-CD24^+$). Since CD24 is almost repressed during maturation of B and T lymphocytes, the reduced percentage of this marker found in untreated mice seems to reflect the very small numbers of immature cells in this group of animals. These results are in concordance with previous findings in which the pre–B cell pool is markedly diminished in a number of autoimmune disorders [223].

To establish whether the phenotype of the B cell subset in the periphery is modified as consequence of treatment with the epitope-oligomer, expression of determinant markers typical for the stage development was analysed in comparison with B cells from untreated and naive mice.

4.7.2.2 Analysis of spleen B cells

Following the exit from the bone marrow, peripheral B cells develop through transitional type 1 (T1 cells that have recently emigrated from the BM) and transitional type 2 (T2) B cell stages. The T1 and T2 subsets can be distinguished by staining for various cell-surface markers. T1 cells are $CD21^{low}$ $CD23^{low}$ $CD24^{high}$ IgM^{high} IgD^{low}, while T2 cells are $CD21^{high}$ $CD23^{high}$ $CD24^{high}$ IgM^{high} IgD^{high}. Activation of specific signalling events in T2 B cells leads

to their further differentiation into either follicular mature (FM) B cells or marginal zone (MZ) B cells. The CD24 marker discriminates immature ($CD24^{high}$) from mature ($CD24^{low}$) splenic B cells, while CD21 (complement receptor type 2 CR2) and CD23 (FcERII) have been widely used to distinguish differentiated B cells in the spleen.

A comparative analysis of the subsets of spleen B cells in the periphery was performed in different group of mice. Firstly, the frequency of B lymphocytes that express IgM, IgD and CD24 was determined in spleen-derived cells from PLP 139-151 16-mer treated, untreated and naive mice.

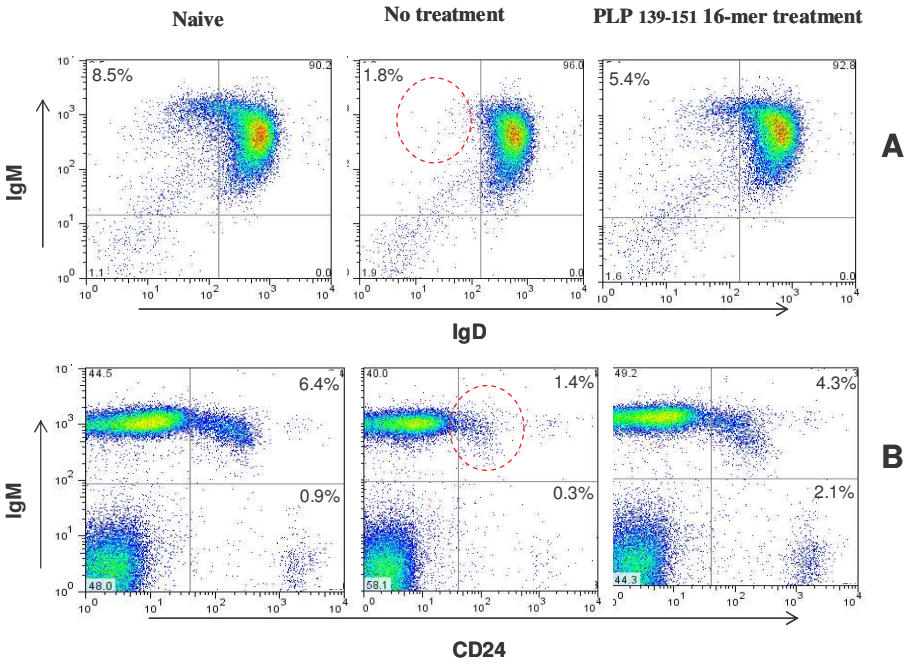


Figure 21. Phenotypic characterization of spleen B cells
Splenocytes were collected on day 13 after disease induction. $B220^+$ gated bone marrow cells were analysed for the expression of: **(A)** IgM versus IgD and total spleen cells for the expression of **(B)** IgM versus CD24. The relative expression of the different markers was analyzed between untreated, PLP 139-151 16-mer treated and naive mice. The percentages of B cell subpopulations are indicated next to each gate. Results in representative mice are shown.

As shown in Figure 21, the deficiency in immature B cells found in untreated mice was not specific to the bone marrow but it was also observed in the spleen. In line with previous observations (Figure 20), the impairment in the production of pre-B cells in the bone marrow of diseased mice is also reflected in the periphery, as noted by the absence of the immature B cell subset in the spleen of mice with active EAE (untreated mice). Analysis of IgM vs IgD expression in $B220^+$ gated cells shows no differences between naive and treated mice. The percentages of IgM^+IgD^- equivalent to the immature "transitional" B cells represent the 5.4% in PLP 139-151 16-mer treated and 8.5% in naive mice. Notably, a very small percentage (1.8%) of immature B cells was found in the periphery of untreated mice.

It is also interesting the reduced expression of CD24 in untreated animals, in which only 1.4% of IgM^+ and 0.3% of IgM^- cells expressed the marker, as compared with treated (4.3% $CD24^+IgM^+$ and 2.1% $CD24^+IgM^-$) and naïve mice(6.4% $CD24^+IgM^+$ and 0.9% $CD24^+IgM^-$) (Figure 21). To characterize better the $CD24^+$ population, additional staining for CD23 and CD21 were included. A large fraction of $CD24^+IgM^+$ (80.6%) in PLP 139-151 16-mer treated and (79.7%) in naive mice stained positively for CD21 and CD23 markers.

Since B cells up-regulate CD23 after activation, it was expected to find higher percentage of CD23 negative cells in naive (10.7%) in comparison with treated mice (6.2%) (data not shown). This population seems to correspond to a B cell subset expressing high levels of CD24, CD21, CD23, and IgM, identified as transitional 2-marginal zone precursor (T2-MZP) B cells. Recent reports have shown an important immunoregulatory role for this B cell population in autoimmune diseases [87].

4.7.3 Effect of naive B cells in the modulation of EAE

As described above, the phenotypic characterization of B cells derived from oligomer treated and naive mice showed a similar pattern in both distribution and surface markers expression. In order to compare the *in vivo* suppressive effect of B cells from mice which have been treated with PLP 139-151 16-mer and B cells isolated from naive mice, adoptive transfer experiments were performed.

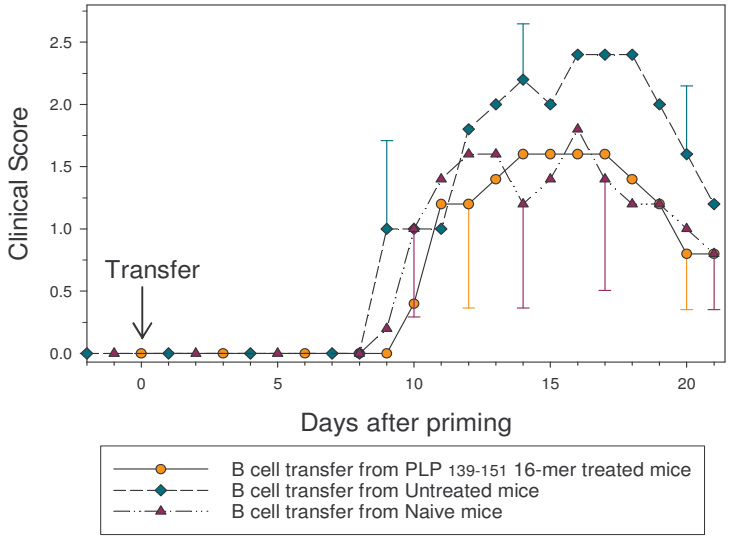


Figure 22. Adoptive transfer of B cells from naive mice
Naive recipient mice were transferred with B cells from different groups of animals: PLP 139-151 16-mer treated, untreated and naive mice. $4x10^6$ purified B cells were intravenously injected and EAE was induced the next day. Mice were daily scored. The results are expressed as mean disease score of a group of five mice. One representative experiment of two performed is shown.

Interestingly, it was observed that adoptive transfer of B cells from PLP 139-151 16-mer treated animals resulted in a level of protection similar to that obtained with B cells from naive mice. Both of them only developed mild disease with a maximal clinical score of 1.6 and 1.8 respectively, whereas control mice that received B cells from untreated mice reached a mean maximal score of 2.4 (
Figure 22).

As mentioned before, mice developing EAE were characterized to have very low number of total cells recovered from both lymph nodes and spleen, in addition to their dramatic deficiency of most $CD24^+$ immature B cells (transitional 2-marginal zone B cells T2-MZP).

The partial protection induced by naive B cells may be explained in terms of "homeostasis", as these cells introduced into diseased mice with lymphocyte deficiency might supply the conditions to recover the stable size of the naive B cell pool. In addition, recent findings have shown a regulatory role for this special subset of B cells (T2-MZP) present in naive mice, since these cells can undergo antigen-independent proliferation after transfer and hence, they produce IL-10 [87].

4.7.4 Protective effect induced by transfer of B cells from PLP 139-151 16-mer versus naive B cells

To further investigate the antigen specificity and the length of the protective effect induced by B cells from PLP 139-151 16-mer treated mice in comparison with B cells from untreated mice, EAE was induced for the second time at different days after the first immunization. The challenge was done at different time points once all the mice in the experimental group have recovered from EAE.

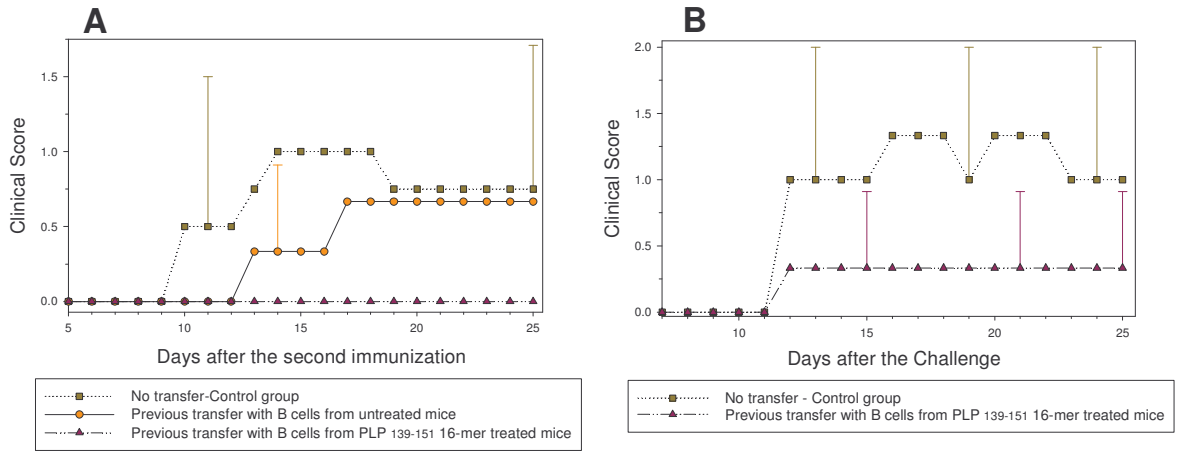


Figure 23. **Second immunization after B cell transfer**
EAE was induced a second time in mice that had been previously transferred with B cells from PLP 139-151 16-mer treated, untreated or naive mice. Mice were challenged with 50 µg of PLP 139-151 peptide in incomplete adjuvant followed by an injection of *Pertussis* toxin. **(A).** Mice challenged 60 days after the first priming. **(B)** Mice challenged 90 days after the first priming. Mice were monitored daily and the mean of the clinical score was plotted. A and B are two independent experiments with similar results; four and three mice per group were assessed respectively.

The challenge of mice was performed at late time points (60 and 90 days) after the first immunization. Adoptive transfer of B cells from PLP 139-151 16-mer treated mice suppressed the subsequent induction of EAE. Figure 23A shows that mice that previously received B cells from treated mice were completely protected, whereas control mice were once again paralyzed. After a second immunization independent of the treatment, mice responded to a much lower degree; they developed a very mild disease (mean maximal score of 1) with an incidence of 50% in the control group**.**

Similarly, 90 days after the first immunization, EAE was reinduced in mice that received or not received adoptively transferred B cells. Mice that previously received adoptively transferred B cells from PLP 139-151 16-mer treated animals were almost completely protected of EAE. They showed very weak symptoms of the disease (mean maximal score of 0.3 with an incidence of 25%) in comparison with control mice that did not receive adoptively transferred B cells and developed mild EAE (mean maximal score of 1.3 and an incidence of 100%) (Figure 23B).

The observation that B cells from treated mice, even at late time points, transferred higher levels of protection against EAE than B cells from untreated mice, leads to see a long-lasting suppressive effect that could indicate resistance transferred by memory immune cells.

To examine further whether the oligomer treatment has an effect in the modulation of regulatory cytokines, specifically in the B cell subset, different groups of mice were analysed

for the IL-10 production. The presence of IL-10 is a determinant factor in tolerance induction of autoimmune diseases and constitutes an important issue to be assessed [85, 86].

4.8 IL-10 production during the PLP 139-151 16-mer treatment

The anti-inflammatory cytokine IL-10 has been showed to play a pivotal role in tolerance induction. Particularly in the EAE model it is classified as a key cytokine in the suppression of the disease [215]. For this reason, it was of interest to look whether PLP 139-151 16-mer treatment could induce the IL-10 production and which cell subtype might be responsible for it. Initially, purified B cells from treated and untreated mice were tested for the release of IL-10 upon activation *in vitro*.

4.8.1 PLP 139-151 16-mer treatment promotes the IL-10 secretion by B cells

Since intracellular detection of cytokines in B cells is sometimes difficult to carry out, Cell-ELISA technique was used in this study as a sensitive probe for cytokine detection [205].

The ligation of CD40 on B lymphocytes by CD40 ligand (CD40-L) provides a key activation signal required for the B cell response that, besides enhancing co-stimulation, induces the secretion of cytokines [224]. Antigen specific B cell activation was carried out by using PLP 139-151 16-mer together with anti-CD40 antibody. Since anti-CD40 simulates the T cell signal, stimulation via this molecule can partially substitute T cells in *in vitro* culture systems.

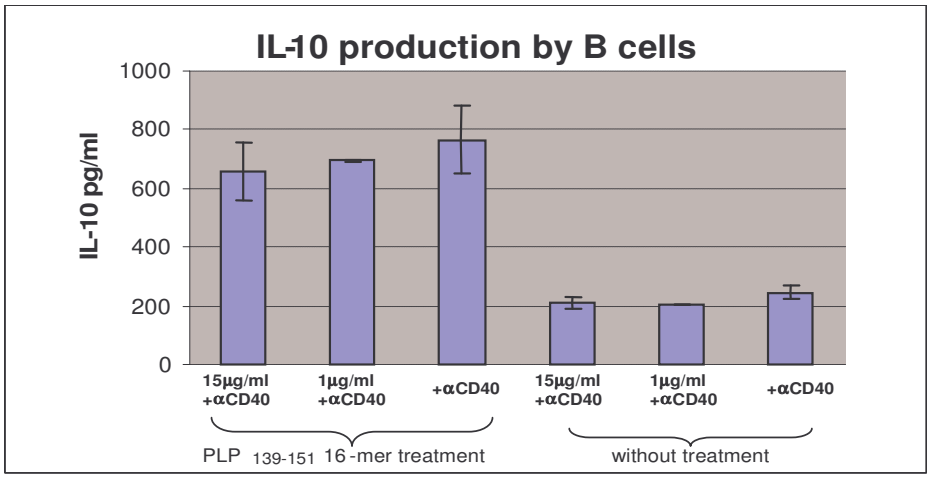


Figure 24. Differential IL-10 production of B cells isolated from PLP 139-151 16-mer treated and untreated mice.

B cells were purified as described in Figure 3. 19 days after disease induction, B cells were analysed by Cell-ELISA for the ability to produce IL-10. At this time point treated mice were completely protected (score of 0) and untreated mice showed a score between 2-3. $4x10^5$ purified B cells from spleen and lymph nodes were activated with the indicated concentration of PLP 139-151 16mer plus anti-CD40 monoclonal antibody (αCD40) (5 µg/ml) or α-CD40 alone. After 4 days of incubation, the cells were transferred to a new plate previously precoated with anti-IL-10 and after 24 hours in culture, the IL-10 ELISA was developed. These results are representative of two independent experiments.

It was observed that after activation with anti-CD40 alone, B cells from mice given PLP 139-151 16-mer for treatment of EAE had increased capacity to produce IL-10 in comparison to B cells of mice that did not received any treatment. In Figure 24, B cells recovered from treated mice produced almost three times more IL-10 than B cells from untreated mice (770 pg/ml vs 244 pg/ml). Remarkably, the antigen-specific response was absent as B cells, particularly those derived after the oligomer treatment produce IL-10 just in presence of anti-CD40. It is possible that these cells are readily activated *in vivo* by the repetitive antigen and the *in vitro* CD40 ligation alters the antigen activation threshold. It is worthy to mention that along these experiments the CD40 stimulation in B cells induced a very strong proliferation (data not shown). Whether B cells from treated mice could produce antigen-specific IL-10 is not conclusive. However, it seems that these cells are particularly activated by CD40 engagement; producing significantly higher levels of IL-10 in comparison with B cells derived from untreated mice.

Several reports have shown that activated B cells with an agonistic anti-CD40 antibody can produce IL-10 in an antigen independent manner [225, 226] and the transfer of anti-CD40 stimulated B cells prevents the development of autoimmune diseases [227].

The identification of the regulatory role of B cells and the characterization of B cell subsets with distinct cytokine profiles have been subject of study [228]. The contribution of B cells to IL-10 production might be relevant during tolerance induction by the oligomer treatment. Secretion of anti-inflammatory cytokines might be one of the mechanisms by which B cells confer protective effect after the *in vivo* adoptive transfer.

4.8.2 *In vitro* antigen-restimulation and IL-10 quantification

Since, intracellular IL-10 was not detectable after *ex vivo* antigen stimulation, neither in treated nor in untreated mice, an alternative approach using long term cultures and ELISA detection was used.

In order to determine the antigen-specific IL-10 production and how it could be related to the kind of antigen used for stimulation, different combinations of the PLP 139-151 (monomer) and PLP 139-151 16-mer (oligomer) were used (Table 3). Total lymph node cells derived from treated and untreated mice were analyzed.

Antigenic Stimulation PLP 139-151 used at 10 µg/ml PLP 139-151 16-mer used at 5 µg/ml	
Primary Stimulation	**Secondary Stimulation**
PLP 139-151	PLP 139-151
PLP 139-151	PLP 139-151 16-mer
PLP 139-151 16-mer	PLP 139-151
PLP 139-151 16-mer	PLP 139-151 16-mer

Table 3. **Combination of peptides for *in vitro* restimulation**

Results demonstrate reduced or undetectable IL-10 secretion in cultured lymph node cells from untreated mice. In contrast, cells derived from oligomer treated mice showed enhanced IL-10 production upon restimulation and it was irrespective of the antigen combination used.

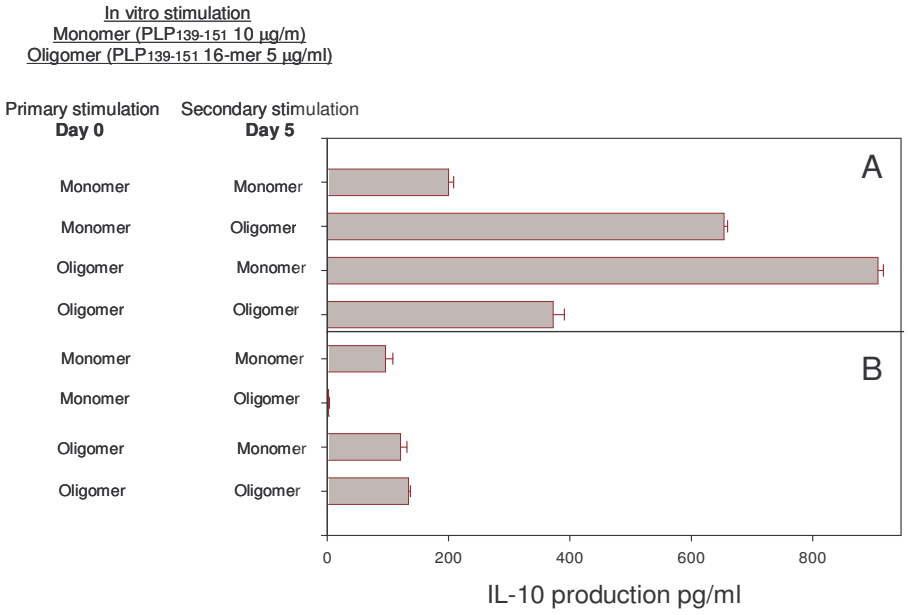


Figure 25. **IL-10 production after *in vitro* restimulation**
Total lymph nodes cells of **(A)** PLP 139-151 16-mer treated mice or **(B)** untreated mice, were collected on day 24 after EAE induction. At this time point untreated mice showed a mean EAE score of 2 and treated mice were completely protected (EAE score of 0).Cultures were stimulated twice (on days 0 and 5) with different combinations of the PLP 139-151 peptide (monomer) or the PLP 139-151 16-mer (oligomer). After 10 days, supernatants were collected for IL-10 measurement by ELISA. Results are expressed as the mean cytokine concentration. SD of duplicate cultures is from one experiment representative of five. These experiments were carried out normally on days 14 and 24 after disease induction and had similar results.

As shown in Figure 25, the IL-10 secretion in PLP 139-151 16-mer treated mice was in the range between 200 and 900 pg/ml in comparison with untreated mice, which produced between 2 and 135 pg/ml of IL-10 depending of the stimulus used. These results seem to indicate that not only B cells, but also T cells might be an important source of IL-10 in oligomer treated mice.

It was clear that independent of the order of stimulation with peptide or 16-mer in all cases, higher IL-10 release was detected in cells derived from treated mice, thus oligomer treatment seems to bias the immune response towards the release of the immunosuppressive cytokine IL-10.

4.9 Neutralization of anti-inflammatory cytokines influence the suppressive effect induced by oligomer treatment

4.9.1 *In vivo* neutralization of IL-10

To test whether IL-10 *in vitro* production in treated mice directly contributes to the curative effect provided by the oligomer treatment *in vivo*, mice were injected with neutralizing antibodies against IL-10 and the IL-10 receptor (IL-10R).

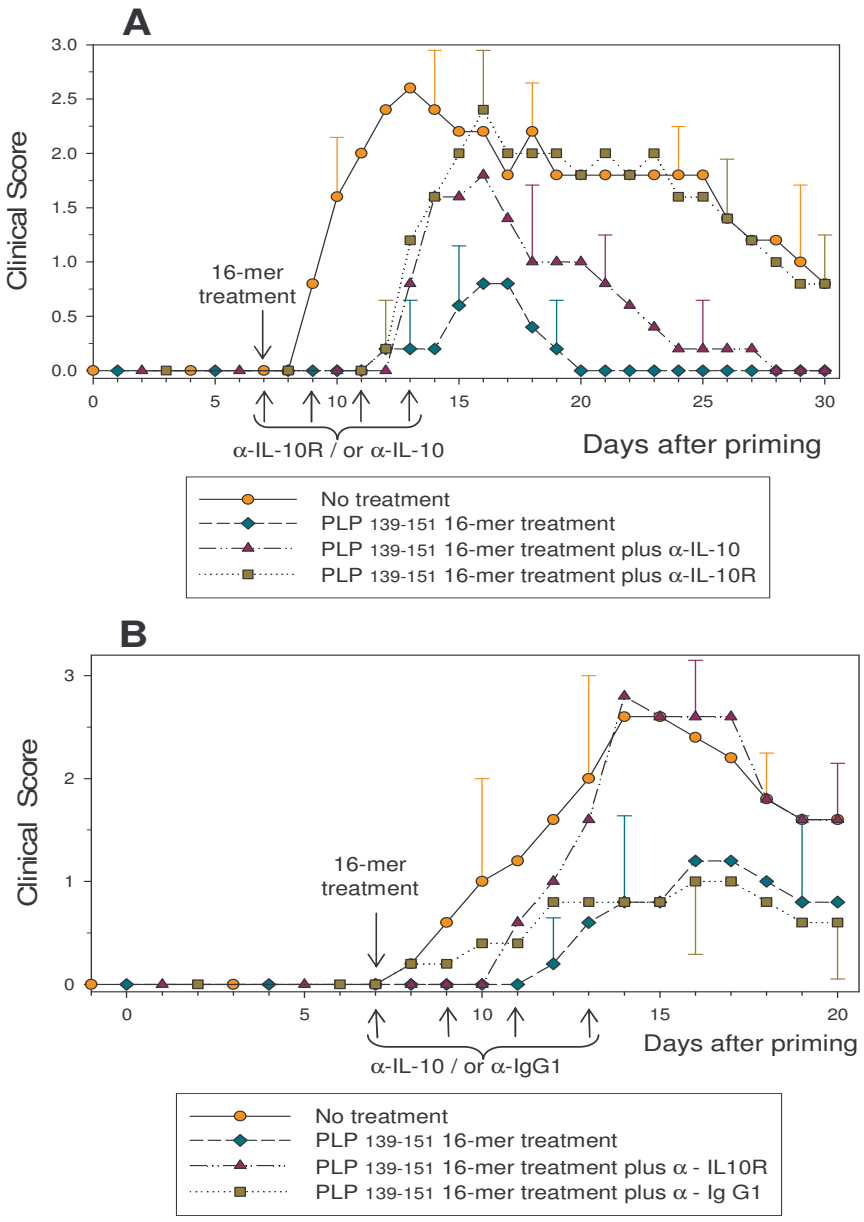


Figure 26. Inhibition of IL-10 reverses oligomer-mediated protection of EAE
Oligomer treated mice were intraperitoneally injected with 0.5 mg of anti-IL 10, anti-IL-10R or IgG1. Injections were performed four times from the day of oligomer-treatment, every two days (on days 7, 9, 11 and 13). A and B represents two independent experiments with similar results. Some of the treated groups received: **(A)** anti-IL-10 or anti-IL-10R antibodies and **(B)** anti-IL-10R or IgG1 antibodies. Untreated mice were included as a control of the experiment. Mice were monitored daily and the mean clinical score was analysed. These results are representative of two independent experiments.

In vivo neutralization of IL-10 with either anti-IL-10 or anti-IL-10R significantly affected the immunomodulatory function of the treatment with PLP 139-151 16-mer. As expected, the clinical disease in treated animals injected with anti-IL-10R was significantly worse than that of animals receiving anti-IL10. It could be explained by a stronger neutralizing effect of anti-IL-10R antibody on IL-10 [229]. Analysis of the clinical EAE score (Figure 26A) shows that oligomer treated mice displayed a peak of relapsing following administration of anti-IL-10R

antibody, reaching similar clinical scores as the untreated animals (mean maximal score of 2.4 and 2.2 respectively). In contrast, oligomer treated mice that did not receive IL-10 blocking antibodies had significantly decreased EAE signs (mean maximal score of 0.8).

Consistent with the previous experiments, the protective effect in oligomer treated mice remained unchanged when mice received the control isotype antibody, and once again it was found that the IL-10 neutralization reversed the suppression of EAE induced during the treatment (Figure 26B).

These results are in line with the enhanced IL-10 secretion upon antigen activation *in vitro* observed in oligomer treated mice in comparison with untreated mice. It indicates that IL-10 plays a critical role in the suppression of EAE induced by PLP 139-151 16-mer treatment, besides IL-10, other cytokines may play important roles during the treatment with the oligomer.

4.9.2 *In vivo* neutralization of TGF-β

To identify the role of other anti-inflammatory cytokines in the PLP 139-151 16-mer induced tolerance to EAE, *in vivo* neutralization of TGF-β was carried out.

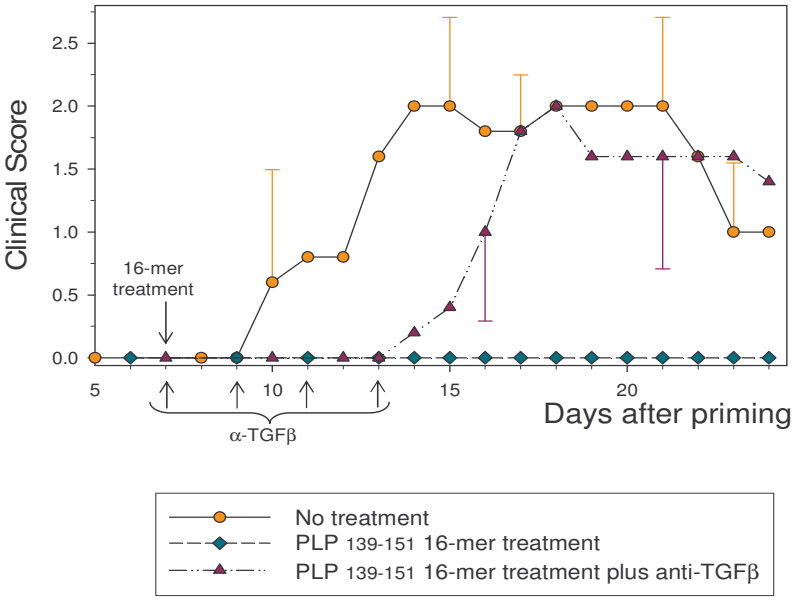


Figure 27. ***In vivo*** **neutralization of TGF-β**
Mice were intraperitoneally injected with 0.5 mg of anti-TGF-β. Injections were performed four times from the day of oligomer treatment every two days (on days 7, 9, 11, and 13 after priming). Mice were scored daily and the mean of the clinical score was analysed. The results shown are from a single experiment representative of two with equivalent results.

Administration of anti-TGF-β completely impairs the ability of the PLP $_{139-151}$ 16-mer to inhibit the EAE development.

Analysis of the clinical EAE score shows that after administration of anti-TGF-β antibody in oligomer treated mice there is a small window of about 4 days in which apparently, it exerts its inhibitory effect and mice start to develop signs of EAE. At this time point (day 13), all treated mice receiving TGF-β blocking antibodies presented EAE symptoms and, at day 18 they reached the same score as the untreated mice (mean maximal score of 2) (Figure 27).

Thus, *in vivo* neutralization of IL-10 or TGF-β reversed the protective effect induced by oligomer treatment. In this sense, the contribution of "regulatory and/or suppressor cells", possibly responsible for the production of IL-10 and TGF-β, might be important.

4.10 Evaluation of the effect of S-Ag fusion proteins in the autoimmune EAE model

Previously, it was shown that repeat antigens consisting of linear copies of a T cell epitope are able to induce tolerance. These compounds have shown to suppress the clinical symptoms in experimental autoimmune model systems of multiple sclerosis, autoimmune neuritis and type I diabetes [155, 156, 170]. The principle of this approach lies in the inherent features of repeated domains within the protein antigens of many protozoan parasites including the genus *Plasmodium* to induce suppression [184, 188]. Based on this characteristic, new constructs containing sequences derived from parasites linked to myelin T cell epitopes were designed.

To determine whether the repeats of the S-antigen have indeed tolerogenic properties, recombinant proteins in which the repeat region was fused to defined CD4 T cell epitopes were generated (Figure 28).

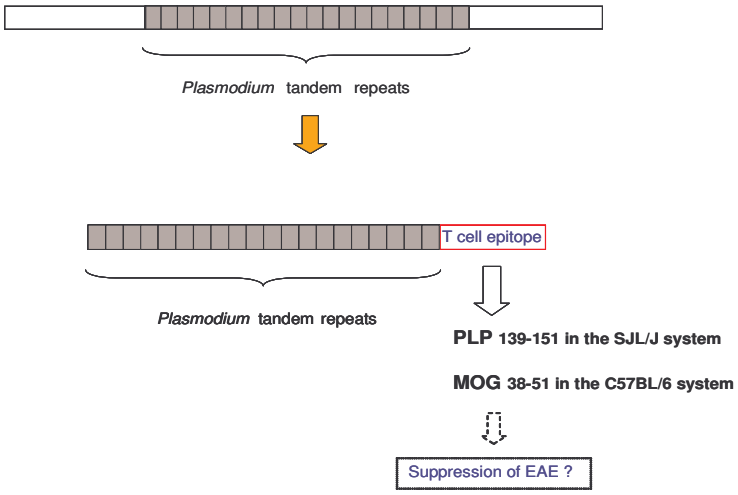


Figure 28. Design of the epitope fusion-proteins with repeat sequences of the S-antigen (S-Ag) of *P. falciparum*
The repeat unit, consisting of 24 repeats of a 8mer unit derived from the NF7 isolate (A(L/R)KSDEAE), was linked to N-terminus of $CD4^+$ T cell epitopes such: PLP 139-151 (S-AgPLP139-151), MOG 38-51 (S-AgMOG38-51), OVA 323-339 (S-AgOVA323-339). Since the natural units contained either L or R in their repeats, building blocks were used in which the two amino acids alternate in tandem repeats.

To evaluate the potential of S-Ag fusion proteins in the context of autoimmune reactions, constructs were generated in which the S-Ag repeat was linked to an epitope derived from the proteolipid protein (PLP) PLP 139-151 /or myelin oligodendrocyte glycoprotein (MOG) MOG 38-51 (Figure 28). SDS-PAGE analysis of the fusion proteins is shown in Figure 29.
PLP 139-151 and MOG 38-51 are encephalitogenic $CD4^+$ T cell epitopes known to induce EAE in SJL/J and C57/Bl6 mice respectively. Analogue to the previous constructs, a fusion protein was generated with the encephalitogenic epitope replaced by OVA 323-339. This fusion protein was used as a control of the experiments.

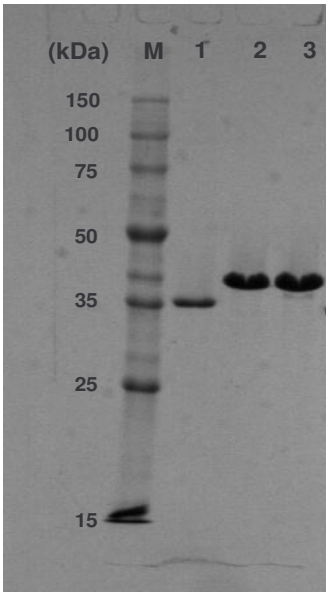


Figure 29. SDS-PAGE analysis of the purified fusion proteins.
Analysis of the expression of epitope fusion-constructs with S-antigen repeats in E. coli. Lane 1: SAg 12-mer (22 KDa); lane 2: SAg 12-mer MOG 38-51 S3 (24Kda); lane 3: SAg 12-mer PLP 139-151 S3 (24 KDa) and lane M: molecular weight markers. The weights calculated from the DNA sequence are lower compared to those estimated from the SDS gel. It is due to abnormal SDS binding (SAg sequence is 38% ASP and GLU) [230].

4.10.1 Amelioration of EAE induced after S-Ag PLP139-151treatment

The fusion constructs were tested *in vivo* using the experimental mouse model of multiple sclerosis EAE. The aim of this study was to determine whether the addition of the repeat region would mediate the induction of antigen-specific tolerance.

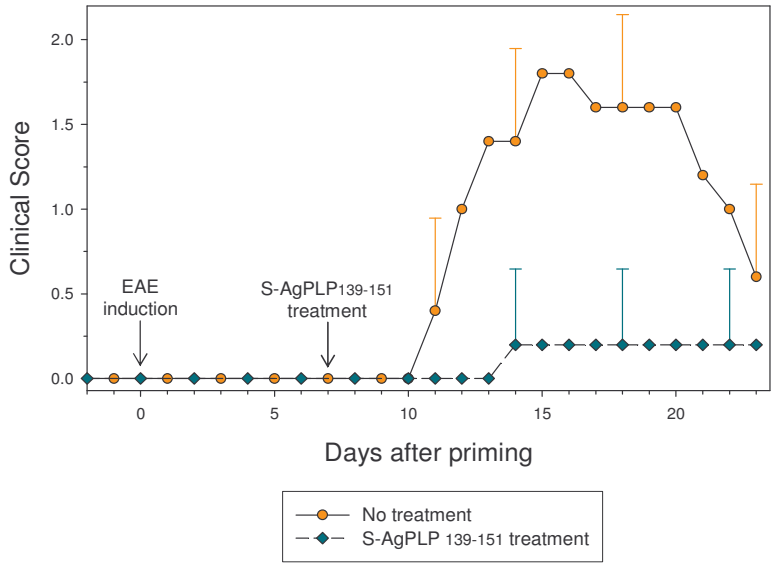


Figure 30. Intravenous treatment with S-AgPLP 139-151 inhibits the progression of EAE in SJL/J mice
Treatment of EAE induced SJL/J mice, was carried out on day 7 after disease induction using 50 µg of S-AgPLP 139-151 administrated intravenously. Disease severity was scored to compare the EAE course between the S-Ag fusion protein treated and not treated mice. The data represent the average of five mice per group. One representative of several experiments performed is shown.

Interestingly, as illustrated in Figure 30 only one injection of the S-Ag fusion construct inhibits the paralysis associated with EAE almost completely. S-AgPLP 139-151 treated mice developed very weak symptoms (with maximal average clinical score of 0.5) at the peak of the disease, and they recovered completely on the next days. In contrast, untreated animals developed EAE with a mean maximal score of 2.7.

Thus, S-AgPLP 139-151 treatment suppressed almost completely the development of EAE, showing the potential of natural repetitive sequences to induce tolerance.

4.10.2 Induced protection against EAE after S-Ag PLP139-151 vaccination is antigen specific

To determine further whether the application of the S-AgPLP 139-151 fusion protein prior to the disease induction could also prevent the EAE development, vaccination experiments were performed. In addition, the antigen specificity was tested by including a fusion protein in which the encephalitogenic epitope was replaced by OVA 323-339 (Figure 31).

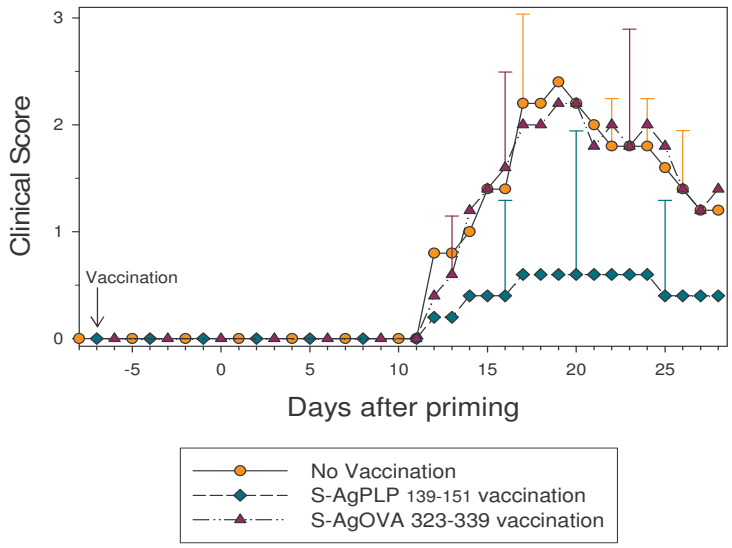


Figure 31. Antigen specific effect of S-AgPLP 139-151 vaccination to suppress the EAE progression
SJL/J mice were vaccinated 7 days before disease induction with 50 µg of S-AgPLP 139-151 or S-AgOVA 323-339. EAE was induced with 50 µg of the encephalitogenic PLP 139-151 peptide and next day animals received 200 ng of *Pertussis* toxin. Mice were monitored daily and the mean clinical score of five mice per group was plotted. One representative experiment of two performed is shown.

S-AgPLP 139-151 vaccination rendered mice tolerant to EAE. There was a significant decrease in the severity of the disease in S-AgPLP 139-151 vaccinated mice as they had a maximal mean clinical score of 0.6 in comparison with untreated mice which showed a maximal mean score

of 2.4. Remarkably, S-Ag fusion constructs that contain non-encephalitogenic epitopes were not able to suppress EAE. S-AgOVA 323-339 vaccinated mice had a score of 2.4 which is comparable to the score seen in untreated mice (Figure 31).

Therefore, both treatment and vaccination with the S-AgPLP 139-151 fusion protein are effective in prevention of EAE. The effect is highly antigen-specific, since the treatment with a fusion protein that contains an unrelated epitope did not have any effect in the modulation of EAE.

4.10.3 Repetitive sequence in a free form from the self-epitope has no effect in the course of EAE

The next step was to test the effect of S-Ag repeat plus free PLP 139-151 peptide compared with the effect induced by S-AgPLP 139-151 fusion protein. Treatment with S-AgMOG 38-51 was carried out to evaluate the antigen specificity of the fusion protein.

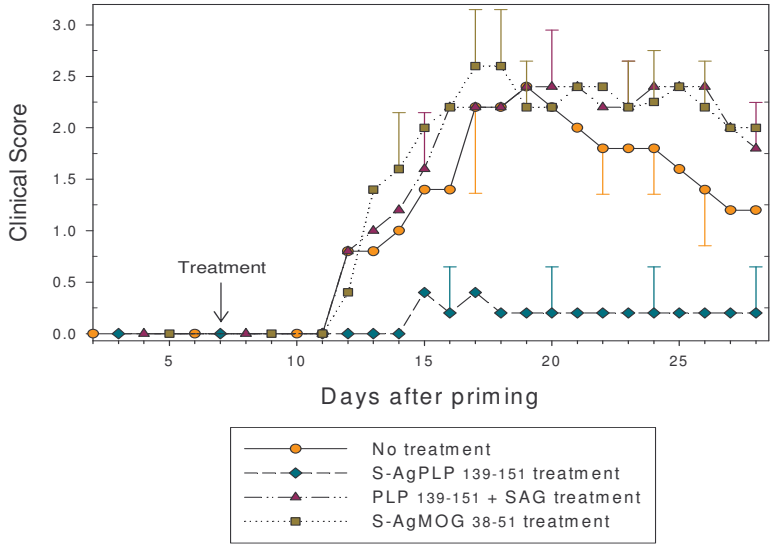


Figure 32. A physical link between the T cell epitope and the repeat structure is required to induce protection against EAE

EAE was induced in SJL/J mice by priming with 50 µg of PLP 139-151 peptide. Mice were treated intravenously with S-Ag repeat only (50 µg) or S-Ag repeat plus free PLP 139-151, using the corresponding amount present in the fusion protein: 45 µg of S-Ag and 3.3 µg of PLP 139-151. Treatment with the S-AgMOG 38-51 was performed as a control of the experiment. Mice were monitored daily and the mean clinical score of five mice per group was plotted. One representative experiment of two performed is shown.

Experiments confirmed that the S-AgPLP 139-151 effectively suppressed the clinical symptoms of the disease. Notably, the effect was evident only with the fusion protein and not with a mixture of S-Ag and free PLP 139-151 peptide indicating that a physical link between the T cell epitope and the repeat structure is required (Figure 32).

Mice treated with the non-antigen specific fusion protein or with the repetitive sequence lacking the self-epitope had a similar course of EAE as untreated controls; they had a mean maximal score of 2.6, 2.4 and 2.4 respectively. In contrast, mice which received S-Ag PLP 139-151 were almost completely protected from EAE, showing a mean maximal score of 0.4.

4.10.4 Control of EAE by S-Ag fusion constructs is also successful in the C57/Bl6 system

To evaluate the potential of S-Ag fusion proteins in the general context of autoimmune reactions, constructs were tested in a different system. C57/Bl6 mice susceptible for EAE induction by an epitope derived from the myelin oligodendrocyte protein (MOG 35-55) were treated with S-Ag constructs in which the repeats were fused to this peptide (Figure 33).

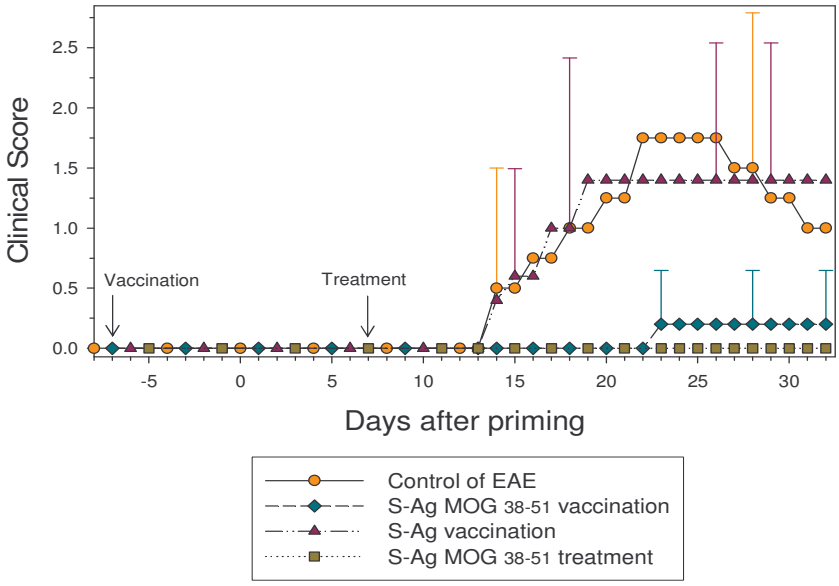


Figure 33. Control of EAE by S-Ag fusion constructs in the C57/Bl6 system

EAE was induced in C57/Bl6 mice by priming with 50 µg of MOG 35-55 peptide. The next day animals received 500 ng of *Pertussis* toxin. Mice were vaccinated 7 days before disease induction with 50 µg of S-AgMOG 38-51 or 45 µg of S-Ag repeat only (not linked to the self-epitope). Treatment was carried out on day 7 after EAE induction with 50 µg of S-AgMOG 38-51. Mice were monitored daily and the mean clinical score was analyzed. One representative experiment of two performed is shown.

EAE control by S-Ag fusion constructs was successful not only in the SJL/J system but also in the EAE-susceptible strain C57/Bl6. The efficacy of the treatment was evident when the antigen was given in form of a vaccination 7 days before priming or as therapeutic intervention on day 7 after disease induction (Figure 33).

The potential unspecific effect of the S-Ag repeat (repetitive sequence lacking the T cell epitope) in the modulation of EAE was ruled out in these experiments because S-Ag vaccinated mice showed a mean maximal score of 1.4, comparable to the 1.8 score seen in the control group (mice which did not receive any treatment or vaccination). In contrast, S-Ag MOG 38-51 vaccinated mice had significantly decreased maximal EAE signs (maximum score of 0.2) and S-Ag MOG 38-51 treated mice were completely resistant to EAE.

4.10.5 Role of IL-10 in the S-Ag MOG 38-51 mediated protection of EAE

To confirm that suppression of EAE is in fact due to the induction of active tolerance, the IL-10 receptor (IL-10R) was blocked in vivo by injection of neutralizing antibodies.

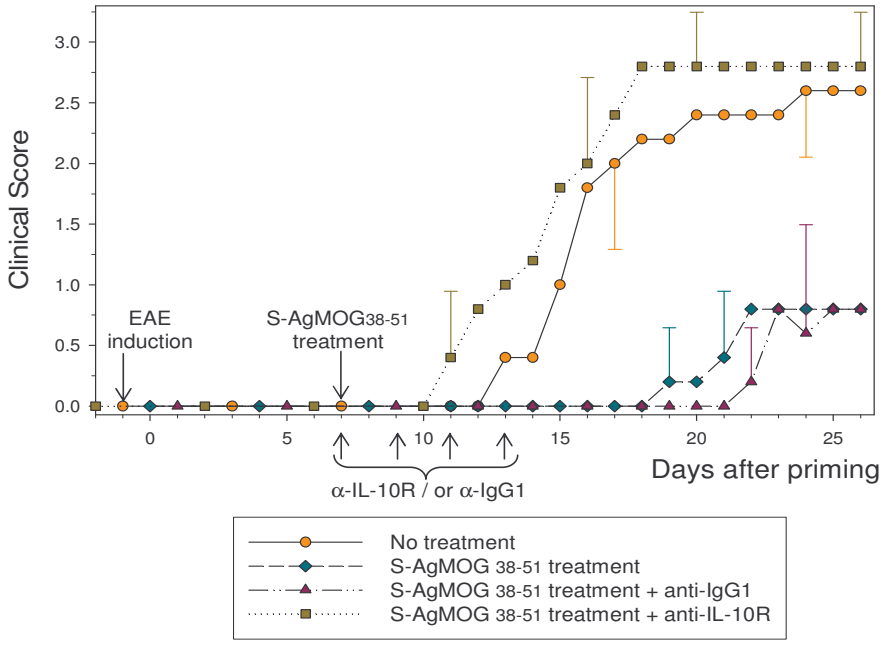


Figure 34. Reversal of S-AgMOG 38-51 induced suppression by anti-IL-10R antibodies
EAE was induced in C57/Bl6 mice by priming with 50 µg of MOG 35-55 peptide. Mice were treated on day 7 with the S-AgMOG 38-51 fusion protein by a single intravenous injection of 50µg. Some of the treated groups were intraperitoneally injected with 0.5 mg of anti-IL-10R or IgG1. Injections were performed four times from the day of treatment, every two days (on days 7, 9, 11 and 13). Disease progression was followed and the mean clinical score was analyzed. One representative experiment of two performed is shown.

Administration of anti-IL-10R completely abrogated the ability of the S-AgMOG 38-51 fusion protein to inhibit the EAE development. In contrast, the protective effect in treated mice remained unchanged when they received the control isotype antibody.

Analysis of the clinical EAE score (Figure 34) shows that treated mice which received neutralizing IL-10 antibodies displayed a maximal average clinical score of 2.8, similar to untreated animals (mean maximal score of 2.6). In contrast, S-Ag MOG 38-51 treated mice

that did not receive IL-10 blocking antibodies and treated mice which received control IgG1 antibody had significantly decreased EAE signs (mean maximal score of 0.8 in both cases).

Using the C57/Bl6 EAE model, the experiment revealed that the injection of anti-IL-10R antibody completely reversed the suppressive effect of the S-Ag fusion protein. Thus, suppression is mediated by the release of the immune suppressive cytokine IL-10. These results highlight an important role for regulatory cytokines in the S-Ag fusion protein induced suppression of EAE.

Repetitive sequences of parasites, which play an important role in the mechanisms for evading host immune responses, are commonly associated with the induction of suppression. This fact could represent an advantage that can be applied in the regulation of autoimmune processes.

5 DISCUSSION

The use of highly repetitive sequences in the design of vaccines and as potent T-cell independent or T-dependent B cells inducers has been subject of investigation. Depending on the conditions and the experimental aim, synthetic peptides in an oligomerized form can either promote strong immune response or halt the immune system [155, 168].

These experiments have shown improvement in the antigenicity of the encephalitogenic T cell PLP $_{139\text{-}151}$ epitope after oligomerization. This effect is provided by the ability to form multivalent arrays of peptide-MHC complexes on the surface of the antigen presenting cells. In consequence, after cross-linking with class II MHC molecules, activation and upregulation of costimulatory molecules relevant for the immune response are induced [168], but on the other hand, high zone tolerance could also be induced with relatively low dosages of the multivalent antigen [155]. Basically, the modulation of the immune response by multimers lies on the dosage, time and route of administration. It has been proposed that a quantitative characteristic determines the induction of either tolerance or immunization [231].

The therapeutic effect driven by repetitive antigens has been proven in different autoimmunity models such as in experimental immune encephalomyelitis (EAE), experimental autoimmune neuritis (EAN) and diabetes with successful results. [155, 156, 170]. The aim of this study is the determination of the EAE protective effect by oligomers when given as a therapeutic treatment, comparing different routes of administration, as well as the characterization of mechanisms underlying the oligomer induced suppression.

The model used in this work was SJL (I-A^s) in which EAE, characterized by relapsing and remitting peaks, is induced by the encephalitogenic T- cell epitope PLP $_{139\text{-}151}$ of proteolipid protein (PLP) [232]. Oligomer 16-mer (PLP $_{139\text{-}151}$ 16-mer), which consists of multiple copies of the T cell epitope (PLP $_{139\text{-}151}$) connected by spacer sequences, was used for treatment of EAE.

Regarding the use of repetitive epitopes in prevention or treatment of autoimmune diseases, therapeutic treatment and vaccination experiments were carried out. These experiments showed that administration of PLP $_{139\text{-}151}$ 16-mer suppresses almost completely the appearance of EAE, which is consistent with previous reports [155]. Different routes of oligomer injection showed also to be successful when used for vaccination or treatment of EAE. The broaden effectiveness of oligomerized peptides to induce tolerance has been discussed in the context of high zone tolerance [168] understood as a threshold level in the number of antigen recognition sites associated by repeating antigenic determinants [231, 233]. Several reports have demonstrated the induction of tolerant effects based on the persistence of

antigen after injection of high-dose antigen or continual injections of low dose soluble antigens [154, 234, 235]. This represents another advantage for the use of oligomerized sequences, as only one injection in a relatively low concentration is able to induce a long lasting suppressive effect.

More importantly, in this study it is also demonstrated that PLP 139-151 16-mer was effective in the treatment of established EAE in SJL/J mice. Oligomer administration after appearance of the first clinical symptoms reversed ongoing EAE. It is noteworthy that soon after oligomer injection, mice entered in a remission phase controlling completely the development of EAE. The therapeutic effect of the oligomer, seen along these experiments, has important implications for its potential use in the control of human autoimmune diseases.

In this work, it was shown that the protection induced by oligomer T cell epitope PLP 139-151 was dependent of antigen specificity. For this purpose, an unrelated oligomer (HA 107-119 4-mer) containing the epitope derived from the influenza virus, which also contains the same spacer sequence as the PLP 139-151 16-mer between repeats, was used. Antigen specificity of the oligomer used in this study could represent a therapy that allows the specific tolerization of auto-reactive immune cells. The importance of antigen specific therapy that involve T cell recognition for the regulation of autoimmune diseases has been matter of investigation and several reports have shown that this is one of the most reliable approaches to treat or prevent autoimmune disorders [236, 237].

The oligomer induced suppressive effect after oligomer treatment was reflected by the absence of clinical disease; however, it did not induce antigen-specific unresponsiveness. Isolated cells from oligomer treated mice readily proliferated after *in vitro* activation with the autoantigen (PLP 139-151). Similar findings have been documented in experimental treatments of EAE, using high doses of the antigen, DNA encoding self peptides and recombinant TCR ligands, in which tolerized animals showed a normal proliferation against self antigens [209, 238]. Nevertheless, tolerance mechanisms that involve anergy or deletion of antigen-specific cells are not consistent with the observation in this study.

A feature of multivalent antigens is the ability to induce B cells for the antibody production with the help of specific $CD4^+$ cells [178, 239]. The results of the present study showed that in fact, treated mice had higher antibody titers than non-treated mice. Even when transfer experiments did not show any protective role for factors present in serum of treated donors (data not shown), the possibility that antibodies generated by the oligomer may have an *in vivo* function is not ruled out. Standardisation of different concentrations of serum for transfer

experiments as well as transfer with purified immunoglobulins of treated mice might represent an alternative approach to be assessed.
Some investigations have driven the interpretation that, similar to the MHC crosslinking with multivalent antigens, a clustering of oligomerized peptides with immunoglobulin receptors may take place [240], competing for the target antigen and rendering to a suppressive status. This, together with formation of immune complexes and its relation with inhibitory Fc receptors [241], is a subject for future research.

One of the important observations was that PLP $_{139\text{-}151}$ 16-mer treatment markedly reduced leukocyte infiltration in the brain and spinal cord, thus indicating its therapeutic potential in the regulation of the migratory capacity of cells into the CNS. This mechanism might lead to inhibition of EAE development in treated mice, possibly modulated by the expression of integrins and chemokines, recruiting autoreactive T cells in the periphery. In line with this observation, the analysis of activation, regulatory and migration markers were carried out in this study. There were not differences in the regulation of any marker or cell subset between oligomer treated and untreated mice, just a consistent slight increase in the expression of CD103 ($\alpha E\beta 7$ integrin) in treated animals. Recent reports support the role of $CD4^+CD103^+$ and $CD8^+CD103^+$ cells as negative regulators of T cell activation in several autoimmune diseases [215, 216]. The general importance of $CD103^+$ expression as a regulatory marker and in the recruitment of lymphocytes in peripheral tissues might be a significant feature for the PLP $_{139\text{-}151}$ 16-mer treatment. It would be interesting to know whether this molecule is directly correlated with the inhibition of migration of pathogenic cells to the CNS. This can be done by the use of immunosuppressive strategies based on adoptive transfer or by the use of CD103-deficient mice. Thus, the relevance of antigen specific CD103-expressing cells in the oligomer induced protection represents an important issue to be investigated.
Many reports have shown that interfering with the process of leukocyte infiltration into the CNS represents one of the most reliable means to modulate inflammation in neurological diseases [242]. The development of novel inhibitors of CNS inflammation that blocks the migration of pathogenic T cells provide a helpful approach currently used for the treatment of EAE and MS [243-246].
In the context of cytokine production, it was demonstrated that PLP $_{139\text{-}151}$ 16-mer treatment induced the IL-10 production, as observed after *in vitro* stimulation in total lymph node cells and in B cells. A similar pattern has been showed after EAE treatment with molecules containing repetitive sequences such as Glatiramer Acetate, in which cell lines established

with copolymers produce high amounts of IL-10 and their adoptive transfer inhibit the EAE development [166]. The role of IL-10 in the protection of EAE has been widely described; it is often up regulated in remission phases of the disease [215] and its deficiency is associated with susceptibility [86].

The outcome of these experimental data, using multimerized epitopes for EAE treatment, reflects the significance of a biased immune response for the secretion of the suppressor cytokine IL-10. The results also showed that suppression is not due to the inability of PLP-specific T cells to respond against the autoantigen, but most likely resulted from the occurrence of suppressive cytokines after antigen specific stimulation.

Interestingly suppression was actively transferred by cells from oligomer treated mice. This finding was consistent after transfer with total lymph node cells, $CD4^+CD25^+$ and B cells. The protective effect observed after adoptive transfer of B cells from treated mice is consistent with previous reports, which have demonstrated a regulatory role of B cells that is mainly mediated by IL-10 [85, 86]. B cells have been involved in the regulation of immune responses in infections and autoimmune diseases, as they are able to tolerize antigen specific $CD8^+$ and $CD4^+$ T cells [247-249]. Some studies indicate that treatment with B cells that produce IL-10, can modulate the course of arthritis, inflammatory bowel disease and EAE [85, 86, 250]. In addition, mice previously transferred with B cells from oligomer treated mice showed no disease symptoms after a second immunization, when compared with untransferred mice, which once again were paralyzed. Memory immune response induced by these means would deserve a more detailed study in long-term experiments.

The results show that B cell *in vitro* activation only with anti-CD40 induced more IL-10 secretion in oligomer treated mice than in the untreated ones. This observation might suggest that these B cells are readily activated and then, they can be stimulated by CD40 costimulation in an antigen-independent manner [226]. *In vivo* IL-10 production is one of the possible mechanisms by which B cells from oligomer treated animals transfer suppression of EAE.

For the phenotypic characterization of B cells, the frequency of $B220^+$ lymphocytes that express IgM, IgD, CD21, CD23 and CD24 was analyzed. The results show that the reduced percentage of $CD24^+$ cells found in untreated mice seems to reflect the very small numbers of immature B cells (IgM^+IgD^-) in this group of animals. This is consistent with previous findings in which the pre–B cell pool is markedly diminished in a number of autoimmune disorders [223]. In this way, CD24 has also been characterized to play an important function in the control of homeostasis and autoimmune diseases in both mice and humans [251].

Analysis of B cells from oligomer treated and naive mice showed the same phenotype. This population, which seems to correspond to a B cell subset expressing high levels of CD24, CD21, CD23 and IgM, is identified as transitional 2-marginal zone precursor (T2-MZP) B cells [87]. Recent findings have shown a regulatory role for this special subset of transitional B cells (T2-MZP) present in naive mice, as these cells can undergo antigen-independent proliferation after transfer and, hence, they produce IL-10 [87]. This could explain the ability of naive B cells to transfer a partial suppression of EAE, since the inflammatory conditions found in the host might induce activation and hence, the production of IL-10.

Given that the comparative analysis of B cells between treated and naive mice showed no significant difference, it is uncertain whether the PLP $_{139\text{-}151}$ 16-mer treatment induces a kind of modulation in the development and/or function of B cell subsets. Thus, it is possible that humoral immune responsiveness detected in treated mice have an effect on the regulation of the production of B lymphocytes in the bone marrow and on the supply of these cells to the peripheral lymphoid tissues.

Results from adoptive transfer experiments regarding the partial protection of EAE induced by naive B cells might be also explained in terms of "homeostasis", as these cells introduced into diseased mice with some sort of lymphocyte deficiency might supply the conditions to recover the stable size of the naive B cell pool. B cell deficient mice will be valuable in future research for a better understanding of the role of B cells in the oligomer-induced suppression.

In several experimental systems, protection from autoimmune diseases has been attributed to a population of $CD4^+CD25^+$ cells and many studies use surface expression of CD25 to distinguish conventional T cells from T regulatory cells [215]. In the present study the transfer of $CD4^+CD25^+$ cells from oligomer treated mice conferred partial protection against the EAE development. Similarly, the *in vivo* suppression mediated by 16-mer was not reversed completely by CD25 depletion. These data suggest that $CD4^+CD25^+$ regulatory T cells are important but may not play a pivotal role during the oligomer tolerance induction. The potential role of $CD25^-$ cells is not ruled out, there exists a population induced in the periphery that expresses $CD4^+CD25^-Foxp3^+$ and has suppressive functions [202]. Similarly, a subpopulation with regulatory functions $CD25^-$TGF-β dependent has been described [220].

In future experiments, a model with DEREG mice in which all $Foxp3^+$ cells are depleted with diphteria toxin would be useful to explore the role of T regulatory cells in the oligomer induced protection. [63].

Furthermore, the suppressive function observed after transfer of $CD4^+CD25^-$ subpopulation could be explained by the fact that upon adoptive transfer, the expression of CD25 in donor-

derived cells is not completely stable [252]. For that reason, some $CD4^+CD25^+$ cells could appear in $CD4^+CD25^-$ T cells of transferred animals and vice versa. Therefore, the possibility that regulatory or suppressor T cells other than those expressing CD25 might be induced during the PLP 139-151 16-mer treatment, cannot be excluded. Both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells could have a role in the production of suppressive cytokines as previously described in oligomer treated animals.

The roles of immunomodulatory cytokines such as IL-10 and TGF-β were examined *in vivo* after blocking with neutralizing antibodies. The effects of oligomer treatment observed *in vitro*, concerning the IL10 production, was reflected *in vivo* in cytokine neutralization experiments. These results suggest that the secretion of suppressive cytokines produced by PLP 139-151 16-mer treated mice is important for the protection against the EAE development. Neutralization of IL-10 or IL-10 receptor and TGFβ in oligomer treated mice resulted in the lost of protection.

According to a number of studies, IL-10 has shown to have several down-modulatory functions that may diminish autoimmune pathologies. Some of these reports illustrate that IL-10 down-regulates p40 synthesis and thus the IL-12 production needed for the formation of the inflammatory T cells response in EAE [253]. Moreover, IL-10-deficient mice develop more severe EAE symptoms than wild-type mice, and some IL-10 promoter polymorphisms are associated with progression of multiple sclerosis [254] [255]. These facts suggest a natural function for IL-10 in disease activity. This cytokine has also been implicated in various immunotherapeutic models in which administration of IL-10 can inhibit EAE symptoms [199, 256].

Likewise, *in vivo* neutralization of TGF-β reversed the protective effect induced by PLP 139-151 16-mer treatment. TGF-β is one key contributing molecule to peripheral tolerance. TGF-β deficient mice have marked inflammation in multiple organs and abrogation of TGF-β signalling in T cells results in spontaneous T cell differentiation and autoimmune disease [257], indicating its essential function in T cell homeostasis and in the prevention of inflammatory autoimmunity [258].

The observation that TGF-β is also critical for the protective activity generated by the oligomer treatment could address a possible synergistic function with IL-10, since blocking just one of these cytokines induced a breakdown of tolerance.

IL-10 and TGF-β are considered key mediators of peripheral tolerance. It is plausible that the regulation of IL-10 depends of the presence of TGF-β and vice versa. There is a noteworthy suppressive synergy between IL-10 and TGF-β. For example, IL-10 renders activated T cells responsive to TGF-β by promoting their expression of the critical RII portion of the TGF-β receptor [259]. It is also plausible that different subpopulations of T regulatory cells (Tr1 cells), which produce both cytokines IL-10 and TGF-β and/or Th3 that mainly produce TGF-β [46, 260], might have an important role during the oligomer treatment.

In contrast to the recent report of type I diabetes, in which $Foxp3^+$ CD4 T cells were accumulated and found to play a function in the tolerance induced by multimerized self peptides [170], oligomer treatment was not correlated with increased numbers of $Foxp3^+$ or $CD25^+$ cells. These observations emphasize a central role of regulatory cytokines Il-10 and TGF-β in the oligomer induced suppression of EAE.

One of the important observations in this study was the finding that PLP 139-151 16-mer could be useful as therapeutic treatment, even when clinical symptoms of EAE have already appeared and this is correlated with the induction of active tolerance. The preventive and therapeutic effects of the oligomer, seen along these experiments, have important connotations for their potential use in the control of human autoimmune diseases. The contribution of "regulatory and/or suppressor cells" during the oligomer treatment could occur through the production of IL-10 and TGF-β. It has been widely demonstrated that active suppression is one of the major mechanisms of peripheral tolerance mediated principally by these suppressive cytokines [78, 261].

Identification of the oligomer target and antigen specific cells that generate a suppressive response, shifting to an anti-inflammatory status, would provide further insight into the precise mode of action of multimerized peptides and the way they control pathogenesis of autoimmune diseases.

The second part of the present thesis described the use of repetitive sequences derived from parasites as a reliable mean to induce tolerance. Proteins containing tandemly repetitive sequences are present in several immunodominant protein antigens in pathogenic protozoan parasites and are important in mechanisms for evading host immune responses, commonly associated with the induction of suppression. However repeat arrays from parasites of the genus *Plasmodium* have been found to be characterized by a particularly high level of

evolution and relatively recent repeat array expansion in comparison with repeat arrays from other organisms [172] [173].

Induced immunosuppression by natural repeat antigens seems to have evolved as a mechanism of evasion that induce a strong but ineffective immune response, allowing the parasite to avoid immune mediated clearance by the host. There are several reports about the relevance of suppressor mechanisms to downregulate effector immune responses in malaria disease, they are mainly characterized by an increased frequency of suppressor cells and production of modulatory cytokines [184, 190].

In this study, repeat sequences of parasites have been identified to induce antigen-specific tolerance when linked to defined T cell epitopes (S-Ag fusion proteins). S-Ag fusion proteins containing encephalitogenic epitopes from myelin antigens could suppress EAE. The physical link between the T cell epitope and the repeat structure showed to be necessary for the efficacy of the treatment. This protective effect was also found to be antigen-specific and mediated by a process of active tolerance that involve the action of suppressive cytokines like IL-10, possibly through the induction of suppressor T cells specific for the epitope linked to the repeat region. Additionally, the efficacy of the treatment was evident when the antigen was given in form of a vaccination or as therapeutic intervention.

Therefore repeat antigens could represent a strategy to trigger immune escape by the induction of active tolerance, inducing the generation of suppressor cells against the self. Since the presentation to the host immune system of parasite-derived repetitive sequences constitute an important evolutionary mechanism of evasion, the coupling of T cell-epitopes to immunogenic repeat antigens can, therefore, be an effective way to induce antigen-specific tolerance o the epitope linked.

The fact that S-Ag fusion proteins have demonstrated to be effective in inducing suppressive responses might have application in the vaccine design directed to the control of many different cell-mediated autoimmune diseases.

6 SUMMARY

The aim of this PhD work was the characterization of the principles of suppression and identification of the cellular mechanisms underlying the oligomer (PLP 139-151 16-mer) induced protection of EAE. The tolerogenic capacity of repeat antigens to control the development of EAE was carried out by using the SJL/J mouse EAE model. It was shown that the protection induced by oligomer T cell epitope PLP 139-151 was dependent of antigen specificity. Importantly, it is also demonstrated that PLP 139-151 16-mer was effective in the treatment of established EAE as its administration after appearance of the first clinical symptoms reversed ongoing EAE. Another important observation was that PLP 139-151 16-mer treatment markedly reduced leukocyte infiltration in the brain and spinal cord. Interestingly, suppression was actively transferred by cells from oligomer treated mice and this finding was consistent after transfer with total lymph node cells, $CD4^+CD25^+$ and B cells. Cytokine analysis of cells during the oligomer treatment showed increased production of IL-10 and suggested that suppression is not due to the failure of PLP-specific T cells to respond against the autoantigen, but resulted from the occurrence of suppressive cytokines after antigen specific stimulation. The importance of suppressive cytokines was demonstrated by the inhibition of the protective effect mediated by the epitope-oligomer after *in vivo* blocking of either IL-10 or TGF-β cytokines. Active suppression provoked by oligomerized peptides was partially mediated by $CD4^+CD25^+$ cells, which could be associated with the occurrence of immunosuppressive cytokines.

The second part of the present thesis describes the use of parasite-derived repetitive sequences containing self epitopes as a reliable mean to induce tolerance. The fusion constructs that consist of a recombinant protein containing repetitive sequences from the *Plasmodium falciparum* S-Antigen linked to myelin T cell epitopes, were evaluated in the autoimmune EAE model for the ability of inducing tolerance. The efficacy of the treatment was evident when the antigen was given in form of a vaccination or as therapeutic intervention. The physical link between the T cell epitope and the repeat structure showed to be essential for the effectiveness of the treatment. This protective effect was also found to be antigen-specific and mediated by a process of active tolerance that includes the action of suppressive cytokines like IL-10, probably by the induction of suppressor T cells specific for the epitope linked to the repeat region.

In summary, this work strongly supports an immunosuppressive role of repeat antigens to control the development of EAE with potential applications to rational vaccine design and treatment of autoimmune diseases.

ZUSAMMENFASSUNG

Das Ziel der eingereichten Doktorarbeit ist die Untersuchung der zellulären Mechanismen, welche der Oligomer-induzierten Suppression von Autoimmunerkrankungen zu Grunde liegt. Die tolerogenen Eigenschaften dieser ‚repeat Antigene' (PLP 139-151 16-mer) wurden hierfür am Beispiel der experimentellen autoimmunen Enzephalomyelitis (EAE) anhand eines SJL/J Maus-EAE-Modells näher charakterisiert. In dieser Arbeit konnte gezeigt werden, dass die Protektion durch das Oligomer antigenspezifisch ist. Zudem konnte gezeigt werden dass eine therapeutische Wirkung bei der Behandlung bestehender EAE möglich ist, da die Verabreichung des 16-mers nach Auftreten der ersten klinischen Symptome zu einer deutlichen Rückentwicklung der EAE führte. Insbesondere auf Grund dieser therapeutische Wirkung des Oligomers könnten repeat-Antigene somit potentielle Bedeutung für die Behandlung von humanen Autoimmunerkrankungen besitzen. Als Grundlage dieses Effekts konnte dabei nachgewiesen werden, dass das 16-mer durch die Aktivierung von Suppressorzellen das Eindringen von Leukozyten in das zentrale Nervensystem zu unterdrücken scheint. Der Nachweis, dass die Suppression aktiv durch Suppressorzellen erfolgte gelang durch den apdoptiven Transfer entsprechender Lymphozytenpopulationen von oligomerbehandelten Mäusen, insbesondere von $CD4^+CD25^+$ Zellen. Die *ex vivo* Zytokinanalyse während der Behandlung ergab dabei eine erhöhten Produktion von IL-10.

Die Ergebnisse deuten somit darauf hin, dass die Suppression nicht durch die direkte Einwirkung des Oligomers auf die autoreaktiven T Zellen ausgelöst wird, sondern indirekt auf der antigen-spezifische Induktion suppressiver Zytokine beruht. Die Bedeutung der suppressiven Zytokine wurde durch die Aufhebung des protektiven Effekts der Oligomere nach *in vivo* Blockade mittels IL-10R- oder TGF-β-spezifischer Antikörper bestätigt. Die aktive Suppression durch oligomerisierte Peptide konnte somit zumindest teilweise durch die Aktivierung von $CD4^+CD25^+$ Zellen erklärt werden, die mit dem Auftreten dieser immunsuppressiven Zytokine in Zusammenhang gebracht werden können.

Im zweiten Teil der Arbeit wurden ‚natürliche' Epitopoligomere untersucht. Grundlage hierfür waren Fusionskonstrukte, welche auf repetitive Regionen von Parasitenproteinen beruhen. In dem untersuchten Fall wurden repetitive Sequenzen des S-Antigens des Malariaerregers *Plasmodium falciparum* an Myelin T Zellepitope gekoppelt und im EAE-Modell in Hinblick auf ihre Fähigkeit zur Toleranzinduktion untersucht. In der Studie konnte dabei gezeigt werden, dass die repeat Region des Malariaerregers in der Tat tolerogene Eigenschaften besitzt, welche Wirkung bei Vakzinierungen oder therapeutischem Anwendungen bei der EAE zeigte. Vorläufige Studien ergaben dabei, das hierfür eine direkte

physikalische Verbindung zwischen dem T Zell Epitop und der ‚repeat' Struktur bestehen muss. Auch hier war der protektive Effekt antigenspezifisch und basierte auf ‚aktiver Toleranz'. Die Wirkung wurde durch suppressive Zytokinen wie z.B. IL-10 vermittelt, welches vermutlich von Suppressorzellen ausgeschüttet wird. Die Charakterisierung dieser Zellen und der genauen Wirkungsweise des S-Antigens würde somit neue Einsichten in parasitäre Toleranzmechanismen geben und könnte Grundlage neuer Werkzeuge für eine mögliche Kontrolle der Pathogenese von Autoimmunkrankheiten sein.

Zusammenfassend lässt sich sagen, dass diese Arbeit neue Erkenntnisse hinsichtlich der immunsuppressive Rolle von ‚repeat' Antigenen liefert und neue Perspektiven zur Entwicklung von Wirkstoffen mit potenzieller Anwendung in der Prävention und Behandlung von Autoimmunerkrankungen liefert.

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8 APPENDIX

8.1 ABBREVIATIONS

APC: Antigen presenting cell

APC: Allophycocyanin fluorochrome

BBB: Blood-brain barrier

CCR: Chemokine (C-C motif) receptor

CNS: Central nervous system

CPM: Counts per minute

CS: Circumsporozoite surface protein

CTLA-4: Cytotoxic T lymphocyte–associated antigen 4

CXCR: Chemokine (C-X-C motif) receptor

DC: Dendritc cells

EAE: Experimental autoimmune ecephalomyelitis

ELISA: Enzyme-linked immunoabsorbent assay

FACS: Fluorescence activated cell-sorting

Fas: Apoptosis stimulating fragment

FITC: Fluorecein isothiocyanate

ICAM-1: Intracellular adhesion molecule-1

IDO: Indoleamine 2,3- dioxygenase

IFN: Interferon

IPEX: Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

JNK: jun N-terminal kinase

LFA-1: Lymphocyte function-associated antigen-1

LPS: Lipopolysaccharide

MACS: Magnetic activated cell sorting

MBP: Myelin basic protein

MHC: Major Histocompatibility Complex

MMP: Multiple matrix metalloproteinase

MOG: Myelin oligodendrocyte glycoprotein

MS: Multiple sclerosis

NK: Natural Killer cells

NO: Nitric oxcide

PBS: Phosphate buffered saline

PE: Phycoerythrin fluorochrome

PLP: Proteolipid protein

PMA: Phorbol 12-myristate 13- acetate

PSGL-1: P-selectin glycoprotein ligand 1

RPMS: Relapsing-progressive form of multiple sclerosis

RRMS: Relapsing-remitting form of multiple sclerosis

STAT: Signal transducers and activators of transcription

TCR: T cell-receptor

mTECs: Medullary thymic epithelial cells (mTECs)

TGF: Transforming growth factor

Th: T helper cells

Th1: T helper 1

Th2: T helper 2

TNF: Tumor necrosis factor

Tr1: T-regulatory

Treg: T regulatory cells

VCAM: Vascular cell adhesion molecule

VLA: Very late activation antigen

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8.3 PUBLICATIONS

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