LOW DOSE TOLERANCE VACCINE PLATFORM, REOVIRUS PROTEIN SIGMA 1 AND TREATMENT OF AUTOIMMUNITY

by

Agnieszka Rynda

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Veterinary Molecular Biology

MONTANA STATE UNIVERSITY
Bozeman, Montana

August 2008

APPROVAL

of a dissertation submitted by

Agnieszka Rynda

This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citation, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

Dr. David W. Pascual

Approved for the Department Veterinary Molecular Biology

Dr. Mark T. Quinn

Approved for the Division of Graduate Education

Dr. Carl A. Fox

STATEMENT OF PERMISSION TO USE

In presenting this dissertation in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this dissertation should be referred to ProQuest Information and Learning, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute my dissertation in and from microform along with the non-exclusive right to reproduce and distribute my abstract in any format in whole or in part."

Agnieszka Rynda

August, 2008

ACKNOWLEDGEMENTS

I would like to thank my advisor and mentor Dr. David Pascual for his tremendous support and guidance throughout my graduate education at Montana State University. Without Dr. Pascual's faith, determination, great knowledge and support, this degree may have never been achieved. I would also like to thank my committee members Dr. Mark Jutila, Dr. Allen Harmsen, Dr. Mark Quinn, and Dr. Wendy Gilmore. Their guidance, ideas and support throughout the years have been invaluable. My research could not have been completed without the help of scientists and staff in the Pascual lab. In particular, Dr. Massimo Maddaloni has taught me anything I know about molecular biology. Dr. Maddaloni and Dr. Dagmara Mierzejewska have been a tremendous help, great colleagues and friends. Finally, I would like to thank my family. I would not be where I am today if it were not for the support, love, and encouragement of my parents, sister, and grandmother.

TABLES OF CONTENTS

TABLES OF CONTENTS - CONTINUED

TABLES OF CONTENTS - CONTINUED

TABLES OF CONTENTS - CONTINUED

TABLES OF CONTENTS - CONTINUED

LIST OF TABLES

Table Page

LIST OF FIGURES

LIST OF FIGURES – CONTINUED

Figure Page

LIST OF FIGURES – CONTINUED

Figure Page

ABSTRACT

Effective treatments for multiple sclerosis (MS) are problematic due to its unknown etiology. Experimental autoimmune encephalomyelitis (EAE) in rodents mimics MS. Mucosal treatment of EAE with antigens to induce tolerance is effective, but requires large and/or multiple administrations, which introduces an allergy risk. We utilized reovirus adhesin, protein sigma 1 (pσ1), to improve mucosal auto-antigen delivery and show that a single low-dose of pσ1-based vaccines induces tolerance and prevents autoimmunity when administered nasally. We engineered three pσ1-based vaccines carrying chicken ovalbumin (OVA-pσ1) and/or myelin antigens (PLP:OVA-pσ1, MOG-pσ1). When mice were nasally immunized with OVA-pσ1, tolerance to OVA was established. This tolerance resisted co-administration of mucosal adjuvants and peripheral challenge with OVA. Pσ1-mediated tolerance relied upon specific IL-10-producing regulatory T (T_{reg}) cells, which inhibited OVA-specific $CD4^+$ T cell proliferation. OVA-pσ1 did not generate tolerance in IL-10-deficient mice presumably by a failure to induce T_{reg} cells. Mucosal, but not systemic pσ1 delivery, induced tolerance, while mice lacking mucosal inductive tissues were resistant to pσ1-mediated tolerance. Likewise, PLP:OVA-pσ1 and MOG-pσ1 protected mice against relapsing-remitting or acute EAE, respectively. Protection against $PLP_{139-151}$-induced EAE was accomplished by PLP:OVA-pσ1, but not OVA-pσ1, implicating antigen-specificity of pσ1-mediated tolerance. Moreover, MOG-pσ1, but not PLP:OVA-pσ1, ameliorated MOG_{35-55}-induced EAE via apoptosis of encephalitogenic $CD4^+$ T cells. The PLP:OVA-pσ1- or MOG-pσ1-mediated protection against EAE depends on specific IL-10^+ T_{reg} cells and is supported by IL-4^+ Th2-type cells. Adoptive transfer of PLP:OVA-pσ1-primed T_{reg} cells entirely prevented EAE development in mice; however, transfer of PLP:OVA-pσ1-specific $CD25^-CD4^+$ Th2 cells significantly reduced and delayed clinical EAE. Aggressive EAE, due to the TGF-β which induced activation of Th17 cells, was observed in mice dosed with PLP:OVA-pσ1 and were functionally depleted of T_{reg} cells. Concomitant inactivation of TGF-β and T_{reg} cells induced Th2 cells bias and re-established PLP:OVA-pσ1-mediated protection against EAE. IL-10-producing B cells supported MOG-pσ1-mediated protection against EAE, as MOG-pσ1-dosed B cell-deficient mice developed attenuated disease. Adoptive transfer of T_{reg} cells, but not Th2 or B cells from MOG-pσ1-dosed B6 mice to diseased IL-$10^{-/-}$ mice, significantly accelerated recovery from EAE. These data demonstrate the feasibility of using pσ1-based single-dose delivery system to prevent and/or treat autoimmunity.

INTRODUCTION

Tolerance and Autoimmunity Background

Peripheral Tolerance and Autoimmunity

It is now well established that thymic expression of self-antigens (self-Ags) during embryonic development induces self-tolerance to these Ags. As demonstrated by Kappler in 1987, the majority of immature T cells, which T cell receptor (TCR) binds to the self-Ags with the affinity exceeding activation threshold is directed to apoptosis in thymic medulla (1). This clonal deletion of potentially autoreactive T cells, commonly known as the negative selection, provides a basic mechanism for central self-tolerance and takes place at the double positive ($CD4^+CD8^+$) stage of T cell development (1-4). Although negative selection within the thymus plays a major role in eliminating autoreactive T cells, evidence accumulated during the last two decades has found that this process is not absolute. It is now well-established that some autoreactive T cells escape the process of negative selection and persist in a normal host either because certain Ags are not expressed during embryonic development or, alternatively, their expression in the thymus is insufficient (3). According to the long lasting dogma, the central nervous system (CNS) develops after the negative selection process, and consequently its Ags may not be exposed during selection, thus CNS is considered to be an immune privilege site sequestered from circulation by the blood brain barrier (BBB) (5, 6). Contrary to this view, myelin basic protein (MBP) was shown to be expressed in the fetal thymus (7) and intrathymic injections of MBP have shown to be protective against experimental autoimmune encephalomyelitis (EAE) (8). Interestingly, MBP NAc1-11 peptide binds

weakly to major compatibility complex class II (MHC II) and forms unstable complexes that are not able to mediate an efficient negative selection (9). Therefore, the deletion of MBP-reactive T cells in thymus seems to be incomplete and results in persistence of MBP-reactive T cells at the periphery (9). The peripheral T cell repertoire in healthy individuals contains autoreactive cells that remain anergic (3). The small fraction of these anergic cells, when activated, can lead to autoimmunity, suggesting involvement of another mechanism for induction of a peripheral tolerance (10). Control of autoimmunity by the thymus is not only by eliminating autoreactive T cells, but also via development of regulatory T cells (9). Additionally, the thymus has been shown to be required for induction of oral tolerance following feeding with chicken ovalbumin (OVA) or MBP (9).

A number of hypotheses explaining the induction and maintenance of peripheral tolerance have been proposed. It has been suggested that peripheral tolerance results from the ignorance of an immune system that simply cannot see an auto-Ag being sequestered from a circulation (11). This hypothesis was challenged by the fact that many auto-Ags from immune privileged sites were shown to be expressed in fetal thymus (7). Additionally, ignorance of an immune system does not explain why autoimmune diseases do not always occur when previously sequestered Ags are released to the environment due to the nonspecific injury, even though autoreactive T cells are present in healthy individuals who do not display autoimmune diseases (3).

During 1990s, deletion by apoptosis of autoreactive cells was described as a mechanism regulating self tolerance at the periphery (3). It has been shown that the

programmed death of autoreactive T cells can occur in Fas-dependent or -independent fashion (12-14) and that suppression of this apoptosis results in exacerbation of autoimmune diseases (15). Additionally, apoptotic cells have been shown to enhance interleukin-10 (IL-10) production and inhibit IL-12-secretion (16, 17), as well as stimulate tolerogenic dendritic cells (DCs) (3, 6, 18). On the other hand, interference with apoptotic pathways does not always prevent autoimmunity (3, 14).

Anergy can be understood as unresponsiveness to the stimulation, or functional inactivation, and can be acquired by T cells undergoing activation in the absence of co-stimulatory signal (19, 20). Anergic cells are unable to proliferate *in vitro* in the presence of Ag and in the absence of IL-2, but the addition of IL-2 reverses their anergic state (21). Rarely, anergic T cells can inhibit naïve and primed T cells in an Ag-specific manner due to competition for IL-2 and interaction with antigen presenting cells (APCs) (3). Lack of B7 co-stimulation is one of the predominant mechanisms by which peripheral tolerance can be established (3, 22), and in fact blockade of CD28-B7 interaction, has been shown to prevent epitope spreading and relapses in EAE (19). Contrary to this discovery, it has been demonstrated that autoreactive T cells in patients with relapsing-remitting multiple sclerosis (RRMS) do not require CD28-co-simulation and display a significantly lower activation threshold (20).

Currently, natural tolerance to self-Ags is considered to be an active suppression that relies on the activity of inhibitory T cells that accounts for 5-10 % of total lymphocytes in a healthy individual (22-24). Peripheral tolerance can be induced via either the peripheral route (systemic tolerance) or mucosal route (mucosal tolerance).

The intravenous (i.v.) or intraperitoneal (i.p.) injection of an Ag results in systemic tolerance. In EAE, systemic i.v. injections of myelin components were shown to effectively prevent disease manifestation, even in inducible costimulator (ICOS) molecule deficient mice (25). Additionally, in the model of hyper-IgE and asthma, i.p. tolerization led to the simultaneous induction of T regulatory (T_{reg}) cells and effector Th2 cells displaying the same antigen specificity (26).

Mucosal Tolerance

Mucosal tolerance is classically defined as a state of hyporesponsiveness to subsequent parenteral injections of proteins to which an individual or animal has been previously exposed via the mucosal route (27, 28). Like peripheral tolerance, mucosal tolerance is believed to be an active suppression of an Ag-specific cellular and/or humoral immune response (22). It is believed that mucosal tolerance, as a form of peripheral tolerance, evolved in response to exogenous agents, such as food, that after gaining access to the body via mucosal sites become part of the host (22, 29).

Either systemic (25, 30-32) and mucosal (24-26, 33-37) routes have been successfully used for induction of tolerance in EAE. The ease and safety of administration and the potential for using smaller amounts and/or fewer doses of an Ag makes the mucosal route more attractive for tolerance. Mucosal tolerance can be induced via the oral or the nasal route, and depending on the amount of an Ag it can occur via a number of mechanisms, including anergy/deletion and/or active suppression of specific effector T cells (26).

Gut-Associated Lymphoreticular Tissue (GALT). Orally administered Ags encounter the GALT, which, aside from providing a physical barrier against ingested pathogens, also has innate properties, preventing the host from reacting to ingested proteins (24, 38). Within the GALT, villus structures are built by intestinal epithelial cells (IECs), intraepithelial lymphocytes (IELs), and lamina propria (LP) lymphocytes (24, 38-41). Peyer's patches (PPs) are localized in the areas in the GALT between villis where the primary immune response is generated (24, 39, 42). It is well-established that GALT favors induction of Th2-type response (24, 43), although prolonged exposure of the gut to oral Ags has been shown to suppress this response (reviewed in ref. 29). Interestingly, it has been reported that PPs in OVA-TCR transgenic (Tg) mice fed with high doses of OVA initially produce interferon gamma (IFN-γ) (24).

Mucosal villi represent 99 % of the small intestine and are surrounded mostly by IECs that are capable of translocating Ag in its native or partially digested form (38, 39). Aside from their role in transporting an Ag, IECs can also play a role of tolerogenic APCs *in vivo* since they express high levels of MHC I and II, but very low levels of co-stimulatory molecules (39). Additionally, IECs have been shown to preferentially expand $CD8^+$ T cells, although they can suppress both $CD4^+$ and $CD8^+$ T cells *in vitro* (39). While effective in presenting supreantigens, IECs are inefficient in presenting a nominal amount of an Ag in the context of the MHC II (39, 40).

PPs are mucosal inductive sites that contain specialized microfold (M) cells localized within the follicular-associated epithelium (FAE) (40, 44). The most characteristic feature of fully differentiated M cells is the intrapepithelial "pocket" which

harbors lymphocytes and shortens the distance for the transcytolic vesicle to reach the basolateral surface. The M cell pocket contains macrophages, B cells that express immunoglobulin (Ig) IgM, but not IgA or IgG, and mostly αβ $CD4^+$ T cells (40). On the basolateral surface, M cells contain processes that extend into the underlying lymphoid tissue (40). Below the FAE is the "dome" area that is filled with the extensive network of APCs, T and B lymphocytes derived from the underlying follicle (40, 44).

It is generally agreed that establishment of oral tolerance occurs at the T cell level (43, 44). Increased numbers of T cells were observed in OVA-Tg mice that have been tolerized by high or low doses of OVA (45). The essential role of PPs in oral tolerance induction has been reported by Fujihashi and colleagues. This group has shown that PP^{null} mice resulted from *in utero* treatment with lymphotoxin (LT) β-receptor Ig fusion protein, were resistant to induction of a high dose oral tolerance, despite having functional mesenteric lymph nodes (MLNs), sacral LNs, and cervical LNs (CLNs) (43). Importantly, augmented splenic humoral and cellular responses to orally administered OVA were generated in PP^{null} mice before systemic challenge, suggesting that PPs may trigger mucosal tolerance by down regulation of an immune response, and that protein uptake in the absence of PPs primes systemic immune responses (43). Additionally to their role in establishment of mucosal tolerance, PPs are the primary inductive sites in the gut that generate Ag-specific IgA responses (43, 44, 46). Along these lines, TGF-β in PPs has been shown to be pivotal for the induction of oral tolerance (45); however TGF-β has also been shown to trigger isotype class switching from IgM to IgA B cells (reviewed in ref. 43).

Early Events in Oral Tolerance Induction. Luminal Ags gain access to the PPs and other GALT via transfer across M cells, which are scattered amongst the other epithelial cells in the FAE above the PPs (42). Ag translocated into the PPs undergoes extensive processing and presentation by local DCs (42, 47). Afterwards, processed Ag is presented by DCs to the T cells that mediate oral tolerance or immune response (42, 47-49). Factors influencing the decision between induction of tolerance or immunity to a given Ag are largely unknown, but they most likely include the type of an Ag, type of the encountered DCs, and type of activated T cells (31, 42, 47-49). It has been demonstrated that PP-associated T cells can secrete TGF-β, and it is believed that these cells are at least partially responsible for induction of oral tolerance (24, 42, 48). Additionally, PPs-associated T cells have been shown to interact with IgM-producing B cells to form germinal centers containing a high proportion of B cells differentiating into IgA phenotype (42, 48, 50, 51). These activated T and B cells traffic to the iLP areas that underlay mucosal surfaces and therefore spread systemically-induced immunity or tolerance (42, 47, 48, 51). Alternatively, Ag, instead of being acquired by PPs M cell, can encounter the host via epithelial cells, utilize alternative immunogenic or tolerogenic pathway, and enter the circulation directly (24, 39).

The pivotal role of liver in the induction of peripheral and oral tolerance has been well established (reviewed in ref. 39). Orally administered Ags were shown to enter the liver and cause apoptosis of Ag-specific $CD4^+$ T cells, as well as the induction of regulatory T cells (22, reviewed in ref. 39). Moreover, hepatic $CD11c^+$ DCs were demonstrated to capture ingested Ags and induce T cell apoptosis and Th2 cell bias (51).

Aside of myloid $CD11c^{+}CD8\alpha^{-}$ and lymphoid $CD11c^{+}CD8\alpha^{+}$ DCs, the liver also contains a large population of $CD11c^{low}$NK1.1 DCs (reviewer in ref. 39) and NKT cells (3) that has been shown to play a regulatory role in diabetes models.

High and Low Dose Tolerance. Mucosal tolerance can be obtained by a number of mechanisms including deletion or anergy of specific effector T cells, Th1/Th2 cell immune deviation, as well as suppression by T regulatory cells (26). Administration of high doses of an Ag induces tolerance via the mechanism of clonal anergy/deletion of effector T cells (36, 52). Clonal deletion most likely occurs via apoptosis and has been shown to be one of the mechanisms for inducing oral tolerance to myelin components in EAE (36, 53). Low doses of mucosally administered Ag induce tolerance via the mechanism of an active suppression of effector T cells (3, 24-26, 36, 54, 55). Importantly, these two forms of mucosal tolerance are not mutually exclusive, as they sometimes overlap (23, 52).

Most of the research on mucosal tolerance in EAE has been focused on oral administration of an Ag (24-26, 36, 54, 56-58). It is believed that large doses of an Ag delivered orally act systemically and can suppress both Th1 and Th2 responses (29). It was shown that oral immunization with protein Ag induces $CD4^{+}$ Th cells that support IgA antibody (Ab) responses in PPs, whereas regulatory T cells are induced in systemic compartments to down regulate Ag-specific IgM, IgG, and IgE Ab responses (reviewed in ref. 44). Interestingly, oral immunization with cholera toxin (CT), a potent mucosal adjuvant, prevents induction of mucosal tolerance, but it does not alter normal Ag-specific mucosal IgA Ab responses (27, reviewed in ref. 44). Moreover, reduction in

OVA-specific mucosal IgA Ab response and OVA-specific proliferation was observed in mice fed with large doses of OVA and subsequently challenged with OVA plus CT as mucosal adjuvant, implicating that CT does not break already established tolerance in mucosal and systemic compartments (44, 46).

High doses of myelin Ags fed to rodents (24, 25, 54, 56, 57) have been shown to induce Ag-specific tolerance and down regulate EAE upon challenge (37, 56). Continuous and serial feeding of small doses of protein Ags has been shown to induce tolerance mediated by IL-10 and transforming growth factor beta (TGF-β)-secreting $CD25^{+}CD4^{+}$ regulatory (T_{reg}) cells more effectively than high doses (23, 56, 59). Aside from T_{reg} cells, Th3 cells have also been shown to be protective against MBP-induced EAE in a TGF-β-dependent manner (54, 60). A study by Benson *et al* demonstrated the mechanism of high dose oral tolerance using MBP TCR Tg mice that underwent thymectomy in adulthood (52). In this study, MBP-Tg mice that were fed high doses of MBP one day prior to EAE induction showed protection from the disease due to the internalization of MBP-specific TCRs. However, 3 days post oral administration of MBP, these MBP Tg TCRs reappeared on a cell surface, and mice challenged with EAE at this time point developed clinical disease. The transient decrease of MBP-reactive T cells was followed by intermediate state of anergy that, by 14 days after feeding, turned into clonal deletion of MBP-reactive T cells (52).

Mechanisms of Low Dose Tolerance. It has been established that even though orally acquired Ags induce predominantly Th2-type response, they also suppresses production of IgE Abs (24, 61). This can occur most likely by generation of TGF-β-

secreting $CD4^+$ T cells or γδ T cells that were shown to suppress IgE production after aerosol administration of an Ag (24). In general, it is believed that all types of soluble proteins, when orally given to animals without mucosal adjuvants, induce mucosal tolerance (24, 44, 46). Complex Ags, such as inactivated microbes, can also induce tolerance, although it is possible that in these cases the actual tolerogens are also soluble proteins released from the microbe during digestion in the gastrointestinal (GI) tract. Additionally, complex Ags are usually associated with natural adjuvants, including LPS, bacterial toxins, polysachcarides, etc., which unable induction of a tolerance (51, 61-63).

In contrast to proteins, substances that are not subjected to oral tolerance include polysaccharide Ags, Ags that strongly bind to epithelial cells, or bacterial toxins (51, 63, 64). Th2-type responses are preferentially generated in the GALT following oral immunization (61, 65), and in general, oral tolerance influences stronger the Th1 cell response than Th2 or humoral responses (3, 41, 51, 62). It is believed that oral administration of a tolerogen is associated with diminished IgA and IgG production (44, 46, 51). However, as demonstrated by Fujihashi *et al,* chronic oral exposure to an Ag may increase IgA levels (66). Additionally, a number of doses of an Ag given orally can influence the IgG Ab level, since as shown by this group, oral administration of an Ag 6 times significantly induces Ab-specific IgG, whereas the same Ag given orally 12 times greatly decreases IgG Ab levels (66).

The strategy of improving mucosal tolerance by chemical coupling of an Ag to CT-B subunit (CTB) has been explored by Czerkinsky's group. This group has demonstrated that oral (67) and nasal (68) delivery of significantly smaller doses of auto-

Ags by chemically altered CTB can be applied to prevent autoimmune diseases. Interestingly, depending on the nature of delivered Ag the mechanism of CTB-induced tolerance was shown to be mediated by various subsets of regulatory T cells (69-71). Furthermore, sublingual (s.l.) administration of CTB-coupled OVA was shown to induce forkhead box transcription factor (FoxP3)-expressing regulatory T cells that suppress OVA-specific delayed type hypersensitivity reactions (72), and induce apoptosis of effector T cells (71). Interestingly, modulation of systemic and mucosal immune responses to dextran challenge was observed after mucosal delivery of CTB-dextran (73, 74); however, the suppression of immune response to CTB-coupled dextran was critically dependent on the presence of preexisting immunity to CTB (74).

Studies of mucosal tolerance exhibit important issues that need to be addressed in order to therapeutically use oral tolerance for autoimmune diseases. A first consideration is that the target Ag is not always known, especially when studying autoimmune diseases. This problem is partially overcome by the process of bystander suppression discussed below. Bystander suppression has been demonstrated in animal and human diseases like EAE and MS, respectively (75-77). For instance, proteolipid protein immunodominant peptide ($PLP_{139-151}$)-induced EAE can be suppressed by feeding mice with MBP (76), or by transferring TGF-β-secreting $CD4^+$ T cell clones isolated from orally tolerized animals (54). Alternatively, oral administration of an unrelated Ag is shown to induce Th2 cell bias and protect and treat mice against EAE (33, 34, 78). It has been reported that naïve animals are generally susceptible for induction of oral tolerance; however animals that have been already primed with an Ag are more resistant to the suppressive effect of orally

administered Ags (22, 56, 79). Finally, the requirement for large Ag doses in oral tolerance can be overcome by the alternative mucosal route of Ag administration, namely by nasal route.

The nasal route for tolerance induction has been receiving increased attention over the last decade. The nasal mucosa provides an alternative route for drug delivery and tolerance induction (25, 80-83), and it has been shown to be equally if not more efficient in suppressing autoimmunity (24, 76, 84). Nasal immunization has been shown to be effective in stimulating mucosal immunity or tolerance to pathogens, soluble proteins, and peptides in the upper (74, 83) and lower respiratory (74, 85) tracts and in the distal mucosa (82). Additionally, the ease of nasal administration and the number of Ag doses make this route very attractive, especially in terms of allergy-safe vaccination (83, 86).

Tolerance induction via the nasal route can be established with much smaller doses of an Ag. This is presumably because protein Ags are not subjected to digestion like they are in the GI tract, and they can reach the nasal-associated lymphoid tissues (NALT) more readily than the GALT subsequent to oral Ag administration. Nasal administration of OVA alone (87, 88) or much smaller doses of OVA delivered by pσ1 (89) has been shown to efficiently establish OVA-specific tolerance. Additionally, nasally administered myelin peptides alone or delivered by vehicle molecules have been demonstrated to significantly reduce clinical EAE (32, 35, 57, 90), diabetes (68), and collagen-induced arthritis (91). Nasal administration of guinea pig MBP_{68-86} and/or MBP_{87-99} peptides to Lewis rats protects them against MBP-induced EAE (92).

Moreover, nasal administration of MBP altered peptide ligands (APLs), or even an unrelated auto-Ag has been shown to protect mice against EAE via induction of cytokine production in the absence of proliferation (92, 93). It has been demonstrated that tolerance to MBP followed APL therapy is dependent on IL-10, but not on TGF-β secretion (94, 95).

The upper respiratory tract resembles the gut mucosa in that they both contain M cells (82), DCs, and lymphocytes scattered in the epithelium (22). The mechanisms involved in tolerance induction via nasal route include suppression of an immune response by IL-10-producing $CD4^+$ T_{reg} cells (87, 90), or $CD8^+$ γδ T cells (96). Additionally, as demonstrated by Ostroukhova and coworkers, tolerance induced in response to inhaled Ag can be associated by induction of $FoxP3^+CD4^+$ regulatory T cells that produce TGF-β (80). It has been shown that given nasally $PLP_{139-151}$ peptide is effective in suppressing clinical EAE induced by systemic administration of either $PLP_{139-151}$ or MBP peptides, further indicating the involvement of bystander suppression is nasally induced tolerance (32).

Even though little is known about the early events involved in tolerance induction via the nasal route, the results described elsewhere suggest that cervical LNs (CLNs), the draining LNs in nasal mucosa, are critical for this process (87). It has been that reported that nasal tolerance cannot be established in the absence of CLNs (97). Moreover, substitution of CLNs by peripheral LNs (PLNs) did not restore the ability to generate nasal tolerance (97). Additionally, this group has shown that that functional regulatory $CD25^+$ and $CD25^-$ T cells are induced in CLNs by nasally administered OVA (87).

T Regulatory (T_{reg}) Cells. It is well-established that low dose mucosal tolerance is mediated by Ag-specific or nonspecific regulatory cells (6, 22, 24, 29, 56). Over the last decade the anti-inflammatory and/or tolerogenic properties have been assign to nearly every type of adaptive and innate cells. Depending on the route of an Ag administration, type of an Ag, its dose and administration regiments, generation of regulatory $FoxP3^+$ or $FoxP3^-$ T, and B cells, DCs, γδ T and NKT cells can occur (3, 24, 31, 47, 49, 51, 99-101). Generation of Ag-specific regulatory cells can also result from presentation of an Ag by APCs at mucosal inductive sites (22).

Recently, it was discerned that activation of the inducible costimulator (ICOS) molecule (CD28 homologue) on T cells (25) is essential for the development of mucosal, but not high-dose peripheral (i.v.), tolerance to encephalitogenic peptide (myelin oligodendrocyte glycoprotein peptide; MOG_{35-55}). The inducible costimulator (ICOS) molecule belongs to the CD28/CTLA-4 (CD152) co-stimulatory molecule family expressed on T cell (25, 102). Unlike constitutively expressed CD28, CTLA-4 and ICOS are induced upon T cell activation and are important for down regulating T cells response and induction of tolerance (25, 102). While evaluating the effector function of ICOS, Dong *et al* have demonstrated that ICOS-deficient mice fail to produce IL-4 and exhibit impaired humoral response after immunization with several peptides (102). On the other hand, development of functional IL-10-producing regulatory $CD4^+$ T is not impaired in the absence of ICOS, but the lack of this molecule prevents induction of a low dose mucosal but not peripheral tolerance, implicating the role of ICOS in controlling the effector functions of regulatory T cells (25).

Tr1, Th3, NKT, and γδ T Cells and Tolerance. A variety of different subtypes of naturally occurring and/or induced T cells has been implicated in suppression of autoimmunity. TGF-β-secreting $CD4^+$ and $CD8^+$ T cells have been isolated and cloned from PPs and MLNs of orally tolerized animals (23, 54, 103) and from blood of humans orally dosed with MBP (22, 59). Induced by oral administration of low doses of an Ag, Th3 cells (24, 59) were shown to down regulate proinflammatory Th1 cells and promote IgA production (24, 54). Th3 cells were shown to develop in the presence of IL-4, and they did not proliferate well in cultures (22, 61). Compared to Th1 or Th2 cells, Th3 cells appear to be resistant to deletion following feeding high OVA or MBP doses to OVA or MBP TCR Tg mice (24, 52, 103, 104). The most important hallmark of regulatory Th3 cells is that these are induced by orally administered Ag and suppress an immune response in Ag-specific fashion via secretion of TGF-β (3, 61). However, some Th3 cells in addition to TGF-β produce also IL-10 and/or IL-4 (23, 54).

In contrast to Th3 cells, regulatory Tr1 (Th10) cells develop in response to chronic exposure to a superantigen and IL-10 and suppress an immune response in an Ag-specific manner via secretion of IL-10 (3, 22, 105). Development of Tr1 cells is especially prominent in response to IL-10-producing DCs induced after nasal administration of an Ag (22). Tr1 cell have been implicated in EAE suppression after soluble peptide therapy (105).

Natural killer T (NKT) cells are a distinct subset of Tαβ cells, $CD4^-CD8^-$, that share receptor structure with NK cells (3, 106). NKT cells are abundant in the liver, thymus, bone marrow, and are found in peripheral lymphoid organs (106). These cells

are activated by glycolipid Ags presented in the context of nonpolymorphyc MHC I molecule CD1d (106). Defects in NKT cell numbers and function have been associated with susceptibility to autoimmunity, and observed in various autoimmune mouse models, and in humans (106). Also, in adoptive transfer experiments, NKT cells can prevent diabetes in NOD mice, an effect mediated by IL-4 and IL-10 (3). Activation of NKT cells via glycosphingolipid α-galactosylceramide (α-GalCer) has been shown to protect wild type (wt), but not IL-4- or IL-10 –deficient mice, against EAE development in CD1d-dependent manner. In contrast, mice injected with β-GalCer that binds CD1d but is not recognized by NKT cells developed the disease (106).

Another population of regulatory T cells expresses CD45RB/C^{lo} (3). These cells have been shown to successfully suppress either Th1- or Th2-mediated autoimmune diseases in rats by TGF-β and/or IL-4-related mechanism (3). This population was further divided into CD38^{-} CD45RB^{lo} CD4^{+} and CD38^{+} CD45RB^{lo} CD4^{+} T cells. Activated, CD38^{-} population was shown to proliferate and secrete cytokines; whereas the CD38^{+}RB^{lo}CD4^{+} T cells were found to be suppressive *in vitro* but did not produce IL-10 or TGF-β (3).

γδ T cells have been shown to mediate oral tolerance to a number of protein Ags (reviewed in ref. 51). Also, tolerance induction via inhalation of aerosolized Ags has been associated with activation of CD8^{+} γδ T cells (51, 96). Administration of aerosolized insulin to NOD mice in the pre-clinical stage of IDDM reduces pathology and clinical manifestation of disease, and this suppression is dependent on the presence of CD8^{+} γδ T cells (96). The CD8^{+} γδ T cells have been found predominantly among the

IEL population and were shown to be activated by Ag presented by epithelial cells in the context of nonconventional MHC molecules (CD1d) (22, 38, 39, discussed in ref. 51). Adoptive transfer-induced diabetes into non-diabetic mice was suppressed by transfer of splenocytes from insulin aerosol-treated mice, however when these splenocytes were depleted of γδ T cells tolerance and protection could not be established in adoptive transfer recipients, suggesting a direct suppressive role of γδ T cells in establishement of oral tolerance (96). Even though production of regulatory cytokines i.e. TGF-β has been associated with suppressive γδ T cells the exact inhibitory mechanism of these cells is yet to be determined (51).

CD25$^+$CD4$^+$ T Regulatory Cells and Tolerance. Naturally occurring CD4$^+$CD25$^+$ T regulatory (T_{reg}) cells have been identified in the peripheral lymphoid tissues as a subset of T cells exhibiting properties of anergic cells when stimulated *in vitro* (3). Even though these naturally occurring T_{reg} cells secrete cytokines upon stimulation, neutralization of these cytokines did not reverse their suppressive abilities, implicating cytokine-independent mechanism of action (3, 107, 108). It was established that naturally occurring CD4$^+$CD25$^+$ T_{reg} cells suppress effector T cells in a cell-to-cell contact-dependent manner by inhibiting IL-2 (108, 109).

In an effort to define the surface molecule mediating the cell-to-cell contact, it has been demonstrated that an adhesion, LFA-I (CD11a; CD18), is crucial for the optimal development of these cells (110). Depletion of T_{reg} cell leads to spontaneous development of various autoimmune disorders, implicating that CD4$^+$CD25$^+$ T_{reg} cells are essential for peripheral tolerance (3, 107). Moreover, it has been reported that allogenic

$CD4^+CD25^+$ $FoxP3^+$ T_{reg} cells suppress autoimmunity and establish transplantation tolerance in IL-2Rβ-deficient mice (111). Naturally anergic T_{reg} cells do not proliferate in response to Ag stimulation *in vitro* and are able to suppress the activation and proliferation of $CD4^+$ and $CD8^+$ T cells in an Ag-nonspecific manner. However, CD28 and TCR stimulation may reverse their anergic and suppressive state (112).

An interesting subset that exhibits characteristics of both naturally occurring and adaptive $CD4^+CD25^+$ T cells has been observed by two independent investigators. Ostroukhova *et al* in 2004 and Mucida *et al* in 2005 describe a T regulatory cell subset that expressed all of the surface phenotypes of $CD25^+CD4^+$, although these cells that express $FoxP3^+$ become adaptive in response to specific tolerogenic stimuli (26, 80, 81). Additionally, it has been demonstrated that naturally occurring $CD4^+CD25^+$ T cells could suppress the inflammatory colitis induced in SCID mice by adoptive transfer of $CD4^+CD25^-$ T cells followed by s.c. infection with *Leishmania major* (113). Moreover, this protection could not be established in SCID mice transferred with T_{reg} cells and *in vivo* neutralized with anti-IL-10R, anti-CTLA-4, or anti-TGF-β Abs (113). Likewise, naturally occurring $CD4^+CD25^+$ T_{reg} cells were also shown to prevent graft rejection and induction of immunity against tumors (114).

While naturally occurring $CD25^+CD4^+$ T cells require cell-to-cell contact (109), suppressive action of adaptive $CD25^+CD4^+$ T_{reg} cells is mediated via surface-expressed TGF-β1 and/or secretion of IL-10 and/or TGF-β (80). In addition to adaptive T_{reg} cells, Th3 and Tr1 cells have also been shown to suppress systemic inflammatory responses (22, 24, 103). Consistent with naturally occurring $CD4^+CD25^+$ T_{reg} cells, deletion of

mucosally induced T_{reg} cells can lead to the development of a variety of autoimmune diseases (107, 112). Ag-specific $CD4^+CD25^+$ T_{reg} cells have been shown to suppress effector cells *in vitro*, and transfer tolerance to naïve animals. Additionally, it has been demonstrated that $CD4^+CD25^+$ T_{reg} cells can directly suppress B cell responses (109).

The exclusive phenotypic markers for $CD4^+CD25^+$ T_{reg} cells are still being investigated. At this time, the expression of forkhead box transcription factor (FoxP3) has been the most consistently associated with these suppressor T cells (22, 26, 114-117). It has been demonstrated that absence or mutation of FoxP3 leads to abrogation of $CD4^+CD25^+$ T_{reg} cell development and resulted in wasting disease and IBD in mice (115, 116). On the other hand, the ectopic expression of FoxP3 in mouse $CD4^+CD25^-$ T cells induced their suppressive activity and protected mice from wasting disease and IBD (114-116). IL-4 and IL-13 have been shown to be required for FoxP3-expression and tolerogenic potential of T_{reg} cells, since *in vivo* neutralization of either of these cytokines in OVA-Tg mice abolished expansion of $FoxP3^+CD4^+CD25^+$ T_{reg} cells that could be induced by OVA feeding (117). Additional phenotypic markers for $CD4^+CD25^+$ T_{reg} cells include IL-2Rα (CD25), activation-induced cytotoxic T lymphocyte associated protein-4 (CTLA-4), and glucocorticoid-induced tumor necrosis factor receptor (GITR) (112, 118). GITR and CTLA-4 may play a co-stimulatory role in T_{reg} cells' activation, since *in vitro* neutralization of these molecules inhibits suppressive effects by T_{reg} cells (102, 112). Recently, the surface expressions of chemokine receptor 6 (CCR6) (119) and Latency-associated peptide (LAP) (120) have been shown to be expressed on $CD4^+CD25^+$ T_{reg} cells. It has been demonstrated that $CD4^+CD25^+LAP^+$ T_{reg} cells show

increased expression of FoxP3 and CTLA-4. *In vitro,* these cells displayed the suppressive function in contact-dependent and soluble factor-dependent manner; although *in vivo* the suppression of EAE by these T_{reg} cells was TGF-β-dependent (120).

While the *in vitro* suppressive role of TGF-β is well-established, its *in vivo* importance for T_{reg} cell-mediated tolerance is questionable (3, 56, 57, 80). TGF-β signaling in T_{reg} cells (81) has been shown to be required for their *in vivo* expansion and suppressive capacity (118, 121, 122) and for protection against diabetes (123), or colitis (81). On the other hand, TGF-β alone (124, 125), or in combination with IL-2 (121), or IL-6 (126) has been shown to stimulate differentiation of Th17 cells in IL-23-independent fashion. TGF-β secretion by $CD25^+CD4^+$ T_{reg} cells has been shown to be responsible for recovery from EAE (33), and presence of TGF-β has been shown to be necessary for conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+FoxP3^+$ T_{reg} cells (118, 121-123). However, others have demonstrated that induction of T_{reg} cells from naïve peripheral $CD25^-CD4^+$ T cells and expression of FoxP3 can be facilitated by IL-4 (117) or IFN-γ (127).

T_{reg} cells can also produce IL-10, as demonstrated by a number of investigators (114, 128, 129). Induction of IL-10-secreting regulatory $CD4^+CD25^+FoxP3^+$ or $FoxP3^-$ T_{reg} cells that exhibit suppressive capacity or down regulated proinflammatory encephalitogenic response have been also demonstrated in autoimmune EAE model (114, 128). Alternatively, induction of T_{reg} cells by administration of IL-10 has been shown to ameliorate clinical and histopathological manifestation of EAE (129). Aside from $CD4^+CD25^+FoxP3^+$ T_{reg} cells, also $CD4^+CD25^+FoxP3^-$ T cells can exhibit regulatory

function (114). We have shown previously that partial protection against EAE has been achieved by adoptive transfer of $CD25^{-}CD4^{+}$ T cells obtained from mice orally dosed with *Salmonella*-CFA/I (33). Also, $FoxP3^{-}$ IL-10-producing T_{reg} cells have been derived from MBP TCR Tg mice repeatedly nasally dosed with MBP (Ac1-9) peptide (114). Despite production of IL-10, these $FoxP3^{-}$ T_{reg} cells were shown to be suppressive *in vitro* in IL-10-independent manner (114).

Role of Cytokines in Mucosal Tolerance. It has been suggested that low-dose oral tolerance is mediated via TGF-β-producing Th3 cells, whereas small doses of an Ag delivered nasally most likely induce IL-10-dependent regulatory T cells or Tr1 cells (23, 87, 90).

Cytokines have been shown to have an important influence on tolerance induction. It is now well-established that IL-4, together with IL-13, defines the Th2-type cells; *in vitro* stimulates growth of T and B cells, but down regulates inflammatory macrophages. IL-4 has been shown to recruit B cells for Ag processing and presentation, which promotes development of anergy (3, 22). Interestingly, IL-4 knockout (KO) mice are not more predisposed to develop autoimmunity than their wt littermates (6, 130). Another Th2/ regulatory cytokine, IL-10, has been shown to be an efficient down regulator of MHC II, ICAM-1, and B7 expression on APCs (3). In fact, IL-10 inhibits activation of both Th1 and Th2-types of immune responses, and DCs *in vitro* incubated with IL-10 significantly reduces their capacity to stimulate $CD4^{+}$ T cells (3, 131). Finally, in contrast to IL-4 KO mice, mice deficient in IL-10 or IL-10R develop more severe EAE and spontaneous inflammatory bowel disease, whereas IL-10 Tg mice are

resistant to EAE induction (131, 132). Lack of protection against EAE in IL-10-deficient mice is due to their inability to induce regulatory T cells (22), but IL-10-secreting MOG-specific $CD4^+$ T cells can transfer tolerance into naïve recipient mice (22).

TGF-β has been described to inhibit Th1 and Th2-type cells, in addition to B cells, NK cells, and macrophages (3). TGF-β has been shown to stimulate the generation of Th3 cells and $CD4^+$ $FoxP3^+$ T_{reg} cells (61, 122). Like IL-10, TGF-β can down regulate expression of MHC II on macrophages, but it also inhibits expression of IL-12R and IL-2R (104). Mice deficient in TGF-β develop spontaneous multi-organ autoimmunity and die early (3, 104). Additionally, deletional events in gut mucosa induce production of TGF-β by phagocytic macrophages and phagocytized T cells (22, 23).

Tolerogenic Vaccines against EAE

Induction and maintainance of Ag specific- or unspecific tolerance has been widely used to prevent and/or treat autoimmune diseases (22, 24) including EAE (3, 6, 8, 33, 34), insulin-dependent diabetes mellitus (IDDM) (11, 68), and collagen-induced arthritis (CIA) (91). The pathology and clinical manifestation of EAE and MS are associated with myelin-specific autoreactive $CD4^+$ T cells that display a proinflammatory phenotype and number of different cell types, including $CD8^+$ T cells, B cells, and DCs, and various infiltrating and CNS resident innate cells have been implicated in pathogenesis of these diseases (6). The treatments directed against encephalitogenic T cells seems to be the most effective (77, 134), and therefore attention has been focused on designing treatment that would inhibit myelin-reactive T cells in direct or indirect manner (33, 78).

Tolerogenic Vaccines Delivered via Systemic Route. An interesting strategy undertaken by a number of investigators has been a development of tolerogenic vaccines by administration of attenuated autoreactive T cells, also known as T cell vaccination (TCV) (135-137). The concept of TCV is parallel to the classical vaccination against infectious agents and it has been introduced by Cohen's group (138). These investigators have demonstrated that systemic injection of attenuated MBP-specific T cells can protect naïve mice against following EAE induction (138). Subsequent to the positive results from animal models, the first pilot clinical trial using irradiated autologous MBP-specific $CD4^+$ T cells was conducted in 1995 by Medaer e*t al* (139). Subcutaneous (s.c.) administration of TCV to RRMS patients induced moderate clinical improvement in majority of patients. The follow-up long term clinical study revealed that in majority of patients MBP-reactive T cells remained undetectable for 1-2 years after TCV (137). After another couple of years MBP-reactive T cells reappeared in few patients but were effectively depleted after another TCV (137). Compiled evidence from three independent studies suggests that TCV is a safe way to induce an immune response resulting in depletion of circulating MBP-reactive T cells (137).

Although the mechanism of TCV remains unclear, it is believed that anti-vaccine T cell response exclusively targets the T cell clones by recognition of a clonotype specific determinant i.e. the idiotype or TCR (137). Alternatively, TCV may induce the autoreactive encephalitogenic cells to switch from Th1 to Th2 phenotype (136). Aside from $CD4^+$ T cells, $CD8^+$ T cells, B cells, γδ T cells, and NK cells also have been implicated in the protective effect of the TCV, as these populations expand following

TCV (136, 137). Additionally, TCV administration induces both anti-idiotypic and anti-ergotypic effects, meaning that inhibition of MBP-specific immune response is induced to specific effector cells based on the clonal identity (anti-idiotypic) and also to the activated cells irrespective of their TCR-specificity (anti-ergotypic) (136); the effect of TCV to a humoral response is still not conclusive (137).

Described elsewhere in this dissertation, suppression of encephalitogenic proinflammatory cells via administration of an APL or injection with DNA encoding the encephalitogenic peptides have been an extensively studied with successful approaches. APLs have been applied therapeutically in animal models of autoimmune disease and also in clinical trials. Even though the exact mechanism of APL-mediated suppression of autoimmune responses is still elusive, combined arguments suggest that rather than acting as antagonists *in vivo*, APLs mediate bystander suppression plausibly via generation of regulatory T cells (133, 135). Additionally, effectiveness of APLs *in vivo* in MHC I-restricted environment has been demonstrated by suppression of $CD8^+$ T cell activation, cytotoxicity, and IFN-γ production by naïve $CD8^+$ T cells isolated from MHC I-restricted $OVA_{357-364}$-specific TCR Tg mouse (140).

Genetic approaches to Ag-specific tolerance have been also extensively studied. In addition to systemic administration of encephalitogenic peptide-encoded DNA in a presence or absence of CpG-ODN and/or IL-4 (141, 142), Tg expression of self-autoAgs on an MHC II promoter in APCs from the thymus has been shown to prevent autoimmune response (143).

Another idea for a tolerogenic vaccine that would prevent and/or treat EAE evolved from the studies on APL. In 1997, a group led by H. Zaghouani demonstrated that Ig chimera containing Ig-coupled APLs for $PLP_{139-151}$ (PLP-RL-Ig; APL-Ig) can improve delivery of APL and suppress proliferation of encephalitogenic $CD4^+$ T cell (144). When evaluated for an ability to inhibit $PLP_{139-151}$-specific effector T cells, free APL was unable to restrain IL-2 production by effector $CD4^+$ T cells (144). In contrast to free APL, APL-Ig chimera suppressed PLP-specific effector cells independently if these were stimulated with $PLP_{139-151}$ peptide, native PLP, or even Ig-$PLP_{139-151}$ (144). According to the authors, enhancement of the anti-encephalitogenic potential of APL-Ig chimera compared to free APL was due to more efficient endocytic presentation of Ig-coupled Ag.

Next, utilizing a similar approach, Zaghouani's group demonstrated that Ig-delivery of encephalitogenic $PLP_{139-151}$ peptide into neonatal mice confers the protection against EAE induced in their adult life (145). Additionally, administration of encephalitogenic peptide-Ig chimera in saline overcomes the requirement for incomplete Freund's adjuvant (IFA)-mediated delivery that could potentially be associated with serious side effects (145). Taking this discovery one step further, in 2000, Legge *et al* showed that aggregated Ig-$PLP_{139-151}$ chimera can successfully ameliorate clinical EAE in SJL mice and induce production of IL-10 by DCs and macrophages, but not B cells (146). *In vitro* aggregated chimera did not inhibit PLP presentation to the T cells by APCs, although due to increased production of IL-10 by APCs, the IFN-γ-production by these T cells was impaired (146). In contrast to mice given soluble $PLP_{139-151}$-Ig chimera

which developed almost normal manifestation of EAE, mice injected with aggregated chimera recovered entirely from the disease. Subsequent investigation revealed that in FcγR- deficient mice, the chimera uptake and IL-10 production are compromised since these mice were not protected against EAE (31). However, reconstitution of FcγR mice with IL-10-producing $CD8\alpha^-$ DCs, but not $CD8\alpha^+$ DCs, restored Ig-chimera–mediated protection against EAE, providing a mechanism for Ig-chimera induced protection against EAE (31).

Tolerogenic Vaccines Delivered via Mucosal Route. Mucosal administration of vaccines has been shown to result in induction of appropriate immune response to environmental Ags in systemic sites, peripheral blood, local, and distal mucosal sites. Due to a large surface area in the gut, oral immunization is able to stimulate mucosa B and T cell response (147). Additionally, in contrast to systemic route of immunization, the mucosal route is needle-free and therefore provides a safe and painless alternative for delivery of large quantities of vaccine Ags for human immunization (22, 24, 56).

Even though protection against EAE by DNA-encoded encephalitogenic peptide is usually conferred via systemic administration (133, 141, 142), it has been demonstrated that nasal delivery of mouse proinsulin DNA in cytomegalovirus-based vector prevents cyclophosphoamide-induced and adoptively transferred diabetes in NOD mice (133).

Oral delivery of Ags using emulsion systems (148) or liposomes (149) has been demonstrated. A shown by Kim *et al*, single oral administration of type II collagen entrapped in poly(lactic-co-glycolic acid) nanoparticles induces oral tolerance and inhibits collagen-induced arthritis (CIA) (148). Induction of oral tolerance to OVA,

evidenced by lack of OVA-specific proliferation, has been demonstrated after its delivery in liposomes (149).

It has been shown that mucosal delivery of an Ag chemically conjugated or genetically fused to cholera toxin B subunit (CTB) induces tolerance to this Ag (35, 67-70, 74, 150, 151). This approach is interesting since cholera toxin (CT) is one of the most potent mucosal adjuvants, and oral administration of CT abrogates oral tolerance induced by feeding unrelated Ag (22, 64, 152).

The pioneer work in studies on CTB-mediated mucosal delivery was done by Czerkinsky's group. In 1994, Sun *et al* showed that oral administration of human γ-globulin (HGG) chemically conjugated to CTB prevents delayed type hypersensitivity (DTH) response after s.c. challenge with horse (HRBC) or sheep (SRBC) red blood cells (150). Moreover, oral administration of conjugated HGG after animal sensitization with HRBC or SRBC prevents systemic response to the challenge, implicating tolerogenic impact of CTB-delivered Ag on memory cells (150). This strategy has been subsequently applied to treat autoimmunity (67, 151). CTB-coupled human insulin effectively suppresses beta cell destruction and clinical manifestation of diabetes in NOD mice (151). Oral administration of significantly smaller amounts of MBP-coupled to CTB rather than MBP alone is able to protect rats from EAE induction, but also sufficiently suppresses ongoing EAE (67). Importantly, MBP-CTB induces production of IFN-γ but suppresses production of IL-2, and is more effective than MBP alone in reducing the CNS inflammation (67). Subsequent investigation of a mechanism behind CTB-induced

tolerance revealed that oral administration of CTB-OVA induces regulatory $CD4^+CD25^-$ $FoxP3^+$, $CD4^+CD25^+FoxP3^-$ and $FoxP3^+$ T_{reg} cells *in vivo* (69).

In the parallel report by this group, the involvement of regulatory T cells was evaluated for CTB-influenza haemagglutinin peptide (HApep). In contrast to the previous studies, HApep has been genetically fused to CTB (70). It has been shown that CTB-HApep induces extensive proliferation of HApep-specific T cells in LNs and spleen, up-regulated gut homing α4β7 integrin, and down-regulated L-selectin (70). The addition of CT to CTB-HApep induced the same events, although to a higher extent, resulting in abrogation of CTB-HApep-mediated oral tolerance (70). Additionally, feeding with CTB-HApep induces oral tolerance in wt mice, as well as in mice *in vivo* depleted of functional $CD25^+$ T regulatory (T_{reg}) cells, implicating that CTB-mediated low dose tolerance does not rely on conventional $CD4^+CD25^+$ T_{reg} cells (70).

Variable results have been observed when CTB-conjugated Ags were delivered nasally. In 1995 Bergquist *et al* demonstrated that nasal delivery of CTB-coupled dextran significantly enhances the systemic and mucosal immune response to dextran (73). However, in another study this group showed that preexisting immunity to CTB induced by nasally given CTB-dextran inhibites systemic and mucosal response to dextran challenge (74). On the other hand, transfer of anti-CTB serum into naïve recipients suppresses the systemic and mucosal Ab response against CTB but does not affect the response to dextran after nasal administration of a conjugate (74).

Nasal administration of chemically coupled CTB-insulin conjugates has been shown to delay the incidence of diabetes when compared to free CTB-treated mice (68).

Co-administration of diabetogenic T cells with the splenic T cell from CTB-insulin-treated mice reduced infiltration of the islets. Nasal tolerization with CTB-insulin induced expression of TGF-β and IL-10 but did not change the levels of IFN-γ and IL-4 transcripts when compared to controls. Interestingly, only a single nasal dose of 1 μg of the conjugate, but not lower or higher doses, conferred protection against diabetes in this study (68).

An elegant study performed by Yuki and coworkers has shown that genetically fused encephalitogenic peptide ($PLP_{139-151}$) to CTB results in a hybrid recombinant protein (rCTB-PLP) that when administered nasally can suppress ongoing EAE in SJL mice (35). Multiple low doses of rCTB-PLP given nasally significantly inhibit CNS inflammation and $PLP_{139-151}$-specific DTH response (35). The genetic approach in CTB-delivery studies is more advantageous than the chemical coupling. First of all, as stated by the authors, chemical coupling procedures may affect the immunogenicity of the chemically modified Ag (35). Also, chemical coupling may induce formation of heterologous population of CTB-conjugates, among which only a fraction will retain the original conformation and ability to bind GM1 receptor (35), which may be associated with different functional outcome following mucosal immunization (73, 74). Mucosal administration of low-dose of an Ag alone has been shown to induce tolerance in naïve animals; this approach is relatively less effective in animas already systemically sensitized with an Ag (67). Despite the successful attempts of tolerance induction via chemically or genetically modified CTB-Ag chimeras, CTB-induced T cell proliferation

and IFN-γ-production demonstrated by some groups (67, 70) could be detrimental in humans.

The strategy of tolerance induction via nasal administration of small amounts of Ag genetically fused to a mucosa-binding molecule has been investigated in our laboratory. In our approach, reovirus adhesion protein sigma 1 (pσ1) is used as a vehicle for the nasal vaccines (153, 154). Developed in our laboratory, recombinant pσ1 has been shown to enhance immunity to delivered DNA when chemically modified (82, 154), however when genetically altered pσ1 induces tolerance to fused protein Ags, as demonstrated elsewhere in this dissertation.

As an indirect approach, orally given *Salmonella*-CFA/I vaccine has been shown to be an effective inducer of tolerance and Ag-unspecific protection against EAE (33, 34, 78). This strategy is especially relevant in designing treatments for autoimmune diseases of unknown etiology, such as MS. *Salmonella*-CFA/I vaccine has been shown to protect (78) and treat (33) mice against $PLP_{139-151}$-induced EAE via induction of myelin-unspecific Th2 cell bias and activation of TGF-β-secreting $CD25^+CD4^+$ T regulatory cells. Moreover, adoptive transfer of vaccine-derived $CD25^+CD4^+$ T_{reg} cell, and to a lower extent also $CD25^-CD4^+$ T cells protects mice from subsequent EAE induction (33). Interestingly, a recently shown modification of *Salmonella*-CFA/I vaccine, in which CFA/I fimbriae instead of being expressed on a cell surface was expressed in the intracellular compartment (*Salmonella* –CFA/I_{IC}) showed the comparable protection level against EAE though via a different mechanism, not including Th2 bias (34).

Adjuvants for Tolerance

Even though vaccination against autoimmune diseases has been extensively studied, very little is known about the suitable adjuvants that could efficiently induce immune tolerance, when co-administered with an Ag.

Recently, Kang and coworkers have demonstrated that s.c. co-injection of a potent glucocorticoid immunosuppressant dexamethasone (DEX) and an Ag can trigger induction of an Ag-specific tolerance (155). In mice with pre-established DTH response to hen OVA, s.c. injection of $OVA_{323-339}$ and DEX induced long lasting unresponsiveness to OVA (155). Subsequent investigation revealed that the OVA-specific tolerance was DEX-dependent and mediated by $OVA_{323-339}$-specific $CD25^{+}CD4^{+}FoxP3^{+}$ T_{reg} cells. Additionally, DEX-mediated inhibition of DCs maturation was considered to be a supportive tolerogenic mechanism. Consistently, co-administration of insulin-derived, MHC II-restricted peptide with DEX prevented development of spontaneous diabetes in these mice, providing an argument for DEX as a suitable adjuvant for tolerance (155).

Enhancement of mucosal tolerance has also been achieved by tolerogen co-administration of certain cytokines, Abs, or microbial products (22). For instance, i.p. administration of IL-4, or oral but not s.c. injection of LPS has been shown to enhance tolerance to MBP via enhancing IL-4-expression in the CNS (41, 142, 156). Mucosal administration of IL-10 and IL-4 with an Ag has been shown to enhance Ag-specific tolerance (22, 57). Additionally, anti-IL-12 given i.p. enhanced oral tolerance via the mechanism associated with increased production of TGF-β and T cell apoptosis, and i.p. injection of orally tolerized mice with IgG enhanced and prolonged the Ag-specific

tolerance (6, 23). Oral co-delivery of certain exogenous agents, including Ags obtained from *Schistosoma mansoni*, or *Kelbsiella oxitoca,* have been shown to enhance oral tolerance (22).

Protein Sigma 1 (Pσ1) as a Mucosal Delivery Vehicle.

Intestinal reovirus infections are mostly evident in children (157). Upon primary infection of the gut, reovirus can spread into the CNS by infecting either neurons (type 3 reovirus) or ependymal cells (type 1 reovirus; T1L) (157, 158). These distinct patterns of disease segregate genetically with the S1 gene that encodes two functional proteins: 45 kDa attachment pσ1 and ~14 kDa nonstructural pσNS protein with overlapping reading frame (49, 154, 157, 159). Reovirus cell attachment pσ1 interacts with surface receptors on a variety of cells, including erythrocytes, lymphocytes, M cells, and neurons (reviewed in ref. 157). The interaction of pσ1 with the cell-surface receptor is thought to mediate reovirus spread via apoptosis of infected host cells (49). While the presence of both pσ1 and pσNS were shown in PPs of T1L-infected mice, only pσ1 was detected in subepithelial dome (SED) area - where it associated with $CD11c^{+}CD8\alpha^{-}CD11b^{lo}$ DCs and fragmented DNA from apoptotic cells (49). Unlike pσNS, pσ1 was previously implicated in the induction of apoptosis, presumably through interaction of sialic acid binding domain (SABD) and/or JAM receptor located in a C-terminal head structure of pσ1 (49, 159, 160). Pσ1 is most likely taken up from apoptotic epithelial cells by $CD11c^{+}CD8\alpha^{-}CD11b^{lo}$ or plasmocytoid (pDCs) DCs subsets previously associated with tolerance induction (47). Therefore, the pσ1-based approach provides an attractive tool for induction of immune response and/or tolerance.

Experimental Autoimmune Encephalomyelitis Background

EAE as a Model for Multiple Sclerosis

Experimental Autoimmune Encephalomyelitis (EAE) is one of the best and most frequently studied models that mimic neuropathology and clinical appearance of MS. EAE was first demonstrated in 1933 in mammals by Thomas Rivers at Rockefeller Institute, who by repeatedly injecting monkeys with rabbit brain and spinal cord induced an autoimmune demyelinating disease (161). EAE is characterized by the specific recognition of myelin epitopes by autoreactive T lymphocytes (162) and can be induced in rodents either by the immunization against specific myelin antigens, including myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG), or their respective peptides (19, 56, 162-164). Additionally, EAE can be adoptively transferred to susceptible mice by intravenous (i.v.) injection of encephalitogenic $CD4^+$ T cells (19, 56, 165).

Myelin-activated $CD4^+$ Th1 cells and their cytokines, including IFN-γ, TNF-α, and IL-2, IL-12, are thought to be responsible for the development of EAE (19, 58, 166, 167). Consistent with this view, IL-12, a cytokine essential for Th1 cell differentiation, was considered central for EAE development. Recent discoveries, however, have demonstrated that even though Th1 cells display encephalitogenic potential, another proinflammatory population, namely Th17 cells, is ultimately required for development and persistence of EAE (168, 169). Peripherally-activated encephalitogenic $CD4^+$ T cells that infiltrate the CNS have been shown to initiate demyelination (165, 170, 171). Neurodegeneration in EAE is further supported by myelin-reactive $CD8^+$ T cells, but also

by peripherally and locally activated macrophage and microglial cells (6, 172-174). Depending on the Ag used for EAE induction, this model can mimic either relapsing-remitting MS (PLP- or MBP- induced; RR EAE) or progressive MS (MOG-induced; acute EAE) (175).

Suppression of EAE has been achieved by administration of anti-CD4 mAbs (176). In addition, passive transfer of myelin-specific Th2 cells (37, 177) or induction of Ag-unspecific Th2 cell bias (33, 34, 78) has been shown to significantly down regulate proliferation of inflammatory $CD4^+$ T cells. Moreover, adoptive transfer of splenocytes from animals orally tolerized to myelin components was shown to induce protection against EAE challenge (24, 29, 37). Mice deficient in IL-23 (IL-23 $p19^{-/-}$ mice) show resistance to EAE induction, whereas overexpression of IL-23 renders them more susceptible to EAE development (reviewed in ref. 167). In addition, it has been shown that mice lacking chemokine receptor 2 (CCR2) expressed on mononuclear cells do not develop acute EAE or the CNS histopathology (170).

Consistent with MS, it is generally assumed that susceptibility to EAE development is associated with appropriate MHC II background (6, 172, 178). Interestingly, B10.S mice are resistant to EAE induction despite shared $I\text{-}A^s$ (MHC II) locus with highly susceptible SJL mice (178). Likewise, NOD mice that spontaneously develop diabetes are susceptible to EAE induced by $MOG_{35\text{-}55}$, and $MOG_{92\text{-}106}$, whereas their MHC congenic strain III, which also express $I\text{-}A^{g7}$ MHC haplotype, is resistant to both diseases (178).

Immune Response in EAE

Consistent with MS, EAE progression is associated with the phenomenon of epitope spreading (19, 179), explained as the diversification of the initial immune response secondary to the initial myelin destruction, which results in reactivity to other endogenous CNS determinants (180). In agreement with this trend, adoptive transfer of PLP-specific encephalitogenic $CD4^+$ T cells can mediate recognition of other myelin proteins, e.g. MBP by primed T cells in the CNS (24).

In addition to proinflammatory T cells, B cell, myelin-specific Abs, and cytotoxic $CD8^+$ T cells, as well as various innate cells have been associated with EAE pathogenesis (6, 172, 174). The combined effect of Abs, complement, reactive oxygen species, cytokines, and chemokines damage myelin and impairs electrical conduction along the axon which is believed to result in clinical manifestation of the disease (6).

Inflammatory $CD4^+$ T Cells and EAE Pathology. It was a largely accepted dogma that Th1 cells driven by IL-12 were the major pathogenic cells in EAE and MS (6, 172). Recent data have revised this view, however, by demonstrating that even though Th1 cells are important in effector phases of inflammatory responses in EAE, they are not pivotal for the disease induction (181-183). Instead, Th17 cells expressing transcription factor orphan nuclear receptor (RORγt) play a critical role in induction and maintenance of EAE (168, 169). Even though it is currently agreed that Th17 and not Th1 cells are pivotal for development and maintenance of EAE, Th1 cell can still play a supportive proinflammatory role after disease induction. Recently, Xiao and coworkers demonstrated that IL-12-activated IFN-γ- producing Th1 cells are necessary for the NO

production and effector phase of the MOG_{35-55}-induced EAE (184). Additionally, cytokines produced by Th1 cells including lymphotoxin α (LT-α; TNF-β) and TNF-α activate macrophages, microglial cells, and astrocytes to produce free radical nitric oxide (NO) involved in killing oligodendrocytes (185). On the other hand, neutralization of major Th1-type cytokine IFN-γ in certain phases of EAE exacerbates clinical manifestation of EAE, which together with the fact that IFN-γ- of IFN-γR-deficient mice are capable of developing EAE, suggests that Th1 cells can also have a supportive role in regulating EAE and its clinical remission (182, 183, 186). Currently, it is now accepted that the major proinflammatory population crucial for EAE pathology secretes IL-17, although it is still largely debated what cytokines are crucial for development of this distinct $CD4^+$ T cell linage.

When the differentiation of T-bet-inducing Th1 cells occurs through the IL-12-activated expression of transcription factor STAT4 (168), Th17 cell differentiation requires expression of transcription factor RORγt (187, 188). Th17 cells express their encephalitogenic potential via the secretion of cytokines, including IL-17A (also known as IL-17), IL-17F, TNF-α, GM-CSF, and IL-6, in response to TGF-β and IL-6 priming in mice (188). The cytokines required for Th17 cell differentiation are still being investigated, and at this point role of IL-6 plus TGF-β, IL-23, and/or IL-21 are each being implicated (126, 168, 169, 187, 189-193). Importantly, proinflammatory Th1 cells and anti-inflammatory Th2 cells, in addition to their suppressive activity against each other, secrete IFN-γ and IL-4, respectively, to inhibit Th17 differentiation (168, 169, 177, 186,

194-198), although already committed Th17 cells were shown to be resistant to IFN-γ and/or IL-4-mediated inhibition of differentiation (168, 169).

Role of Cytokines in EAE. The positive and negative roles of cytokines in EAE development have been well-established. Proinflammatory cytokines, including IFN-γ, IL-2, TNF-α, OPN, and recently also IL-23 and IL-17 (168, 169, 192, 199, 200), have been implicated in clinical manifestation and pathology observed in EAE, whereas anti-inflammatory (Th2-type) IL-4, IL-5, IL-13, and suppressive cytokines IL-10, TGF-β have been associated with EAE inhibition (3, 36, 56-58). Produced by encephalitogenic $CD4^+$ T cells Lymphotoxin (LT-α, TNF-β) and TNF-α induce production of free radical NO by macrophages, microglial cells, and astrocytes involved in killing oligodendrocytes (185).

Additionally, a number of cytokines, including IFN-γ, TNF-α, IL-21, IL-22, IL-23, IL-25, IL-27, and IL-28, has been implicated to have both inductive and/or inhibitory influences on EAE mediated by Th17 cells (130, 182, 183, 199, 201, 202). Among the cytokines able to play dual role in EAE pathogenesis, IFN-γ and TNF-α are the best studied. Neutralization of IFN-γ by anti-IFN-γ mAb (203), or by using IFN-γ - or IFN-γ R-deficient mice (182, 204) has been shown to exacerbate the clinical manifestation of EAE (169, 182, 183), which further implies that instead of Th1 cells, perhaps the Th17 subset is responsible for the majority of EAE related inflammation (169, 192). Additional support for this view was provided by studies demonstrating that IL-23, not IL-12, is the major proinflammatory cytokine responsible for EAE pathology (181, 205). On the other hand, IL-12 is known to induce Th1 cell differentiation and has been found in MS lesions (17) and shown to promote effector $CD4^+$ T cell activation in the presence

of regulatory T cells (206). Also, TNF-α, contrary with its proinflammatory role in the CNS, has been shown to promote proliferation of oligodendrocyte progenitors and re-myelination (199). However, TNF-α co-produced with IL-17A, and GM-CSF by Th17 cells is considered proinflammatory (186).

Recently, attention has refocused on the role of IL-23 in the induction and maintenance of Th17 cells in EAE. The role for IL-12 and IL-23 in EAE has been studied by Cua *et al* (205). Heterodimeric IL-12 and IL-23 share the common p40 subunit, whereas p19 is exclusively expressed by IL-23, and p35 by IL-12 (205). In the elegant study using IL-23p19$^{-/-}$ and IL12p35$^{-/-}$ mice, Cua and coworkers determined that IL-23 that is pivotal for autoimmune inflammation in the brain, since mice lacking IL-23p19 were shown to be resistant to EAE induction. In contrast, IL12p35$^{-/-}$ mice lacking IFN-γ were susceptible for EAE development and displayed even more severe clinical signs than the wild-type (wt) mice due to increased IL-17 production (205). Moreover, disease resistance in mice lacking IL-23 correlated with the absence of IL-17-producing CD4^{+} T cells but normal production of IFN-γ (186). Therefore, it is believed that IL-23 predominantly secreted by DCs and phagocytic cells (207) is necessary for differentiation of Th17 cells, but dispensable for their effector function, as evidenced by IL-23 requirement for induction, but not effector phase of EAE (191). Alternatively, IL-1, IL-6, and TGF-β seem to be essential for Th17 differentiation (186).

It is a general assumption that TGF-β produced by regulatory CD4^{+} T cells plays an important role in EAE prevention and/or treatment via inhibition of inflammatory response and induction of tolerance (3, 33, 36, 56). Contrary to this view, in 2006,

Mangan and coworkers found that exogenous TGF-β sufficiently suppresses intracellular expression of IFN-γ, but induces production of IL-17 (124). Moreover, as shown in this study, TGF-β is sufficient to induce Th17 cell development in the absence of IL-4, IFN-γ, and even IL-23. Additionally, this group discovered that TGF-β induces FoxP3 expression on Th17 cell, and the effect is abolished by IL-6 (124).

Another two proinflammatory cytokines, IL-6 and IL-1, have been shown to stimulate differentiation of Th17 cells in EAE. In 2006, Sutton and coworkers showed that, in contrast to Th1 and Th2 cells, induction of Ag-specific Th17 cells cannot occur in IL-1R-deficient mice (208). In this study, IL-1R-deficient mice were protected against EAE until adoptively transferred with Ag-specific Th17 cells from wt challenged mice. In 2007, Kimura *et al* demonstrated that, unlike TNF-α or IL-1β, IL-6 in combination with TGF-β can induce Th17-cell differentiation from naïve T cells and inhibit TGF-β-induced FoxP3 expression (126). IL-6 was also shown to be important for EAE induction since IL-6-deficient mice were resistant to MOG_{35-55}-induced EAE (209).

Interleukin-21 (IL-21) is a cytokine produced by activated $CD4^+$ T cells (189). The involvement of IL-21 in development of encephalitogenic Th17 cells and EAE pathogenesis is to this date controversial. Monteleone and coworkers reported that IL-21 induces development of suppression-resistant pathogenic Th17 cells and blocks differentiation of TGF-β-induced regulatory T cell (189). These results are in agreement with previous results by this group demonstrating the inability to suppress colitis by the naïve $CD4^+$ T cells activated in the presence of IL-21 and TGF-β, implicating IL-21 as a key modulator of TGF-β signaling cascade (210). Partially consistent with this view are

results obtained by group led by F. D. Shi. In 2005, this group showed that IL-21 plays a dual role in MOG_{35-55}-induced EAE in C57BL/6 mice. According to the investigators, administration of IL-21 prior to EAE induction in these mice enhances the inflammatory influx into the CNS and disease severity. Also, autoreactive T cells purified from IL-21-treated mice transferred more severe EAE than did the control encephalitogenic T cells, and this effect was mediated by activated NK cells producing IFN-γ. However, when IL-21 was administered to mice with progressing disease, this effect was not observed (211). In 2008, the same group showed contrasting results. This time a blockade of IL-21 in SJL/J mice before and after induction of EAE enhanced the influx of inflammatory cells into the CNS. Also, $CD4^+$ T cells isolated from mice with blocked IL-21 were capable of causing more rigorous EAE in adoptively transferred recipients, and T_{reg} cells from these mice lost their ability to prevent $PLP_{139-151}$-induced EAE (212). The already controversial role of IL-21 in Th17 induction and EAE has been recently questioned by Coquet and colleagues, who have shown that even though TGF-β in combination with IL-21 can drive the differentiation of Th17 cell *in vitro*, *in vivo* IL-21 is not required for Th17 differentiation and consequently for EAE (190). Experiments performed on IL-21- and IL-21R-deficient mice confirmed their high susceptibility to EAE and showed that these mice develop even more restricted EAE than their wt littermates.

IL-22 is a cytokine secreted by Th17 cells, which were shown to be the pathogenic population of self-reactive T cells in EAE (213, 214). Moreover, the IL-23-induced expression of this cytokine was demonstrated for the particularly pathogenic $CD4^+$ population in the CNS (213, 214). Interestingly, a study by Kreymborg *et al* has

shown that IL-22-deficient mice are fully susceptible to MOG-induced EAE, implying that IL-22 is not directly involved in autoimmune pathogenesis of the CNS. Interestingly, IL-22 association with anti-inflammatory response has been previously reported by Zenowicz *et al* who demonstrated that IL-22-deficient mice are susceptible to ConA-induced hepatocyte inflammation (215). Along these lines, IL-22 production by $CD25^{-}CD4^{+}$ Th2-type cells was reported recently in mice protected against EAE by orally administered *Salmonella* CFA/I and CFA/I_{IC} vaccines, that express enterotoxigenic *E. coli* fimbria colonization factor Ag I (CFA/I) subunit either on the cell surface, or intracellularly, respectively (34).

Interleukin-25 or IL-17E, even though it is a member of IL-17 family, has been shown to promote Th2 cell bias and expression of IL-4, IL-5, and IL-13 (216). As demonstrated by Kleinschek and coworkers, IL-25 also regulated the Th17-cell-mediated autoimmune inflammation in EAE (130). They have shown that EAE in IL-25-deficient mice is much more pronounced than in wt mice, since deficiency of IL-25 promotes production of IL-23-driven proinflamamtory response. Consistently, treatment with IL-25 subsequent to EAE induction but prior to clinical onset entirely suppresses relapsing-remitting EAE in mice. Interestingly, IL-25-mediated protection against EAE could be established in IL-4-deficient mice but not in IL-13- of IL-4R-deficient mice which could not respond to IL-13 (130), suggesting that IL-13 is required for IL-25-mediated suppression of EAE via inhibition of IL-23, IL-6, and IL-1β production by DCs (130).

The relevance of IL-13 in EAE has been evaluated by only a few studies (130, 217). A study examining the therapeutic role of recombinant TCR ligand ($I\text{-}A^{S}\text{-}PLP_{139}$-

$_{151}$; RTL 401) in RR EAE has indicated induced secretion of IL-13 and inhibited production of other anti- and proinflammatory cytokines (especially IL-6) by $PLP_{139-151}$-specific cells *in vitro* pre-treated with RTL401-treated mice (217). In a recent study, IL-13 induced by oral treatment with *Salmonella*-CFA/I_{IC} vaccine was essential for protection against $PLP_{139-151}$-indiced EAE, and *in vivo* neutralization of IL-13 abrogated protection against EAE delivered by vaccine-derived regulatory T cells (34). Also, *in vivo* application of human recombinant IL-13 to Lewis rats followed EAE challenge and significantly ameliorated the clinical disease due to inactivation of macrophages (218). IL-13 together with IL-10 are potent suppressors of NO in the CNS lesions (6). In contrast to the anti-inflammatory role of IL-13, this cytokine via induction of MHC II on macrophages can also direct the Th1-type immune response (219). In fact, the recent study evaluating the IL-13-mediated gender differences in susceptibility to EAE revealed that females lacking IL-13 exhibit a lower incidence and ameliorated MOG_{35-55}-induced EAE with reduced infiltration of $CD11b^+$ cells into the CNS than males.

IL-4 and IL-13 are considered to be the major cytokines produced by Th2-type cell and hallmarks of anti-inflammatory responses (6, 196, 197). In fact, IL-4 secreted by Th2 cells has been shown to support an inhibition of proinflammatory response to $PLP_{139-151}$ peptide (33). Importantly, IL-4 and IL-13, as well as TGF-β, can facilitate induction of T regulatory cells from naïve, peripheral $CD25^-CD4^+$ T cells (81, 117, 122, 220). IL-4 and/or IFN-γ were shown to negatively regulate differentiation of Th17 cells, but had no effect on inhibiting already differentiated Th17 cells (168, 169). Also, according to the Th1/Th2 cell paradigm, IL-4 produced by Th2 cells and IFN-γ secreted by Th1 cells

negatively regulate each other (194). However, depending on the source of IL-4 and the time of its expression, it can also promote Th1 response, as demonstrated by Gregory and coworkers for the IL-4 produced by mast cells (221).

Another potent inhibitor of Th17 cells, the IL-27 was originally described as a proinflammatory cytokine that induces Th1 cells (186, 202). However, later work demonstrated that mice deficient in IL-27 receptor (IL-27Rα) exhibit exacerbated inflammatory responses to a variety of challenges, suggesting that IL-27 may be immunoregulatory and suppress development of Th17 cells *in vivo* (202). Stumhofen and coworkers demonstrated that *Toxoplasma gondi*-infected WSX-1-deficient mice develop more severe EAE and augmented Th17 response (222). Also, IL-27Rα-deficient mice were shown to be hypersusceptible to MOG_{35-55}-induced EAE, and this susceptibility was associated with elevated numbers of Th17 cells (202). IL-27 contains a p28 subunit that is structurally similar to p40 subunit of IL-12 and IL-23 (202, 222). IL-27 inhibits Th17 in a STAT1-dependent, although IFN-γ -independent manner (202, 222), and suppresses IL-6-mediated T cell proliferation (202). Interestingly, it has been noted that the highest expression of IL-27 and IL-27R genes in the CNS occurs during the effector phases of relapsing-remitting EAE, and IL-27p28 protein can be secreted by astrocyte cultures especially when stimulated with endogenous IFN-γ (201). Moreover, exogenous IL-27 potently suppresses the ability of encephalitogenic cells to transfer EAE, and it ameliorates the effector phase of EAE *in vivo* (201).

The potential role of IL-28 (or interferon lambda; IFN-λ or type III IFN) in EAE has not been yet established; however its anti-virial activity, structural similarity to

successfully used in MS patients type I IFN (IFN-β), and genetic similarity to IL-10 (223) allow for prediction of its suppressive role against encephalitogenic T cells. Additionally, DCs *in vitro* treated with IFN-λ exhibit increased expression of MHC I and MHC II but inhibited expression of co-stimulatory molecules, suggesting that IL-28 induces tolerogenic potential on DCs, since these IL-28-treated DCs induce $FoxP3^{+}CD25^{+}CD4^{+}$ T_{reg} cells that suppress effector T cells in cell-to-cell contact-dependent manner (224).

It was recently reported that Epstein-Barr virus (EBV), often linked to the development of MS (6, 225), can form a heterodimeric cytokine with p35 subunit of IL-12 in human and in mouse (226). This protein, known as IL-35 (226), was recently shown to suppress the differentiation of Th17 *in vitro* and ameliorate collagen-induced arthritis (CIA) in mice. Additionally, this *in vivo* suppression of CIA was associated with IL-35-mediated inhibition of IL-17, but not IFN-γ, supporting the anti-inflammatory potential of IL-35. Moreover, it has been demonstrated that Ebi3 gene and p35 are highly expressed by mouse $FoxP3^{+}$ regulatory T cells, but not by rested or activated effector $CD4^{+}$ T cells (227). Consequently, IL-35 is constitutively expressed by regulatory $CD4^{+}$ T cells even when co-cultured with effector $CD4^{+}$ T cells, and Ebi3- or p35-deficient T_{reg} cells exhibit significant reduction in suppressive capabilities *in vitro* and *in vivo* (227).

<u>$CD8^{+}$ T Cells in EAE.</u> The relevance for $CD8^{+}$ T cell in EAE and MS has just recently been explored. According to the two step model proposed by Friese and Fugger in 2005, $CD4^{+}$ T cells are necessary for the induction phase of autoimmune inflammation, whereas the pathogenic role for $CD8^{+}$ T cells enters at the chronic phase of

disease progression (174). The importance of $CD8^+$ T cells in EAE pathogenesis has been suggested by a number of investigators. In 2001, Goverman's group reported induction of severe autoimmunity in mice by adoptive transfer of myelin (MBP)-specific $CD8^+$ T cells (228). Another group in 2005 demonstrated induction of EAE in recipient mice by adoptive transfer of MOG-specific $CD8^+$ T cells (229). Since then a number of studies have reported involvement of $CD8^+$ T cell in EAE pathogenesis (174, 230). A more complex picture of the role for $CD8^+$ T cells in EAE was presented by Abdul-Majid *et al*. A study performed by this group demonstrated that mice deprived of either $CD4^+$ or $CD8^+$ T cells exhibit substantially reduced susceptibility to EAE (231). The protection against EAE was more pronounced in CD8- than in CD4-deficient mice. However, when *in vivo* depletion of $CD4^+$ T cell in CD8-deficient mice protected them entirely from MOG-induced EAE, only 80 % protection was observed in CD4-deficient mice *in vivo* depleted of $CD8^+$ T cells (231). Consistent with animal studies and theory of molecular mimicry, the $CD8^+$ T cell response against Epstein Barr virus (EBV) protein was shown to be elevated in MS patients compared to healthy controls, whereas there were no differences between patients and controls in EBV-specific responses mediated by $CD4^+$ T cells (174).

In opposition to long lasting dogma on the CNS being an immune privileged site, it has been reported that all glial cells and neurons can be induced to express MHC I (232). Notably, MHC I is highly up regulated on microglial and endothelial cells in MS lesions (174). In order to specifically target MHC I-restricted $CD8^+$ T cells in EAE, Goverman's group designed a new approach for disease induction. Instead of classical

immunization with complete Freund's adjuvant (CFA)-delivered myelin peptide, they employed viruses to deliver MBP encoded cDNA to ensure the efficient priming of $CD8^+$ T cells in MHC I-restricted manner (230).

$CD8^+$ T cell were originally described in EAE as "predominantly suppressive cells, with a minimal effector function" (233). Consistent with this view, numbers of IL-17, IFN-γ, and TNF-α-producing $CD4^+$ and also IL-10-, IFN-γ-, and TNF-α-producing $CD8^+$ T cells have been shown to increase in the CNS along with the progression of EAE, further suggesting the suppressive role for $CD8^+$ T cells in disease pathogenesis (234). In 2005, the group led by H. Suzuki reported naturally occurring $CD8^+CD122$ $(IL\text{-}2R\beta)^+$ regulatory T cells are able to suppress effector $CD4^+$ and $CD8^+$ T cell in the IL-10-dependent, but APC-independent, manner (100). More recently this group has demonstrated that *in vivo* depletion of $CD8^+CD122^+$ T cells by administration of anti-CD122 mAbs results in increased duration of EAE symptoms, whereas adoptive transfer of naïve or pre-activated $CD8^+CD122^+$ T cells to EAE-induced mice at the peak of clinical manifestation significantly improves disease symptoms, suggesting an important role for $CD8^+CD122^+$ T cells in the remission phase of EAE (101). Additionally, it has been demonstrated by Miller *et al* that suppression of EAE in Lewis rats after feeding with MBP is mediated by MBP-specific $CD8^+$ T cells (75). These cells isolated from MBP-fed rats transfer protection into naïve recipients. Moreover, they have demonstrated that rats fed with OVA or adoptively transferred with $CD8^+$ T cells from OVA-fed mice are protected against MBP-induced EAE but only when OVA is also injected at the time of challenge (75).

B Lymphocytes in EAE. The role of B cells in pathogenesis and recovery from EAE remains very controversial. Significant amounts of Abs secreted by terminally committed plasma cells are directed against myelin proteins and lipids (6). Production of MOG-specific Abs that contributes to EAE pathology has been reported both *in vitro* and *in vivo* (235). Additionally, like other APCs, B cells express CD40 and B7 co-stimulatory molecules that provide an activation signal for T cell (236), and it has been reported, that B7-deficient mice develop ameliorated EAE (237). MOG- induced EAE displays a much more restricted and severe onset in mice lacking B cells, compared to wt C57BL/6 mice (236). Recently, it has been reported that B cell deficient mice (B10.PL background) also develop more severe and prolonged EAE in response to immunization with MBP_{1-11} peptide compared to their wt littermates (238). The severity of normally acute disease in these H-2^u mice is due to reduced and delayed production of IL-10 and decreased expression of $FoxP3^+$ regulatory T cells (238). In another report, B cell-deficient (B10.PLxSJL) F1$\mu MT^{-/-}$ mice challenged with MBP developed normal onset and manifestation of RR EAE, suggesting a minimal role for B cells in initiation and effector phase of the disease (239). Consistent with Dittel *et al,* Hjelmström and coworkers reported that B cell-deficient μMT mice displaying H-2^b haplotype are susceptible to MOG_{35-55}-induced EAE and develop acute EAE characterized by both inflammatory lesions and primary demyelination (235). In contrast to these results, the requirement for B cells in MOG-mediated EAE was demonstrated by Cross's group. They have shown that H-2^b μMT mice are resistant to MOG-induced EAE; however, passive transfer of serum or B cell isolated from MOG- but not MBP-primed or naïve wt

mice is able to reconstitute the ability to develop EAE in MOG-challenged μMT mice (240). Unlike the majority of studies that induce EAE in C57BL/6 mice with immunodominant MOG_{35-55} peptide, EAE induction by this group was performed with 120 amino acids of extracellular domain of MOG (rMOG). Interestingly injection of MOG-specific serum to healthy μMT mice 30 days after MOG challenge was associated with rapid appearance of clinical symptoms and inflammation of the CNS of these mice, implicating an important role for myelin-specific Abs in pathogenesis of EAE (240).

It was recently discovered that B cells are capable of producing cytokines and therefore can play a regulatory role in EAE (98, 236). Fillatreau and colleagues found that recovery from MOG-mediated EAE requires autoAg-reactive B cells producing IL-10 (236). Moreover, bone marrow chimeric mice that exhibited B cell restricted IL-10- or CD40- deficiency did not recover completely from MOG-induced acute EAE, showing important role for B cell-derived IL-10 in controlling autoimmunity.

In addition to Ab production, B cells can also produce various cytokines and act as secondary APCs (98, 241, 242). Recent studies have indicated that regulatory B cells observed in several models of chronic inflammatory diseases perform the regulatory function either by producing regulatory cytokines (IL-10 and/or TGF-β) or by direct cell-to-cell contact-dependent inhibition of pathogenic T cells (98, 241-243). Additionally, it has been noted that IL-10 –producing B regulatory cells appear under the inflammatory condition, and only negligible amounts of these cells can be detected in normal mice (98). Therefore, the inflammatory environment seems to be required for activation/ differentiation of B regulatory (B_{reg}) cells and their effector function (98). In their study,

Fillatreau's group demonstrated that TLR-signaling in B cells is responsible for their suppressive function toward Th1 and Th17 cells and for recovery from EAE (244). Using mice with B cells deficient for MyD88, TLR9, or TLR2/4, they demonstrated that TLRs are required for B cell-mediated recovery from EAE. According to their proposed mechanism, during EAE onset, certain TLR agonists (like *M. tuberculosis* present in CFA used as a medium for EAE induction) control suppressive functions of B cells. Since B cell suppression of T cell-mediated autoimmunity is MyD88-dependent (244), antagonized MyD88 signaling between B cells and other cells that induce differentiation of Th17 cells can be responsible for EAE pathogenesis (244).

Innate Immune Cells in EAE. Unlike adaptive immune cells that recognize and respond to an Ag via highly specific T or B cell receptors (6), innate immune cells recognize common and evolutionary conserved structures through pattern-recognition receptors i.e. toll-like receptors (TLRs). Innate cells have a potential to initiate or regulate autoimmune responses by production of inflammatory cytokines and chemokines, and also immunomodulatory molecules, like those currently approved to use in RRMS IFN-β (245).

Monocyte-derived macrophages are the major CNS-infiltrating population during MS and EAE (6, 246), and their phagocytic potential and production of reactive oxygen species (ROS) are central to demyelination and axonal damage (246). Additionally, through secretion of IL-1β, macrophages are also potent supporters of NO production (6). Osteoponin (OPN) produced by macrophages and T lymphocytes and also glial cells and

neurons further induce production of Th1 cell cytokines (IFN-γ and IL-2) and the downregulation of suppressor cytokine IL-10 (200).

Extensive infiltration of neutrophils into the CNS during progression of EAE and MS has been implicated in their pathologic role in these diseases (6, 172). However, in 2005, a group led by Owens demonstrated that infiltrating CNS population of Gr-1^{high} cells dominated by neutrophils can play a suppressive role in EAE, and that the neutrophil-mediated inhibition of T cells requires IFN-γ and NO synthase activity (247).

The most potent professional APCs, DCs can stimulate primary immune response or exhibit tolerogenic potential (47). According to the general view, all subsets of DCs can be involved in response to the pathogen; however, certain subsets are implicated in tolerance induction (47). $CD11b^+$ DCs and plasmocytoid DCs (pDCs) are potent inducers of regulatory T cells, whereas $CD8\alpha^+$ DCs can induce clonal deletion of autoreacive T cells (47). Additionally, as demonstrated by Kelsall's group, IL-10-producing $CD11b^+CD8\alpha^-$ DCs can stimulate differentiation of IL-4-, and IL-10-producing Th2 cells *in vitro* in contrast to $CD8\alpha^+$ or $CD8\alpha^-$ $CD11b^{low}$ cells that predominantly produce IL-12 and stimulate Th1 cell bias (248). Further, the role for $CD11b^+$ cell in tolerance has been confirmed by a recent study by Ehirchiou *et al*, demonstrating the inability to induce oral tolerance in mice lacking this molecule (99). Interestingly, as shown by this study, CD11b deficiency has led to differentiation of Th17 cells via IL-6-dependent mechanism. Evaluated by Zaghouani's group, the potential of $CD8\alpha^-$ and $CD8\alpha^+$ DCs to treat EAE revealed that while the IL-12-producing $CD8\alpha^+$ DCs are unable to reverse EAE in FcRγ-deficient mice, $CD8\alpha^-$ DCs pre-incubated with MOG-

IgG chimera are able to cross link-FcγR on APCs, produce IL-10, and mediate recovery from EAE of these mutant mice (31). Additionally, apoptosis of DCs has been shown to play a pivotal role in maintaining of self-tolerance, since mice defective in DCs apoptosis (DC-p35) develop spontaneous systemic autoimmunity due to DCs accumulation and chronic activation of lymphocytes (249). In EAE, pDCs are the major CNS-infiltrating DCs and in contrast to myeloid DCs (mDCs), pDCs play a negligible role in T cell activation and epitope spreading (250). Moreover, as shown by Miller's group, pDCs actively suppress production of IL-17, IFN-γ, and also IL-10 by mDCs in the CNS, and pDCs depletion results in exacerbated EAE due to increased $CD4^+$ T cells activation in the CNS and production of IL-17 and IFN-γ (250).

In contrast to pDCs, the proinflammatory myeloid DCs seem to support a pathogenesis of EAE (207, 251). It has been demonstrated that $CD11c^+$ myeloid DCs isolated from patients with SP MS express significantly higher levels of CD80 and produce more TNF-α and IL-12 compared to RRMS patients and healthy controls (251). In another study, it has been shown that DCs isolated from MS patients secrete elevated levels of IL-23 and express increase levels of IL-23p19 mRNA (207). Interestingly, when the potential of different DCs subsets in EAE were evaluated, it was shown that in contrast to B cells, both $CD8\alpha^+$ and $CD8\alpha^-$ DCs are very potent stimulators of proliferation of naïve and memory-Ag-specific T cells (243).

The anti-inflammatory role of natural killer (NK) cells has been demonstrated in EAE. *In vivo* neutralization of NK cells by administration of anti-NK1.1 Ab prior to MOG_{35-55} challenge exacerbates acute EAE in C57BL/6, RAG-2 KO, and SJL mice (252,

253). Additionally, adoptive transfer of NK cells isolated from mice immunized with immunoprotective heat shock protein (Hsp)-70 peptide complex, prevented development of clinical EAE in $PLP_{139-151}$-challenged mice (254). In contrast, one recent report implicated NK cells in pathogenesis of EAE based on the diminished clinical EAE and associated T cell response observed in mice *in vivo* depleted of NK cells (255).

The γδ T cells have been reported to play either pathogenic or regulatory role in EAE. Among the studies advocating for regulatory role of these cells in EAE pathogenesis, one that stands out was published by Ponomarev and Dittel (256). In this study, investigators suggested the pivotal role of γδ T cells in regulating the CNS inflammation. They showed that γδ T cell-deficient mice are unable to recover from acute disease due to prolonged presence of infiltrating cells in the CNS. However, reconstitution of γδ T cell-deficient mice by wt γδ T cells mediated recovery from EAE (256). These findings are in agreement with their previous discovery indicating that presence of γδ T cells is needed to promote the production of IFN-γ by both $CD4^+$ and $CD8^+$ T cells in the CNS before the onset of EAE. This regulation was shown to be independent of the ability of γδ T cells to produce IFN-γ and specific to T cells in the CNS, since no alterations in IFN-γ production were detectable in γδ T cell-deficient mice in the spleen and LNs after EAE induction (256).

In contrast to these reports, it has been shown that *in vivo* depletion of γδ T cell from lymph node (LN) cells results in ameliorated clinical and histopathological EAE in mice due to reduction of IL-12 production by LN cells (257). This result was supported by Rajan *et al* who implicated the contribution of γδ T cells in EAE via promoting the

inflammatory environment that accelerates inflammatory responses in the CNS (258, 259). According to this group, *in vivo* neutralization of γδ T cells by GL3 mAbs immediately before disease onset or during its chronic phase significantly ameliorates clinical severity of EAE (258).

Apoptosis in EAE. The disease-promoting and disease-regulation role of programmed cell death (apoptosis) has been extensively studied over the last decade, using a number of various Tg models. For instance, apoptotic death of oligodendrocytes is one the most prominent mechanisms for EAE neuropathology (260). Hövelmeyer *et al* demonstrated that mice lacking the "death receptor" Fas exclusively on oligodendrocytes and/or are entirely deficient in TNF-R1 are protected against EAE (260). Additionally, double mutant mice exhibited virtual absence of oligodendrocyte apoptosis, although with a normal degree of lymphocyte infiltration into the CNS, further suggesting that expression of Fas on oligodendrocytes is important for EAE induction and pathogenesis. Consistent with these results, adoptive transfer of MOG-specific oligodendrocytes from the wt mice to *lp*r mice harboring the natural mutation of *fas* gene, or to mice with mutated *tnf-r1* gene proved that expression of "death receptors" Fas and/or TNF-R1 is required for full pronunciation of EAE (12). Mice harboring the FasL mutation (gld mice) demonstrated a significant delay in clinical manifestation and considerable reduction in demyelination compared to wt mice (12, 260). The blockade of Fas-associated death domain (FADD) on T cells rendered mice unsusceptible to EAE challenge and suppressed both Th1 and Th2 cell responses in these mice (261). Induction

of EAE in complement (C5)-deficient mice resulted in higher axon conservation, formation of new myelin, and prevented apoptosis of oligodendrocytes (262).

Aside from the disease-promoting approach, apoptosis can also support recovery from EAE. Fas-mediated apoptosis of encephalitogenic T cells has been shown to be necessary for EAE recovery, since Fas-deficient SJL mice exhibit a significantly more severe course of EAE (13). Further analysis of the levels of Fas/FasL transcripts in the CNS revealed that in wt SJL mice, the highest level of Fas and FasL mRNA can be found at the peak of clinical EAE, and it gradually decreases following clinical recovery (13). To further address the role of T cell apoptosis in EAE, a group led by Bernard employed the Tg mouse over expressed with survival gene bcl-2 (15). No difference was observed in clinical manifestation of EAE or pathology between bcl-2 Tg mice and their wt littermates during the acute disease. However, more severe disease was observed in bcl-2 Tg mice during the chronic phase of EAE, suggesting that T cell apoptosis in the CNS is just one of the naturally evolved regulatory mechanisms during EAE and cannot explain the spontaneous recovery from acute disease (15).

Immunization with excessively activated DCs induces tissue-specific and systemic autoimmune symptoms (249). To investigate if apoptosis of DCs also regulates self-tolerance, mice deficient in DCs, T or B cell apoptosis were generated (249). Results obtained by this group confirmed that lack of DCs apoptosis results in their significant expansion and over activation of T and B cells, and the development of autoimmunity despite normal numbers and functional $FoxP3^{+}CD4^{+}C25^{+}$ regulatory T cells (249). Interestingly, γδ T cells also can induce apoptosis of encephalitogenic T cells during EAE

(256). It has been shown that compared to wt mice, mice deficient in γδ T cells exhibit exacerbated EAE associated with enhancement in proliferating encephalitogenic T cells and a reduction in caspase activity. Moreover, adoptive transfer of wt γδ T cells but not Fas-deficient γδ T cells causes recovery from EAE and resolves the CNS inflammation in γδ T cell-deficient mice (256).

A number of cytokines, including TGF-β, IL-10, and IL-4, have been implicated in mediating apoptosis (263-265). The pivotal role of TGF-β-mediated apoptosis has been established for the maintenance of B- and T-cell homeostasis (263). An important role in apoptosis of the T cell isolated from individuals affected with systemic lupus (SLE) has been assigned to IL-10. In a study performed by Georgescu *et al*, neutralization of IL-10 *in vitro* reduced T cell apoptosis by 50 % in the active SLE patients (264). Recently, IL-4's ability to induce apoptosis of human endothelial cells was demonstrated (265). Treatment of endothelial cell cultures with recombinant human IL-4 significantly increased caspase-3 activity. Consequently, inhibition of caspase-3 suppressed IL-4-induced apoptosis in a dose-dependent manner (265).

Anti-Inflammatory $CD4^+$ T Cells in EAE. It has been well documented that adoptive transfer of myelin-specific proinflammatory $CD4^+$ T cells, but not anti-inflammatory Th2 cells, induces disease in naïve recipients (19, 56, 165). IL-4, a major Th2-type cytokine, was shown to promote downregulation of EAE symptoms and support recovery from the disease (3, 36, 56). Additionally, it has been shown by Gajewski *et al*, that induction of T cell anergy is associated with downregulation of Th1- and induction of a Th2-like phenotype (21), and IL-4 was shown to promote development of anergy (3,

22). The immunomodulatory role of Th2 cells in EAE has been well established, and in the last decade many efforts have been focused on designing treatments against autoimmune diseases by inducing the Ag-specific (141, 266-269) or -nonspecific (33, 34, 78, 147, 270, 271) Th2 cell bias.

Pro- and Anti-Inflammatory Balance in EAE. It has been a general understanding that activation of naive T cells can result in their differentiation into either Th1-type or Th2-type cells (194-197). Accordingly, the pro- and anti-inflammatory cell types counter-regulate each other via production of cytokines. Th1 cells produce high levels of pro-inflammatory cytokines, i.e. IFN-γ, TNF-α and support delayed-type hypersensitivity (DTH) reactions and cell-mediated cytotoxicity. On the other hand, Th2 cells produce high levels of IL-4, IL-5, and IL-13 and support predominantly humoral responses. The "type 1/type 2" paradigm has been implicated in the etiology of autoimmune diseases such as EAE (3, 19, 58, 94, 200). It has been postulated that the deviation of potentially autoreactive T cells to a type 1 phenotype triggers autoimmunity (19, 166, 177, 200). Th1-type cells have been suggested as playing a major role in mediating EAE (166, 198), MS (6, 94, 200, 272), collagen-induced arthritis (273), and diabetes mellitus (274). By contrast, cytokines secreted by Th2 cells are implicated in the suppression of autoimmune response and the promotion of tolerance (6, 33, 78, 198, 200). Systemic tolerance abolished by deletion or inactivation of TGF-β or IL-10 (56) can be reversed by administration of Th2-type cytokines (58), and prevention of a Th1 cell response is critical for this effect. The addition of exogenous IFN-γ and/or anti-IL-4 mAbs has been shown to be responsible for abrogation of tolerance (3).

An important mechanism by which Th2-type cytokines suppress autoimmunity is a process of bystander suppression (272). Bystander suppression in EAE refers to the induction of myelin-specific Th2 cells that cross-react with the native auto-Ag and reduce the proinflammatory immune response whenever autoAg is released (75). In fact, the bystander suppression of MBP-specific encephalitogenic cells is considered the mechanism of action for GA (77). Clinical manifestation and pathology of EAE can be also inhibited by nonspecific Th2 cell bias of the immune response. Previously, we have shown that oral immunization of SJL mice with Th2 cell–imprinting *Salmonella*-CFA/I vaccine is able to inhibit clinical and histopathological manifestation of EAE (33, 78).

The discovery of Th17 cells upended the prevailing dogma of the Th1/Th2-type paradigm (168, 169). Th1 and Th17 cells, even though proinflammatory, utilize different developmental pathways, and often have opposing functions (168, 169). Additionally, the major Th2- and Th1-type cytokines, respectively, can inhibit differentiation of naïve $CD4^+$ T cells into Th17 cells (169).

Antigen-Independent Th2 Cell Bias in EAE. In contrast to induction of Ag-specific Th2 cells bias, the suppression of encephalitogenic responses and protection against EAE induced by treatment with myelin-nonspecific Ags have been studied significantly less. About a decade ago, an important discovery was published by Falcone and Bloom. These investigators tested the hypothesis that a Th2-type immune response induced against an exogenous non-self Ag could shift the proinflammatory cytokine profile observed in EAE to a protective Th2-type (270). They demonstrated that SJL mice pretreated with keyhole limpet hemocyanin (KLH) in IFA prior to EAE induction

and then given a second dose of KLH four weeks later in a challenge mixture with guinea pig myelin and CFA developed significantly reduced clinical EAE. In contrast to these mice, the group of mice that received KLH only for the first dose of KLH in IFA, but was not given KLH at the time of EAE induction, developed normal onset and clinical manifestation of EAE that was undistinguishable from unprotected control mice (270). All mice given KLH in IFA produced IL-4 in response to KLH restimulation, although in contrast to mice pre-dosed with KLH, T cells isolated from mice that received KLH also at the time of challenge showed significant Th2 bias in response to MBP (270).

Recently, a soluble egg antigen (SEA) isolated from *Schistosoma japonicum* has been shown to naturally induce strong a Th2 cell response able to prevent MOG_{35-55}-induced EAE in mice (271). They have demonstrated that i.p. injection of SEA prior to EAE induction or in its pre-clinical stage, but not after clinical manifestation of EAE, significantly reduced demyelination and mononuclear cell infiltration into the CNS and ameliorated the disease severity (271). This immunomodulatory effect, according to investigators, was attributed to the increased production of IL-4 and reduction of IFN-γ observed in spleens and the CNS of SEA-pretreated mice in the chronic disease phase.

It has been reported that silibinins, a major pharmacologically active compound of silymarin or *Silybum marianum* fruit extract, can induce anti-inflammatory responses and stimulate amelioration of EAE (275, 276). Lee *et al* had investigated the influence of silibinin on DC's ability to stimulate immune responses. They concluded that silibinin significantly down regulated expression of co-stimulatory molecules B7.1 and B7.2, as well as MHC I and II on DCs. DCs incubated with silibinin were unable to express IL-12

in response to LPS, and despite impaired Th1-type response, they displayed normal cell-mediated responses (276). A study by Min *et al* focused on the ability of silibinin to protect mice against EAE development. They showed that silibinin significantly reduced the demyelination and inflammation of the CNS in mice induced with EAE (275).

The influence of orally delivered Ag non-specific protein suppressor of immune response (SIRS) on the clinical EAE has been evaluated by Brod and Hood. SIRS was produced by immune-modulatory T cells *in vivo*, and was also shown to be secreted by these cells *in vitro* in response to ConA and type I IFN stimulation (277). Synthesized N terminal SIRS peptide 1-21 that previously was shown to be anti-inflammatory was either injected or orally administered to mice in a course of two weeks, starting one week prior to EAE induction with MOG_{35-55} peptide. Mice parenterally injected or fed with SIRS peptide displayed the decrease in EAE severity and increased production of IL-13, but only mice orally dosed with SIRS showed also a delay in clinical onset of EAE (277).

Finally, an anti-inflammatory effect of live diarrheal vaccine has been shown to significantly ameliorate clinical manifestation and neuropathology of EAE via induction of myelin-independent Th2 cell bias (78). Using an attenuated *Salmonella*-vector vaccine expressing enterotoxigenic *Escherichia coli* (ETEC) fimbriae, colonization factor Ag I (CFA/I), when given to mice prior EAE induction or during the pre-clinical phase of EAE greatly reduces clinical disease and histopathology of the CNS when compared to the unprotected PBS-dosed mice. Moreover, though to a lesser extent, even oral administration of *Salmonella* empty vector alone is able to confer a partial protection against EAE when compared to PBS-dosed diseased mice. Further analysis reveals that,

in contrast to normal anti-*Salmonella* Th1-type responses, the attenuated *Salmonella* vaccine induces a biphasic Th cell response in which Th1-type response is quickly replaced by a strong Th2-type bias that accompanies the induced TGF-β-secreting regulatory T cells and is able to suppress an inflammatory environment caused by immunization with $PLP_{139-151}$ peptide (33, 78).

Antigen-Specific Th2 Cell Bias in EAE. Much attention has been focused on developing an EAE treatment that would induce bystander suppression and myelin-specific Th2 cell bias. Currently approved as an immunomodulatory treatment for relapsing remitting MS, GA (copolymer 1) is believed to induce MBP-specific Th2 cell responses in treated patients (77). Since GA has been successful in preventing and suppressing EAE and has been effective in clinical trials, it was approved in 1996 for use in humans. It has been shown that initial strong and promiscuous binding of GA to the MHC and its competition with various myelin Ags for the presentation to T cells (134) inhibits T cell response to several myelin Ags. In fact, GA acts as a T cell receptor antagonist for the MPB_{82-100} peptide. *In vivo,* GA induces a shift of an immune response from Th1 to Th2-type cells, which is followed by a production of anti-inflammatory cytokines (134). The bystander suppression theory, in which GA-induced Th2 cells cross react with myelin-specific epitopes and inhibit encephalitogenic T cells, was recently challenged by Vollmer's group. Mice challenged with MOG_{35-55} and treated with GA, showed only moderate protection against EAE, and lymphocytes isolated from these mice failed to produce significantly more Th2-type cytokines than controls (268). Additionally, the suppressive effect of GA against EAE was also observed in IL4-and/or

IL-10–deficient mice, suggesting an alternative, Th2-independent mechanism for GA-mediated protection against EAE (268). Also, since GA can prevent EAE induced by immunization with MBP, PLP, or myelin preparation and is also shown to prevent experimental autoimmune uveitis, some investigators qualify GA as an Ag-independent immune modulator (133).

APL strategy has received a lot of attention as a therapeutic approach against autoimmunity (135). APLs designed for EAE research are molecules of modified native encephalitogenic peptide in which one or a few critical amino acids are substituted for contact with TCR, and depending on the substitution, APLs can protect or reverse EAE (135). For example, GA is considered to be a special kind of APL (77). APLs to $PLP_{139-151}$ described by Kuchroo *et al* in 1994 were sufficient to inhibit EAE in mice (278). An interesting approach related to APL-mediated therapy was exploited by Offner and colleagues (217). Constructed by this group, recombinant TCR ligand (RTL) containing soluble MHC II domains linked to $PLP_{139-151}$ encephalitogenic peptide (=RTL401) was shown to reverse clinical severity of passively transferred EAE and reduced, but did not prevent the CNS infiltration (217). According to the authors, the mechanism of action of RTL401 was the inhibition of autoreacttive $CD4^+$ T cells via induction of Th2 cell that predominantly secreted IL-13 (217).

Ramirez and Manson performed a study in which MBP-specific cell lines were incubated with IL-4 and glucocorticoid dexamethasone (DEX) to effectively induce a Th2 cell response (269). These MBP-specific Th2 cell lines were subsequently evaluated for their ability to induce or treat mice against EAE. Even though MBP-specific Th2 cell

lines did not transfer EAE to naïve mice, they were also insufficient in protecting mice against EAE challenge, when MBP-specific Th2 and Th1 cell lines were co-transferred to mice (269). However, a high proportion of mice injected with Th2 cell lines alone were resistant to EAE induction by MBP injection, suggesting induction of MBP-specific Th2 bias (269).

A very interesting approach of induction of Ag-specific Th2 cell bias via DNA vaccination has been explored by number of investigators. Induced protection against EAE following immunization with a naked DNA encoding encephalitogenic peptide has been documented for both MOG- and PLP-mediated EAE (142, 266, 279). Co-delivery of naked IL-4 gene and DNA vaccine encoding $PLP_{139-151}$ immunodominant peptide was shown to provide protective immunity against EAE in mice (266). Moreover, a similar approach of co-delivered IL-4 gene and DNA vaccine encoding myelin oligodendrocyte glycoprotein was shown to reverse already established EAE (266).

Bacterial DNA and immunostimulatory CpG oligodeoxynucleotides (ODNs) activate the innate immune system to produce proinflammatory cytokines. Stimulatory CpG motifs are potent adjuvants inducing Th1-type of the immune response and are currently used as effective therapeutic vaccines for various animal models of infectious diseases, tumors, allergies, and autoimmune diseases (141). CpG-ODN can efficiently replace *M. tuberculosis* in CFA used for induction of EAE in rodents (280). Paradoxically, with its proinflammatory role, DNA vaccines with inserted CpG motifs (141, 142) or even repeated injections of CpG-ODN (281) alone during EAE induction were shown to significantly ameliorate EAE in rodents. Even though successful, the use

of an agent that induces proinflammatory responses against proinflammatory disease can be risky and may be detrimental in a long run. To overcome this potential problem, Ho and coworkers created a "suppressive" CpG in which a single base substitution of a guanine for a cytosine down regulated CpG-mediated Th1-type response and created the "suppressive" CpG that provoked Th2 cell bias (141). The resulting "suppressive" CpG was able to moderately ameliorate clinical EAE when i.p. injected at the time of the $PLP_{139-151}$-challenged, but also improved the disease manifestation when given to SJL mice at the peak of the clinical disease (141). To enhance the effectiveness of this vaccine, the group applied a tolerizing myelin cocktail/IL-4 DNA vaccine mixture with "suppressive" CpG for their further experiments (142). They showed that the improved vaccine was more efficient in suppressing clinical EAE by shifting toward Th2 cell bias and significantly reducing auto-aggressive anti-myelin Abs from spreading due to induction of protective IgG1 isotype (142).

In a recent clinical trial, a DNA vaccine encoding full length human MBP (BHT-3009) was tested in MS patients and was found to be safe and well tolerated by the subjects (282). It has been shown that immunization with BHT-3009 induces anti-inflammatory Ab-specific Th2 cell bias that inhibits Th1-driven production of IFN-γ and markedly reduces myelin-specific auto-Abs (282).

Multiple Sclerosis Background

Multiple Sclerosis

Multiple Sclerosis (MS) is the most frequently diagnosed inflammatory demyelinating disease of the CNS in Northern Europeans and North Americans, affecting

as many as 400,000 people in United States and over 2 million people worldwide (283-285). MS was first observed in 1868 by the French neurologist Jean Martin Charcot, who gave it the name *sclérose en plaques disseminées* (multiple sclerosis) after observing repeatable perivescular accumulation of inflammatory cells in the CNS of patients with sporadic incidences of neurologic dysfunction (172). The frequency of MS is thought to be age-, and gender-dependent, since it most frequently affects young and middle-aged adults and occurs twice as often in females than in males (6, 283, 286, 287). The first manifestation of clinical symptoms is often seen during young adulthood leading to substantial disability in over 50 % of the patients (283). MS is generally not a fatal disease, although very rarely it can become malignant, leading to considerable neurologic disability and/or death shortly after the disease onset (285, 288).

Pathology of Multiple Sclerosis

Examination of brain and spinal cord tissue obtained from individuals with MS demonstrates multiple distinct plaques in the CNS white matter associated frequently with optic nerves, the white matter zones in the periventricular region, brain stem, and spinal cord (172, 285). Substantial axon damage and axonal loss, proliferation of astrocytes and production of glial fiber are important manifestations of MS pathology (285, 289). Additionally, paralysis and the inability to walk are characteristic for the chronic phase of MS and are strongly associated with the axonal loss in the spinal cord and subsequent spinal cord atrophy (6, 289).

The presence of demyelinating plaques is associated with perivascular infiltration of inflammatory cells, including αβ and γδ T cells and monocytes as well as B and

plasma cells (290). Macrophages primarily infiltrate into the center of the plaques, and their presence correlates to the reduction in numbers of oligodendrocytes (172). Currently, four distinct pathological patterns leading to demyelination have been distinguished in active MS lesions. Among them, type 1, evident in 19 % of lesions, is believed to be T cell-mediated with demyelination due to phagocytosis by macrophages, or is caused by macrophage-released toxins. Type 2 lesion (T cell- and antibody-mediated) is the most common pathology and is seen in about 53 % of MS lesions. In type 2 lesions, demyelination is believed to occur via myelin-specific Abs and complement. Type 3 pathology, observed in 26 % of lesions, is related to oligodendropathy followed by apoptosis. The fourth type of demyelination patterns occurs rarely and is associated with only 2 % of lesions and is observed only in a small subset of patient with primary progressive MS (PPMS). Type 4 lesions are the secondary demyelinations resulting from the primary oligodendrocyte damage (172, 285, 291).

Symptoms of Multiple Sclerosis

Variable clinical manifestation and diverse symptoms in MS correlate with CNS pathology (285, 286). During the more advanced stages, MS patients experience alterations of sensory, motor, cerebellar, brain stem, and autonomic functions (285). The pattern and timing of MS lesions are rarely predictable, and often the clinical symptoms show a discrepancy between MS-affected individuals. However, some of the symptoms are more common among MS patients, including fatigue, described in more than 50 % of MS patients, and the presence of bilateral internuclear ophthalmoplegia (172, 292). From the clinical perspective, the majority of MS cases can be categorized as relapsing-

remitting MS (RRMS), though some individuals develop primary progressive MS (PPMS) (6, 135, 172). Over 85 % of patients experience RRMS where episodes of clinical signs of the disease are followed by partial or full recovery within a few weeks or a few months, depending on the stage of the disease (172, 285). Among patients with RRMS, for at least 20 years, approximately 20 % develop benign MS characterized by clinical stability (285). On the other hand, over 40 % of RRMS patients develop secondary progressive MS (SPMS) characterized by progressive neurodegeneration of the CNS caused by chronic CNS inflammation (293). The chronic phase responsible for the majority of MS pathology (200) is due to degeneration of the both the myelin sheath synthesized by oligodendroglial cells and the underlying axons (6). Approximately 20 % of MS patients develop PPMS, characterized by gradual clinical progression (294, 295). In contrast to RRMS, PPMS develops mostly in older individuals and affects both men and women with a similar frequency (285). Importantly, a high percentage of individuals with PPMS develop unresponsiveness to any form of available immunotherapy for MS (296).

Genetic Susceptibility to Multiple Sclerosis

Data obtained from animal models suggest that MS is a T cell-mediated autoimmune disease and may be triggered by an unknown exogenous agent in genetically susceptible individuals. Even though the etiology of MS at this point remains largely unknown, the genetic susceptibility has been consistently linked to development of the disease (297-299). It is well-documented that the most common RRMS affects females twice as often as males, especially those between the ages of 20 to 40 (6, 172, 283).

Additionally, it seems that people of Caucasian descent, especially Northern European, are more susceptible to MS (reviewed in ref. 300). Even though it is well understood that MS is not simply an inherited disease, it does tend to cluster within families, with a 1-5 % of risk for MS development in a first-degree relatives and an increased likelihood between identical twins (25-30 %) (285, 301-303).

Almost every chromosome has been linked with susceptibility to MS development (reviewed in ref. 304). However, the most consistent association has been reported with the chromosome 5p13 in Canadians and Scandinavians and with 10p15 in several European populations (304).

A number of genome-wide linkage studies have been performed over the last decade that consistently reveal the association of MS susceptibility with extended haplotypes of the MHC II genes located on chromosome 6 (297-299). In particular, human leukocyte antigen (HLA)-DRB1*1501 allele (reviewed in ref. 304, 300) has been associated with about 50 % of MS cases (300, 302, 303). Additional MHC II molecules linked to MS development include DQB1*0602 in Sardinian MS cohort and DRB1*1501, DQB1*0602, and DRB5*0101 in Norwegians and African Brazilians (300).

Among non-HLA-genes, polymorphism of two cytokines receptor genes, namely interleukin 7 receptor gene α (IL-7RA) located on chromosome 5p13 and IL-2RA (CD25) on chromosome 10p15, have been consistently linked to MS susceptibility (300). Interestingly, it has been estimated that single nucleotide polymorphism rs6897932, located in the alternatively spiced exon 6 of IL-7RA, is associated with about 30 % cases of MS (300).

In the early 1970s, three independent groups described association of HLA-A3 allele and MS development (reviewed in ref. 300). Recently, the predisposition to MS development has been examined for individuals harboring MHC I HLA-A303 allele (305, 306). It has been suggested that expression of HLA-A3 (A*0301) nearly doubles the risk for MS development in an HLA-DR-independent manner (306). Moreover, HLA-A2 (A*0201) allele was strongly associated with protection against MS, implicating that presence of HLA-A2 cuts in half the risk for MS development in individuals harboring HLA-A3. In contrast, it is believed that individuals expressing HLA-A3 in combination with HLA-DR2 have more than combined risk for development of MS (305, 306).

Environmental Susceptibility to Multiple Sclerosis

Aside from genetic association, MS development has been linked with certain environmental factors. In 1984, an association has been suggested between the frequency of MS and the geographical latitude of where the patients resided and their ages (307). According to migration studies, relocation during early adulthood from the areas exhibiting lower frequency of MS to the high-prevalence areas is associated with the higher risk of MS development (307). In contrast to this study, the migration- related risk of MS development could continue in the Australian population until the third decade of life (308). Migration studies of refugees from East Asia to the United States demonstrated generally low risks for MS development, whereas immigrants from India exhibited a higher risk of MS in the second generation (308).

The association between geographical location and the frequency of MS is believed to be strongly dependent on the amount of the sunlight. Consistent with this

belief is the co-occurrence of the lower MS incidence and higher UV exposure for persons living on the sunny Atlantic coast (308). Interestingly, the frequency of MS in a sunlight-limited Northern Norway is much lower than in the lower latitude and sunnier Scotland or England (308).

The relationship between physical activity, vitamin D sufficiency, and lower incidence of MS has also been suggested (308). The geographic incidence of MS implies that an increase in MS correlates with the decrease in light exposure (309, 310). Insufficient production of vitamin D occus under low-sunlight conditions, and therefore, a role for vitamin D has been proposed in preventing MS (308-310). It has been demonstrated that exogenous 1,25-dihydroxyvitamin D3 (vitamin D3) entirely prevents clinical manifestation of EAE, and treatment of mice with 1,25-(OH)2D3 hormone inhibited EAE progression by inducing synthesis of IL-4 and TGF-β (309). Though not yet proven, the therapeutic potential for vitamin D in MS is currently being evaluated (310).

Combined evidence suggests that when the genetic factors are taken out of the equation, the relationship between latitude, sunlight, vitamin D, and photobiology are the major factors influencing the susceptibility to MS development. Other theories, including the hygiene hypothesis, suggesting that incidence of MS can be associated with the high level of sanitation (308), and birth order hypothesis, that correlate with the susceptibility to MS at this time remain inconclusive (308, 311). In addition, recently, cigarette smoking has been positively associated with MS progression (308). Environmental factors may contribute to MS susceptibility via the mechanism of

molecular mimicry in which inappropriate cross-reactivity occurs between foreign and self-antigen (225, 312).

Molecular Mimicry Hypothesis. The ability to distinguish between self and foreign Ags is one of the most important features of an immune system. Epidemiological studies combined with neuropathology as seen in MS and infectious demyelinating diseases have resulted in the speculation regarding the infectious etiology of MS (283, 307, 313). According to this theory, the immune system can mistakenly recognize self-Ags as foreign if the infectious pathogen shares common structural motifs (6). This theory is supported by the sequence similarities seen between some microbial and myelin proteins (6), as well as by the fact that complete sequence homology between self- and foreign peptide is not required for molecular mimicry (283, 312). A high degree of T cell receptor (TCR) degeneracy, defined as a ability of peptide-specific TCR to tolerate multiple amino acids substitutions in the peptide sequence (296), observed in MS patients that correlated with cross activation of autoreactive $CD4^+$ T cell by an infectious agent has been reported (225, 296, 312). It is believed that relapses in MS are often triggered by infection with common viruses (225). Influenza, human herpes virus (HHV) 6, human papillioma virus (HPV), and Epstein-Barr (EB) virus all have been shown to encode sequences mimicking those found in myelin sheet, stimulating anti-myelin Abs cross-reacting with the sequences isolated from these microbes (6, 225, 314). Moreover, viruses including EBV and HHV-6, have been shown to contain binding motifs for HLA-DR2, and many HLA-DR2-bound microbial peptides can cross-stimulate MBP-reactive T cell clones (315). It has been reported in EAE model that TCR MBP Tg mice

spontaneously develop EAE only when kept in a nonsterile environment, suggesting an important role for the infectious agent in facilitating autoimmune disease (316).

Disease Mechanism

Even though the etiology of MS remains unknown, the current data suggest that it develops in genetically susceptible individuals but may require additional environmental triggers (285). Based on information obtained from experimental rodent models, i.e., autoimmune encephalomyelitis (EAE), peripheral autoreactive $CD4^+$ T cells undergo Ag-specific activation, which results in the loss of immunologic self-tolerance and the development of an autoimmune response (296). The auto-activation of $CD4^+$ T cells may occur by at least two mechanisms. It has been reported that T cell receptor (TCR) of the autoreactive $CD4^+$ T cells are promiscuous, allowing for the activation of these cells outside the CNS in the periphery by the mechanism of molecular mimicry (296). Alternatively, activation of autoreactive $CD4^+$ T cells can occur in the absence of co-stimulatory signals, which combined with a lower threshold for the activation of these $CD4^+$ T cells in both periphery and the CNS, can lead to an autoimmune response (20, 296, 317). These autoreactive T cells penetrate the blood brain barrier (BBB) and recognize autoAgs within the CNS parenchyma in the context of MHC II expressed by DCs and local microglial cells. As a consequence, the autoreactive $CD4^+$ T cells develop proinflammatory phenotypes and secrete proinflammatory cytokines such as IFN-γ and TNF-α, and chemokines that together recruit additional innate inflammatory cells as well as myelin-specific B cells, which amplify tissue injury (5, 296, 318). Additionally, autoreactive T cells can on their own cause disruption of myelin and release additional

potential autoAgs (6, 285, 318). Apoptotic death of autoreactive T cells and T cell conversion toward Th2 cell phenotype trigger partial re-myelination of MS lesions in the early phases of the disease (319).

Epitope Spreading. Another important mechanism for MS pathogenesis was derived from EAE studies. In 1992, Lehmann *et al* observed that induction of EAE in mice by administration of a single epitope to MBP resulted in the activation of T cells against other MBP-epitopes and also against other myelin proteins (179). Epitope spreading requires B7/CD28 costimulation (19). Conveniently, the CNS white matter of MS patients has been shown to express a high degree of co-stimulatory molecule B7.1 (17). Additionally, it has been reported that TCR specificity is not as essential as previously believed for the initiation of autoimmune response (225). A number of amino acid substitutions in the MBP_{87-99} immunodominant peptide was shown to be tolerated by MBP_{87-99} TCR, suggesting a high degree of degeneracy for the TCR MBP_{87-99} recognition (296).

Lymphocyte Penetration through Blood Brain Barrier (BBB). IFN-γ and TNF-α released during inflammatory responses activate expression of MHC II and the vascular cellular adhesion molecule (V-CAM) in the outer layer of the BBB consisting of specialized capillary endothelial cells (320). Activated, clonally expanded lymphocytes express α-4-integrin which binds V-CAM while CD4 molecule interacts with MHC II. Activated T cells expressing very late antigen (VLA)-4 interact with adhesion molecules expressed by the inflamed endothelium (6) and facilitates $CD4^+$ T cell adhesion to the

endothelial layer. It has been shown in EAE that a blockade of VLA-4 reverses clinical manifestation of the acute disease and prevents relapses in chronic EAE (6). Interestingly, MRI data have indicated that macrophage recruitment into the CNS is not fully compromised after administration of anti-VLA-4 Abs (321). Humanized anti-VLA-4 Abs (alpha-4-beta-1 integrin; natalizumab) are currently FDA approved for use in MS patients who do not respond to other MS treatment. Alternatively, T cells may recognize an Ag presented by endothelial cells and attack BBB (reviewed in ref. 283, 322, 323).

After crossing the endothelial layer of BBB, activated lymphocytes produce matrix metalloproteases (MMP) 2 and 9, which degrade collagen IV surrounding the inflamed brain endothelium (324). MMPs also degrade myelin components during the active phases of MS and are found in cerebrospinal fluid (CSF) obtained from MS patients (324). Moreover, immunoreactivity to MMP-9 is observed in MS lesions (324, 325).

Local Antigen Presentation within the CNS. Local inflammatory conditions in the CNS induce microglia and local macrophages to upregulate MHC II and costimulatory molecules, CD86 and CD80, and present Ags to the T cells (296, 326). Low specificity of autoreactive TCRs and stimulation of lower-activation threshold memory T cell in a CD28-independent fashion by local APCs are likely to support the chronic inflammation in the CNS (296, 327). In fact, analysis of the early lesions revealed expression of endothelial cell-activation markers and adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1), MHC II Ags, intracellular adhesion molecules 1 and 2 (ICAM-1 and ICAM -2), etc. (reviewed in ref. 283).

Humoral Immune Response in MS Patients. Mostly what is known about the role of B cells and Abs for MS has been directly translated from the results obtained in animal studies. Early studies on EAE showed the possibility of transferring disease into naïve animals using Th1-type $CD4^+$ T cells but not humoral factors (reviewed in ref. 283). Conflicting results have been published regarding the ability to induce EAE in B cell-deficient mice (235, 240). The most probable scenario is that both cellular and humoral immune components contribute to immune-mediated demyelination and are critical for various steps in MS pathogenesis.

The first discovery implicating an important role for the humoral immune response in demyelination has come from EAE study showing that sera isolated from EAE-diseased mice cause demyelination *in vitro* (328). Additionally, it has been demonstrated that a large number of MS patients produce Abs to immunodominant MBP_{85-99} peptide (314, 329), which is also recognized by MBP-specific T cells obtained from HLA-DR2-expressing patients, implicating a T-cell driven Ab response (283). Elevated Ab titers against various myelin components, including surface-exposed myelin component MOCK or MOG, have also been reported (283, 329, 330).

Among the distinct patterns of pathology and demyelination observed in MS, the second type is believed to be mediated by the combination of Abs and T cells (172, 283, 285). The presence of intrathecally synthesized oligoclonal IgG Abs also known as "oligoclonal bands" is detected in over 90 % of MS patients, and since their activity is not affected by treatments with IFN-β, this is often used as one of the disease markers (reviewed in ref. 283). Sequence analysis of various heavy chains of Abs isolated from

MS patients reveals extensive somatic mutations indicating auto-Ag-specific B cell selection (331).

Even though MOG-specific Abs were shown to cause demyelination in EAE, just recently the pathogenic potential of these Abs have been investigated in MS in primates (330). Anti-MOG Abs have also been shown to specifically bind to disintegrated myelin surrounding axons in acute MS lesions (329).

Even though not confirmed in MS, B cells and Abs can play a supportive role in recovery from demyelinating disease. In 2000, Warrington and colleagues described human mAbs against oligodendrocytes that promoted re-myelination in a virus-mediated model of MS (332). Also, regulatory B cells secreting anti-inflammatory and/or regulatory cytokines, able to suppress inflammatory and induce regulatory responses, have been described in animal models of MS, rheumatoid arthritis and inflammatory bowel disease (IBD) (98).

T Cell Response in MS Pathogenesis. MS-dependence on T cells has been well established in animal models and implicated in human studies (6, 77, 172). T lymphocytes play a central role in the pathogenesis of multiple sclerosis (MS) (333), since increased frequencies of $CD4^+$ and $CD8^+$ T cells have been demonstrated in MS lesions (172, 188, 283, 284). However, when $CD4^+$ T cells predominate in acute lesions, $CD8^+$ T cells are more frequent in chronic lesions (172, 188, 283, 284, 334). Despite the consistence presence of oligoclonally expanded T cells in MS lesions, their action and primary targets are still undefined (173, 174). Aside from Tαβ cells, recently described

$CD16^+$ γδ T cells were found to be cytotoxic to the CNS cells *in vitro* via Ab-dependent cellular cytotoxicity (335).

$CD4^+$ T Cells. Based on results from animal studies, and human clinical trials, it is commonly believed that at least the initial phase of MS is mediated by $CD4^+$ T cells autoreactive to myelin Ags (78, 172, 286, 336). T cell reactivity against myelin components has been extensively studied in both EAE and MS (283), and very similar T cell epitopes have been demonstrated as being encephalitogenic in both diseases. Among these T cell epitopes, MBP_{83-99} has been the best studied and shown to be immunogenic in MS patients expressing various MS-associated DR alleles, including HLA-DR15, DR4, and DR6 (reviewed in ref. 283). However, other MBP-specific T cell epitopes have also been implicated in disease development (283). Interestingly, similar MBP-specific T cell epitopes are immunodominant in healthy controls and in MS patients, although their frequency is significantly higher in MS affected individuals (283). Based on the results obtained from the EAE research, MS is mediated by inflammatory $CD4^+$ Th1 and/or Th17 cells that initiate and propagate proinflammatory responses via the secretion of proinflammatory cytokines, including IL-17, IL-21, IFN-γ, and TNF-α (6, 172, 337). Direct evidence for the role of $CD4^+$ T cells in MS pathogenesis was derived from the phase II of clinical trial on MBP_{83-99} altered peptide ligand (APL). APLs have been designed to reduce the myelin-specific inflammatory response by inducing the Th2 cell bias (77, 172, 283). Even though the desired Th2 response was induced by MBP_{83-99} APL, three out of eight patients experienced exacerbation of symptoms following APL administration. This aggressive MS onset was linked to enhanced inflammatory activity

in MS lesions, as well as to a strong immune response against both APL and MBP_{83-99} peptide, and this effect was dose-dependent (283, 338). In a recent study, Hedegaard *et al* analyzed the MBP-specific response of $CD4^+$ T cells isolated from MS-patients and age/sex matched healthy controls (339). Results obtained by this group have shown that MBP-specific $CD4^+$ T cells from MS patients proliferated *in vitro* in response to MBP stimulation and produced significantly more of IL-17, IFN-γ, but also IL-4 and IL-5 comparing to the healthy controls.

PLP-specific $CD4^+$ T cell epitopes have been also extensively studied in MS. Full length PLP is expressed abundantly and exclusively in the CNS (6, 283). Immunodominant $CD4^+$ T cell epitopes have been described in MS patients and in a considerably lower frequency in healthy individuals in the context of HLA-DR4, DR15, etc, (283).

Based on the EAE studies, Th2-type $CD4^+$ T cells, as well as different types of regulatory $CD4^+$ T cells, can play a supportive role in disease remissions and amelioration of clinical relapses (33, 60). MBP- and PLP- specific $CD4^+$ regulatory T cells that produce TGF-β were found in a blood of patients with RRMS who were previously treated with bovine myelin preparation (59). However, the double-blind phase III clinical trial in RRMS patients orally treated with a single-dose of bovine myelin did not reduce the numbers of relapses in these patients comparing to placebo group (22).

<u>$CD8^+$ T Cells.</u> The role for $CD8^+$ T cells in pathogenesis of MS has been long underestimated. Physiologically, $CD8^+$ T cells are predisposed to eliminate abnormal cells that are infected or neoplastic. Even though strong genetic association between

MHC I in MS has not been established, $CD8^+$ T cells together with $CD4^+$ T cells has been found in the CNS lesions, and their clonal expansion has been documented from CSF obtained from MS patients (173, 174). It has been shown that $CD8^+$ T cell-mediated demyelination displays similar distributions of lesions within the CNS to those seen in MS, where the majority of lesions are located in brain, as opposed to the spinal cord, which is the case in $CD4^+$ T cell-mediated EAE in SJL mice (174). In 2005, Friese and Fugger proposed the two step model for MS pathogenesis where $CD4^+$ T cell are required to initiate the disease and for its acute phase, whereas $CD8^+$ T cells are responsible for CNS damage during MS relapses and the chronic phase of MS (174). Interestingly, it has been reported that neurons and glial cells can be induced to express MHC I when *in vitro* stimulated with appropriate cytokines, especially with IFN-γ (232).

Although the majority of $CD8^+$ T cell epitopes found in the CNS are in the context of the abundant anti-inflammatory HLA-A2 allelle (305, 306), many of the HLA-A2-restricted $CD8^+$ T cell responses studied to date appear to be cytotoxic (174). Cytotoxic clones were isolated from MS patients against a number of epitopes, including $MBP_{110-118}$ and PLP_{80-88}, and these clones lysed MBP-transfected $HLA\text{-}A2^+$ target cells and secreted IFN-γ and TNF-α (174). In addition, depressed numbers and activity of suppressor $CD8^+$ T cells, but not $CD4^+$ T cells, have been reported in MS patients (174), and since these suppressor $CD8^+$ T cells naturally reduce IFN-γ production and proliferation of activated $CD4^+$ T cells, their reduction has been linked to MS relapses.

Effector Molecules in MS. Proinflammatory T cells produce in the CNS large amounts of lymohotoxin alpha (LT-α; TNF-β) and TNF-α that induce macrophages, T lymphocytes, local microgial cells and astrocytes to produce NO and OPN (200). Cytokines, chemokines and their receptors play an important role in clinical relapse and remissions in MS (6, 283). It has been shown that blood samples obtained from RRMS patients prior to the clinical relapse contained elevated levels of secreted TNF-α and increased IL-12p40 transcript (283, 340). Additionally, analysis of blood-isolated PBMCs obtained from untreated patients with RRMS revealed increased expression of IL-17 mRNA (188).

Among the chemokines, IFN-γ- inducible protein 10 (IP-10), monokines induced by IFN-γ (MIg), were shown to be elevated in CSF of MS patients during relapse. Additionally, macrophage inflammatory protein-1 alpha (MIP-1α) was shown to be strongly expressed by macrophages and microgial cells in MS lesions (283). In contrast, Th2-type and regulatory cytokines TGF-β, IL-10, and IL-4, were increased in remitting MS patients (340).

Cell-Mediated Cytotoxicity and Axonal Damage. Axonal damage occurs in both chronic and acute MS lesions, but also in the normal appearing CNS white matter and therefore it is already present in the early stages of MS (341). Chronic infiltration of proinflammatory cells into the CNS triggered by myelin-specific T cells induces release of free oxygen radicals and NO by microglial cells, which leads to myelin destruction (285). It is believed that increased concentration of NO can mediate axonal injury via mitochondrial damage. This view is supported by the study in which NO-mediated

axonal injury was prevented by sodium channel blockers (342). During CNS inflammation, macrophages can release excessive amounts of excitatory neurotransmitter glutamate which activate alpha-amino-3-hydroxy-5-methyl-4-isoxazoleppriopionic acid (AMPA) receptors (6, 285) and induce oligodendroglial cell damage most likely due to increased fluxes of calcium (6, 343).

Treatment of Multiple Sclerosis

MS is one of the best-studied human neurological diseases. Even though the primary cause of MS has yet to be discovered, MS pathogenesis is very well-characterized, mostly due to availability of animal models. Currently used and approved by Food and Drug Administration (FDA), immunomodulatory therapies for MS aim at specific steps in disease development. Available treatments target activation of autoreactive T cell on the periphery (glatiramer acetate, GA) migration of the inflammatory cells into the CNS (IFN-β and natalizumab) or suppress proliferation of inflammatory cells (mitoxantrone) (77). Reduced inflammation following administration of these drugs does not stop MS progression, suggesting that broad immune deregulation during MS is in the best case scenario only partially blocked by these therapies.

The most common immunomodulatory therapies for RRMS include IFN-β (IFN-β1a – Avonex and Rebif; IFN-β1b-Betaseron) and/or GA (Copaxone). IFN-β was initially used in MS due to its anti-viral effects (77), and its strong anti-proliferative and anti-inflammatory potential was discovered later. Additionally, IFN-β inhibits expression of MHC II and co-stimulatory molecules (CD80 and CD86) on APCs in periphery and in the CNS and thereby suppresses Ag presentation and inhibits the proinflammatory

response (77, 172, 338). Moreover, IFN-β is a potent inhibitor of IL-12 production and initiator of IL-10 production (77). IFN-β1a inhibits cell trafficking into the CNS by suppressing adhesion molecules (VCAM-1, ICAM-1), inhibiting expression of chemokines (MIP-1α) and chemokine receptors (CCR5), and blocking metalloproteases 2 and 9 (77).

Glatiramer acetate (GA) is a mixture of short peptides containing four amino acids over-expressed in MBP immunodominant peptides: glutamic acid, alanine, lysine, and tyrosine (77). Human *in vitro* studies demonstrated binding of GA to MHC II DR, suggesting competition between GA and myelin peptides that results in the less frequent presentation of myelin Ags to autoreactive T cells (77). Additionally, *in vitro* GA inhibits activation and proliferation of MBP-specific T cells and suppresses IFN-γ secretion by these cells, causing their unresponsiveness in a dose-dependent manner (77, 338). GA-reactive $CD4^+$ cells exhibit strong Th2 cell phenotypes, and the GA-induced Th2 cell bias has been shown to be neuroportective in animal model (77). In addition to $CD4^+$ T cell responses, GA induces also suppressor/cytotoxic $CD8^+$ T cell responses that *in vivo* modulate EAE during ongoing therapy (284). Even though IFN-β and GA are commonly used individually or in combination for treatment of RRMS, they are effective only in about 30% in reducing relapse rate and exhibit only moderate long-term benefits (338). Interestingly, GA prescribed currently as an injection to RRMS patients has been successfully used in animals as an oral alternative (37, 56).

Mitoxantrone (Novantrone) is an anti-neoplastic agent and chemotherapy drug that inhibits synthesis of nucleic acids and suppresses T and B cell proliferation (338).

Due to the significant toxicity of mitoxantrone that can lead to infections and cadriomypathy, it is prescribed only to MS patients with severe attacks and rapidly advancing disease who do not respond to other MS therapy (77). Mitoxantrone, like Betaseron, Avonex and Rebif is often prescribed to patients exhibiting secondary progressive MS (SPMS).

Natalizumab (Tysabri) is a humanized anti-VLA-4 mAb that blocks α4-integrin on monocytes and lymphocytes, inhibiting their interaction with VCAM-1 and blocking their infiltration into the CNS (6). FDA-approved to be used in RRMS, it was subsequently withdrawn from the market due to three lethal cases of progressive multifocal leukoencephalopaty (PML) in two MS patients and one patient with Crohn's disease. Natalizumab was reapproved in 2006 but is being prescribed for no longer than 2-3 years and with a number of restrictions.

Potential Treatments for Multiple Sclerosis. An open-label study involving i.v. injection of humanized anti-CD25 mAb (daclizumab) to 10 MS patients with incomplete responses to IFN-β therapy revealed almost 80 % reduction in new contrast enhancing lesions and was very well tolerated by the patients (344). Currently daclizumab seems to be successful in phase II of clinical trials (345).

Statins are inhibitors of 3-hydroxyl-3-methylglutarylcoenzyme A (HMG-CoA) reductase that act in the early steps of cholesterol biosynthesis (77) and currently are being tested as a potential therapy for MS. Statins are successfully used as a cholesterol lowering drugs and in prevention of cardiovascular disease (188). Recently the immunomodulatory function of statins has been extensively investigated (77, 346) and

has been demonstrated in EAE model to target multiple steps in the disease pathogenesis. Aside from statin-mediated inhibition of autoreactive $CD4^+$ T cell activation, proliferation and proinflammatory cytokine production, they can also enhance remyelination, promote neuroprotection and possibly neuroregeneration by inhibiting axonal and oligodendrocyte loss (reviewed in ref. 77). Statins inhibit Ag presentation at both the periphery and the CNS, but also suppress migration of activated lymphocytes into the CNS.

Purpose of Study

The purpose of this study is to determine if genetically altered pσ1 can mucosally deliver tolerance to fused protein antigen/s, and to investigate the mechanism of this tolerance. Previously our group has reported the *ex vivo* binding of pσ1 to nasal-associated lymphoid tissue (NALT) M cells suggesting the applicability of a pσ1-based vehicle for vaccination of mucosal sites (82). Additionally, the pσ1-based vehicle was able to exhibit targeting properties of live vector delivery systems, but lacked the risk of producing disease. The possibility of administrating low, and/or single doses of pσ1-based tolerogen may omit the risk of developing allergies.

Our preliminary data described in Charper 2, and previously published in *The Journal of Immunology* (Rynda *et al*., 2008. 180: 5187-5200), confirmed that nasal administration of pσ1-based Ag is able to successfully modulate immune responses, which adds to the success of the described here tolerogenic vaccine. When low doses of recombinant OVA-pσ1 were administered mucosally, tolerance to OVA and to pσ1 was

induced. Generation of a low dose mucosal tolerance was associated with active suppression of an antigen-specific effector T cell. This suppression was shown to be mediated by antigen-specific $CD25^{+}CD4^{+}$ T regulatory (T_{reg}) and supported by $CD25^{-}$ $CD4^{+}$ T cells that secrete IL-10 and IL-4, respectivelly. Therefore, we hypothesized that immune tolerance induced by mucosally delivered pσ1 can be generated through activation of specific T_{reg} cells that actively suppress effector T cells via secretion of regulatory cytokines. Induction of tolerance may also be complemented via stimulation of Th2 cells.

Considering the ability of pσ1 to bind M cells, we questioned whether this protein could be used to mediate tolerance to myelin Ags. Designing effective treatments for MS, a human demyelinating autoimmune disease of the CNS, is problematic due to the unknown etiology of this disease. In animal studies, mucosal administration of myelin components was proven succesfull to treat EAE, a rodent model of MS, though many of these approaches cannot be translated into human treatments either due to considerable toxicity of the delivery agents, and/or the risk of allergy development after administration multiple and/or large doses of Ags.

Data presented in Chapter 3 and 4 confirm that recombinant pσ1 can be engenered to deliver antigen-specific tolerance to fused myelin antigens, after just a single nasal administration to mice, and therefore,can provide protection and/or treatment against two strains of experimental autoimmune encephalomyelitis (EAE), induced by administration of myelin peptides to susceptible mice. In Chapter 3 we questioned whather a single dose protection against $PLP_{139\text{-}151}$-mediated relapsing-remitting EAE can

be delivered by a PLP:OVA-pσ1 fusion protein. We have determined that PLP:OVA-pσ1-mediated protection against EAE is dependent on $PLP_{139-151}$-specific T_{reg} cells that predominantly secrete IL-10, and in the absence of these T_{reg} cells protection against EAE is compromised.

Data presented in Chapter 4 demonstrate that a single very low dose of nasally administered MOG-pσ1 confers complete protection against MOG_{35-55}-induced acute EAE, and that this protection is mediated by IL-10-producing T_{reg} cells and supported by IL-4-producing Th2 cells. We have also questioned if other cell types are involved in this protection, and determined that IL-10 and/or IL-4-producing B cells, as well as antigen presenting cells are likely to play a supportive role in pσ1-mediated tolerance and protection against EAE.

LOW-DOSE TOLERANCE IS MEDIATED BY THE M CELL LIGAND, REOVIRUS PROTEIN SIGMA ONE

The data in this chapter has been published previously in *The Journal of Immunology* (89)

Introduction

Mucosal tolerance is a dose-dependent process (29, 92, 347) requiring either high doses or multiple administrations of Ag typically applied orally. The former mode induces tolerance by clonal anergy/deletion of effector cells, whereas the latter, based on repeated low-dose administration, causes active suppression of effector cells (24, 44, 37, 78, 92). Such suppression occurs via activation of specific regulatory cells, among which $CD25^+CD4^+$ T regulatory $(T_{reg})^3$ cells have been best described (29, 44). Although the phenotypic characteristics of T_{reg} cells are still being investigated, currently, this naturally occuring peripheral $CD25^+CD4^+$ T cell population is thought to normally constitute not more than 5-10 percent of total peripheral $CD4^+$ T cells (348, 349). Specific T_{reg} cells are known to express the nuclear forkhead box P3 (FoxP3) transcription factor and suppress the immune response in an IL-10- and/or TGF-β-dependent fashion (56).

Peyer's patches (PPs) are thought to be required for induction of oral tolerance (43, 44, 347) based upon the findings that treatment of female mice with soluble lymphotoxin-β receptor-Ig fusion protein during gestation results in the disruption of the peripheral lymph nodes (LNs) and PPs in their offspring, leaving the mesenteric, sacral, and cervical LNs intact (350). Offspring of such PP-null mice subjected to a high dose

OVA oral tolerance regimen do not respond to peripheral OVA challenge (43). The PPs (43, 44, 347) and an analogous structure in the upper respiratory tract, the nasopharyngeal - associated lymphoid tissue (NALT; 82, 351), actively facilitate immunity or unresponsiveness by luminal Ag sampling (29, 49, 82) via a specialized epithelium containing microfold (M) cells (47). Ags are subsequently transported from the luminal surface via M cells, which localize in the follicle-associated epithelium (FAE) to the subepithelial dome area for eventual presentation to mucosal B and T cells (46, 49). A number of pathogens, such as reovirus and *Salmonella*, specifically target this specialized FAE to infect the host (38, 157, 352-354). Reovirus infects the host via its cell adhesin, protein sigma one (pσ1) that is responsible for reovirus attachment to M cells (82, 352-354). Previous studies demonstrated binding of recombinant fusion pσ1 to NALT, suggesting that a pσ1-based vehicle can be applied for genetic vaccination of mucosal tissues (82, 154). A modified version of pσ1 obtained by chemically conjugating pσ1 to poly-L-lysine significantly enhanced immunity to the encoded DNA vaccine following nasal administration (82, 355). Due to these targeting capabilities, we queried if recombinant pσ1 could ferry soluble proteins to mucosal tissues, as well.

To test this possibility, a cDNA encoding for the model Ag, OVA, was cloned 5' to pσ1 as a histidine-tag fusion protein and termed OVA-pσ1. We demonstrated here that mice nasally immunized with the OVA-pσ1 fusion protein become tolerogenic to OVA and resistant to peripheral challenge as a result of the generation of Ag-specific T_{reg} cells, which, in turn, actively suppress effector T cells. Immunization with OVA-pσ1 significantly increased the numbers of IL-4-producing $CD25^{-}CD4^{+}$ T cells, together with

IL-10-producing T_{reg} cells, which inhibited the proliferation of OVA-specific $CD4^+$ T cells *in vivo* and successfully suppressed production of proinflammatory cytokines. In the absence of IL-10, OVA-pσ1-mediated tolerance was lost. Thus, these studies show that nasal pσ1-Ag delivery is an effective means to induce systemic tolerance.

Materials and Methods

Preparation of OVA-pσ1

Protein σ1 was recloned without its fusion maltose-binding protein partner (154) to allow expression in the yeast *Pichia pastoris* vector pPICB bearing an his-tag carboxy terminus for protein purification (Invitrogen Corp., Carlsbad, CA), referred to as pσ1. To obtain the fusion protein OVA-pσ1, OVA was amplified with appropriate pairs of primers from an OVA cDNA. The 5' primer encoded an *EcoR*I site and an ATG initiation codon embedded into an optimal Kozak's sequence. The 3' primer provided a *Sal*I site. The PCR product was gel-purified and cloned into an intermediate topocloning vector. The insert was excised by cutting with *EcoR*I and *Sal*I and gel-purified again, resulting in *EcoR*I and *Sal*I ends. The upstream primer for pσ1 contained a *Sal*I site designed to frame the OVA *Sal*I end; the downstream primer contained a *Kpn*I primer designed to frame the fused protein to the his-tag present in the *P. pastoris* expression vector pPICB. The PCR products were gel-purified and cloned into a topocloning vector. The inserts were then excised by cutting with *Sal*I and *Kpn*I and gel-purified again, thus having *Sal*I and *Kpn*I ends. Finally, the yeast expression vector, pPICB, was cut with *EcoR*I and *Kpn*I. A tripartite ligation was set up to join together these components: 1) the

"Antigen" (OVA) as an *EcoR*I-*Sal*I fragment; 2) the "Transporter" (pσ1), as a *Sal*I-*Kpn*I fragment; and 3) the vector cut with *EcoR*I and *Kpn*I. The junction between the "passenger Ag" and the "transporter" featured a flexible linker (Gly-Arg-Pro) to minimize steric hindrance between the components. The resulting construct was sequenced and expressed in the yeast *P. pastoris*, according to the manufacturer's directions (Invitrogen Corp.). Recombinant proteins were extracted from yeast cells by a bead-beater (Biospec Products, Bertlesville, OK) and purified on a Talon metal affinity resin (BD Biosciences, Palo Alto, CA), according to manufacturer's instructions. Proteins were assessed for purity and quality by Coomassie-stained polyacrylamide gels and by Western blot analysis using a polyclonal rabbit anti-pσ1 (produced in-house) or a polyclonal rabbit anti-OVA Ab (Sigma-Aldrich, St. Louis, MO). All recombinant proteins migrated as a single band with the expected MW.

Mice

Female BALB/c and C57BL/6N mice (Frederick Cancer Research Facility, National Cancer Institute, Frederick, MD) were used throughout this study. DO11.10, and IL-10$^{-/-}$ breeder pairs were obtained from The Jackson Laboratory (Bar Harbor, ME) to establish our colonies. Lt$\alpha^{-/-}$ mice (356) were a generous gift from Dr. A. Harmsen at Montana State University, Bozeman. All mice were maintained in the Montana State University (MSU) Animal Resources Center under pathogen-free conditions in individually ventilated cages under HEPA-filtered barrier conditions and were fed sterile food and water *ad libitum*. The mice were free of bacterial and viral pathogens, as determined by antibody screening and histopathologic analysis of major organs and

tissues. All animal studies were approved by the MSU Institutional Animal Care and Use Committee.

Immunizations, Tolerance
Induction, and OVA-Specific Challenge

For tolerance induction, mice (5 mice/group) were nasally dosed up to three times, as described in the text, with 50 - 100 μg of OVA-pσ1 alone or in combination with cholera toxin (CT; List Biologicals, Campbell, CA): 5 μg of CT for the initial dose and 2.5 μg with each boost (357). OVA-pσ1 was administered nasally in a volume of no more than 20 μl / dose up to four times a day, with no less than 2.5 hour intervals between each administration. Mice were nasally dosed with OVA or OVA-pσ1 plus 25 μg of CpG oligodeoxynucleotide cDNA (CpG-ODN) (Sigma-Aldrich), TCCATGACGTTCCTGACGTT (358). As a tolerance control, a group of age-matched mice received a single oral 25 mg dose of OVA (Grade V, Sigma-Aldrich) in a 200 μl of saline (46). To stimulate anti-OVA immunity, mice were nasally dosed with 100 μg OVA (10 mg/ml) with or without adjuvant (CT or ODN). For peripheral challenge, mice were given a subcutaneous (s.c.) injection at 1:1 ratio of 100 μg OVA in IFA (Sigma-Aldrich) for a total volume of 100 μl per mouse.

Sample Collections and Ab ELISA

Serum (saphenous vein) and fecal samples were collected weekly from each mouse. Fecal extractions (78, 82, 357) and vaginal washes (357) were performed, as previously described. OVA-specific endpoint Ab titers were measured by ELISA, as

previously described (46), using purified OVA (Grade V) as coating Ag. Specific reactivity to OVA was determined using HRP conjugates of goat anti-mouse IgG-, and IgA-specific Abs (1.0 μg/ml; Southern Biotechnology Associates, Birmingham, AL), and ABTS (Moss Inc., Pasadena, CA) enzyme substrate. The absorbences were measured at 415 nm on a Kinetics Reader model ELx808 (Bio-Tek Instruments). Endpoint titers were expressed as the reciprocal dilution of the last sample dilution, giving an absorbence of 0.1 OD units above the OD_{415} of negative controls after 1 h incubation (78, 357).

Measurement of Delayed-Type
Hypersensitivity (DTH) Responses

To measure OVA-specific DTH responses *in vivo* (46), 10 μg of OVA were injected into the left ear pinna, and PBS alone (20 μl) was administered to the right ear pinna as a control. Ear swelling was measured 24 h later with an electronic digital caliper (World Precision Instruments, Sarasota, FL). The DTH response was calculated as the increase in ear swelling after OVA injection following subtraction of swelling in the control site injected with PBS.

Isolation of $CD4^+$ T Cells and T Cell ELISPOT

Lymphocytes were isolated from mesenteric lymph nodes (MLNs), head and neck LNs (HNLNs), and spleens. LNs and spleens were obtained and lymphoid cells isolated, as previously described (33, 78). Splenic mononuclear cell suspensions were subjected to Lympholyte-M (Accurate Chemical & Scientific Corporation, Westbury, N.Y.) density gradient centrifugation, and $CD4^+$ T cells were isolated by negative selection (Dynal Mouse CD4 Negative Isolation Kit, Invitrogen). An aliquot of 2×10^6 $CD4^+$ T cells were

cultured for 72 h (37° C, 5 % CO_2) with an equal number of splenic feeder cells (T cell-depleted, mitomycin C-treated) in the presence or absence of 1 mg/ml OVA (46) or 1 μg/ml of $OVA_{323-339}$ peptide. For T cell ELISPOT analysis, $CD4^+$ T cells were added to cytokine-coated, nitrocellulose-bottom microtiter plates (Millipore, MutliScreen, MA). For detection of IFN-γ, IL-2, IL-4, IL-10, IL-13, and IL-17, the plates were coated with 5 μg/ml of purified mAbs (BD Pharmingen, San Diego, CA), and the reaction was detected using 0.5 μg/ml of appropriate biotinylated mAbs (BD Pharmingen). For TGF-β1 ELISPOT, an anti-TGF-β1 mAb (10 μg/ml; Clone 1D11; R&D Systems; Minneapolis, MN) was used for coating, and a biotinylated chicken anti-human TGF-β1 Ab (5.0 μg/ml; R&D Systems) was used for detection. The color reaction was developed using a HRP-conjugated goat anti-biotin Ab (Vector Laboratories) and AEC reagent (Moss, Inc.) and enumerated, as previously described (33, 82).

FACS Analysis

Lymphocytes from CLNs, HNLNs, MLNs, PPs, and spleens from different immunization groups following OVA plus IFA challenge were cultured at 5 x 10^6 cells/ml in medium alone or in the presence of OVA (1 mg/ml). Cells were then stained for FACS analysis with Abs to cell surface molecules: FITC-anti-mouse CD4 (GK1.5; BD Pharmingen), Streptavidin-PE-Cy5 (BD Pharmingen) for biotinylated chicken anti-hTGF-β1 IgY (R&D Systems), and PE-Cy5- or APC-anti-mouse CD25 (both clones PC61) (eBioscience). Intracellular staining was performed following standard protocols with 2 % paraformaldehyde and 0.2 % saponin to stain with PE or APC-anti-mouse/rat

FoxP3 (clone FJK-16s; eBioscience) or PE-rat anti-mouse IL-10 (BD Pharmingen), as previously described (33). Fluorochrome-conjugated rat IgG2a (clone eBR2a), IgG2b (clone A95-1), and IgG1 (clone R3-34) were used as isotype control Abs. FACS analysis was performed on FACSCalibur™ (BD Bioscience). At least 50,000 events were collected for each sample.

Adoptive Transfer of $CD4^+$ T Cell Subsets

Adoptive transfer presented in Figure 2.2*A*: aliquots of 1 x 10^7 $CD4^+$ T cells isolated from spleens of naïve DO11.10 TCR transgenic (Tg) mice were adoptively transferred via i.v. injection into naïve BALB/c mice, which, after 24 h, were dosed nasally with sterile PBS (sPBS), 400 μg OVA, or 80 μg OVA-pσ1 or i.m. (tibialis anterior muscle) with 400 μg of OVA. Three days later, total CLN $CD4^+$ T cells (2 x 10^5) isolated by cell-sorting were adoptively transferred into naïve BALB/c mice, which, 24 h later, were challenged s.c. with 100 μg of OVA in IFA. $CD4^+$ T cells were isolated from the CLNs and spleens 5 days later and subjected to an *in vitro* proliferation assay.

Adoptive transfer experiments are presented in Figures. 2.2*B*, *C*, and 2.3: naïve BALB/c mice were adoptively transferred with Vybrant CM-Dil (Molecular Probes, Eugene, OR) -labeled DO11.10 $CD4^+$ T cells (1 x 10^7) isolated from naïve OVA-Tg mice and with 6 x 10^5 of one of the following T cell subsets: $CD25^+CD4^+$, $CD25^-CD4^+$ or total $CD4^+$ T cells from OVA-pσ1-dosed mice or with total $CD4^+$ T cells from OVA-dosed mice. Control mice received Tg DO11.10 $CD4^+$ T cells and PBS (positive proliferation control). All recipients were challenged 24 h later with OVA, as described above, and

four days later, HNLNs, MLNs, and spleens were evaluated by FACS for proliferation of total and Vybrant^{+} CD4^{+} T cells.

In vitro T Cell Assays

Cell-sorted CD4^{+} T cells isolated from CLNs, HNLNs, MLNs and spleens were cultured, as described elsewhere (33). Cells were pulsed with ^{3}H-TdR (0.5 μCi/well), and ^{3}H-TdR incorporation by proliferating CD4^{+} T cells (triplicate cultures) was measured and expressed as a stimulation index (SI) (33). To assess cytokine production by T_{reg} cells and effector T cells, CD25^{+}CD4^{+} and CD25^{-}CD4^{+} T cells (2 x 10^{5}) were stimulated *in vitro* with anti-CD3 mAb-coated wells (10 μg/ml; BD Pharmingen) and a soluble anti-CD28 mAb (5 μg/ml; BD Pharmingen) for 5 days (final volume of 300 μl in a 48-well plate). Capture ELISA was used to quantify triplicate sets of samples to measure cytokine production (33).

Cytokine secretion by DO11.10 T cells cultured for 24 h or 72 h with or without OVA, OVA-pσ1, OVA + pσ1, or pσ1 stimulation was assessed by cytokine ELISA assay. Cytokine ELISA was conducted, as previously described (33), to determine the levels of IFN-γ, IL-4, IL-10, IL-17 in each sample.

Apoptosis Assay

Lymphocytes isolated from spleens of naïve DO11.10 mice were cultured with or without stimulation for 24 h or 72 h in 37° C with 5 % CO_2. Cells were stimulated with one of the following per 1 ml of culture: 1 mg OVA, OVA-pσ1, pσ1, 1 mg OVA + 1 mg pσ1, 50 μg OVA-pσ1, 50 μg pσ1, or 1 mg OVA + 50 μg pσ1. Percentage of apoptosis

of $CD4^+$ T cells was determined by FACS based on the co-staining of $CD4^+$ T cells with Cy-7-AAD (BD Pharmingen), and PE-Annexin V (BD Pharmingen) according to the manufacturer's instructions.

Statistical Analysis

One-way analysis of variance (ANOVA) ($p < 0.05$) followed by a post hoc Tukey test was applied to evaluate statistical differences between groups in Figure 2.1*F*, Figure 2.4, and Figure 2.7*A*. The remaining groups were analyzed by a Student's *t*-test to evaluate statistical differences between groups or treatments in performed experiments, and p values < 0.05 are indicated.

Results

Nasal Immunization with OVA-pσ1 Induces B and T Cell Unresponsiveness to OVA

Our past studies (82, 154) have shown that chemically modified pσ1 can be successfully used for mucosal delivery of DNA vaccines. To further exploit pσ1 as a mucosal vaccine delivery platform, OVA cDNA was genetically fused 5' to the pσ1 gene so as not to disrupt pσ1's binding capacity. The resulting fusion protein was termed OVA-pσ1 (Figure 2.1*A*). This modified OVA retained its Ag integrity when detected using an anti-OVA mAb (Figure 2.1*B*), and the pσ1 portion retained its binding activity to L cells (Figure 2.1*C*). To test its immunogenicity, OVA-pσ1 was applied nasally with or without the potent mucosal adjuvant CT (Figure 2.1*D, E,* and *F*). BALB/c mice dosed with OVA-pσ1 or OVA-pσ1 plus CT were unresponsive, as evident by the depressed or

lack of OVA-specific IgG and IgA Abs, when compared to mice given OVA alone or OVA plus CT (Figure 2.1*D,* and *E*). Even immunization with OVA alone, which is a poor mucosal immunogen, elicited greater Ag-specific IgG Ab titers than did OVA-pσ1-dosed mice (Figure 2.1*E*). To test unresponsiveness to OVA challenge, mice dosed with OVA, OVA plus CT, or OVA-pσ1 and CT as mucosal adjuvant were peripherally challenged with OVA. Results showed significantly lower OVA-specific DTH responses by OVA and OVA-pσ1 plus CT-dosed mice when compared to mice given OVA plus CT as mucosal adjuvant (Figure 2.1*F*). To discern whether OVA-pσ1-induces tolerance to pσ1, serum samples collected from BALB/c mice nasally dosed with OVA-pσ1 or with DNA complexes with pσ1-poly-L-lysine were tested by Ab ELISA. Virtually no pσ1-specific IgG Ab titers were detected in mice dosed with OVA-pσ1 compared to mice given DNA complexes with pσ1-poly-L-lysine, which exhibited IgG titers in excess of 2^{13} (Figure 2.1*G*). Thus, OVA-pσ1 tolerizes the host to pσ1- and OVA-specific B and T cell responses, even in the presence of co-administered CT, and these results show the feasibility of using pσ1 as a mucosal vaccine delivery platform for induction of mucosal tolerance, rather than immunity.

A

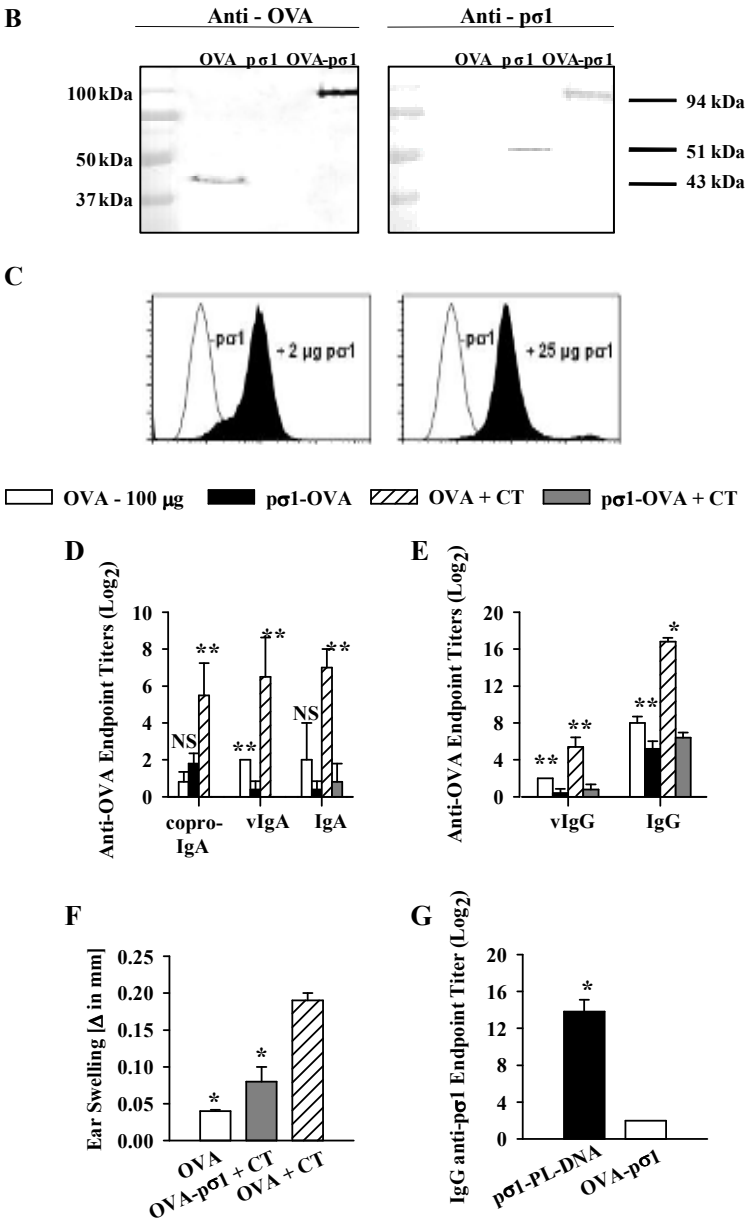


Figure 2.1. Nasal administration of OVA-pσ1 suppresses OVA-specific immune responses. (*A*) Schematic representation of OVA-pσ1 protein: tolerogen, OVA and pσ1 (shaft and head), 6 histidine-tag, and Myc Ag-tag (components are not drawn to scale). (*B*) Western immunoblot of OVA-pσ1, pσ1, and OVA probed with mouse IgG anti-OVA mAb (left panel), or rabbit anti-pσ1 polyclonal Ab (right panel). (*C*) Recombinant pσ1 binds to L cells. L cells were incubated with 2 μg (left panel) or 25 μg (right panel) of his/myc tagged pσ1, followed by an addition of a biotinylated anti-pσ1 mAb and streptavidin-PE to detect pσ1 by FACS; thus, his/myc tag on pσ1's C terminus does not interfere with L cell binding. (*D, E, F*) BALB/c mice were nasally dosed with OVA or OVA-pσ1, with or without CT. (*D, E*) Serum, vaginal, and fecal samples collected on day 28 post-immunization (p.i.) were analyzed for IgA and IgG OVA-specific titers by ELISA. Mean of 10 mice ± SEM is depicted. * $p < 0.001$, ** $p < 0.05$ for OVA-pσ1- vs. OVA-, and OVA-pσ1 + CT- vs. OVA + CT- dosed mice, calculated by Student's *t* test. (*F*) DTH reactions to OVA were measured at day 35 p.i. Mean ± SEM of 6 mice per group.

Figure 2.1. – continued. Statistical significance between groups was calculated by one-way ANOVA followed by post hoc Tukey test. * $p < 0.05$ vs. OVA + CT; OVA vs. OVA-pσ1 were not significantly different. (*G*) Anti-pσ1 Ab ELISA was performed on serum samples collected from BALB/c mice dosed nasally with OVA-pσ1 or pσ1-poly-L-lysine-DNA. Mice dosed with pσ1-PL-DNA, but not OVA-pσ1, showed the pσ1-specific IgG response. * $p < 0.001$ for OVA-pσ1 vs. pσ1-poly-L-lysine-DNA was calculated by Student's *t* test.

Tolerance Can Be Adoptively Transferred with OVA-Specific $CD4^+$ T Cells

Using $CD4^+$ T cells isolated from OVA-transgenic (Tg) DO11.10 TCR mice, we hypothesized that OVA-pσ1 would induce tolerance in an OVA-specific fashion. Splenic $CD4^+$ T cells isolated from DO11.10 Tg mice were adoptively transferred into naïve BALB/c mice. Twenty four h after adoptive transfer, the recipient mice were dosed nasally with 400 μg of OVA to induce tolerance (87) or with only 80 μg of OVA-pσ1. Control mice received an intramuscular (i.m.) injection of 400 μg of OVA as a positive immunized control group (87) or nasal sPBS (negative control group). Since T_{reg} cells are highly enriched in CLNs (87), $CD4^+$ T cells were isolated from recipient CLNs and were adoptively transferred 3 days after immunization into a second group of naïve recipient BALB/c mice, which, 24 h later, were challenged s.c. with OVA. Five days post-peripheral challenge, the second recipient mice were evaluated for tolerance induction by an *in vitro* OVA-specific proliferation assay (Figure 2.2*A*). Contrary to the i.m. immunized group, $CD4^+$ T cells originating from mice dosed nasally with OVA-pσ1 or with a high dose of OVA failed to proliferate following *in vitro* stimulation with OVA (Figure 2.2*A*). The $CD4^+$ T cells originated from the i.m. OVA-dosed mice showed a ~5-fold increase in OVA-specific proliferation. Thus, these data show that OVA-pσ1 can stimulate OVA-specific tolerance via $CD4^+$ T cells.

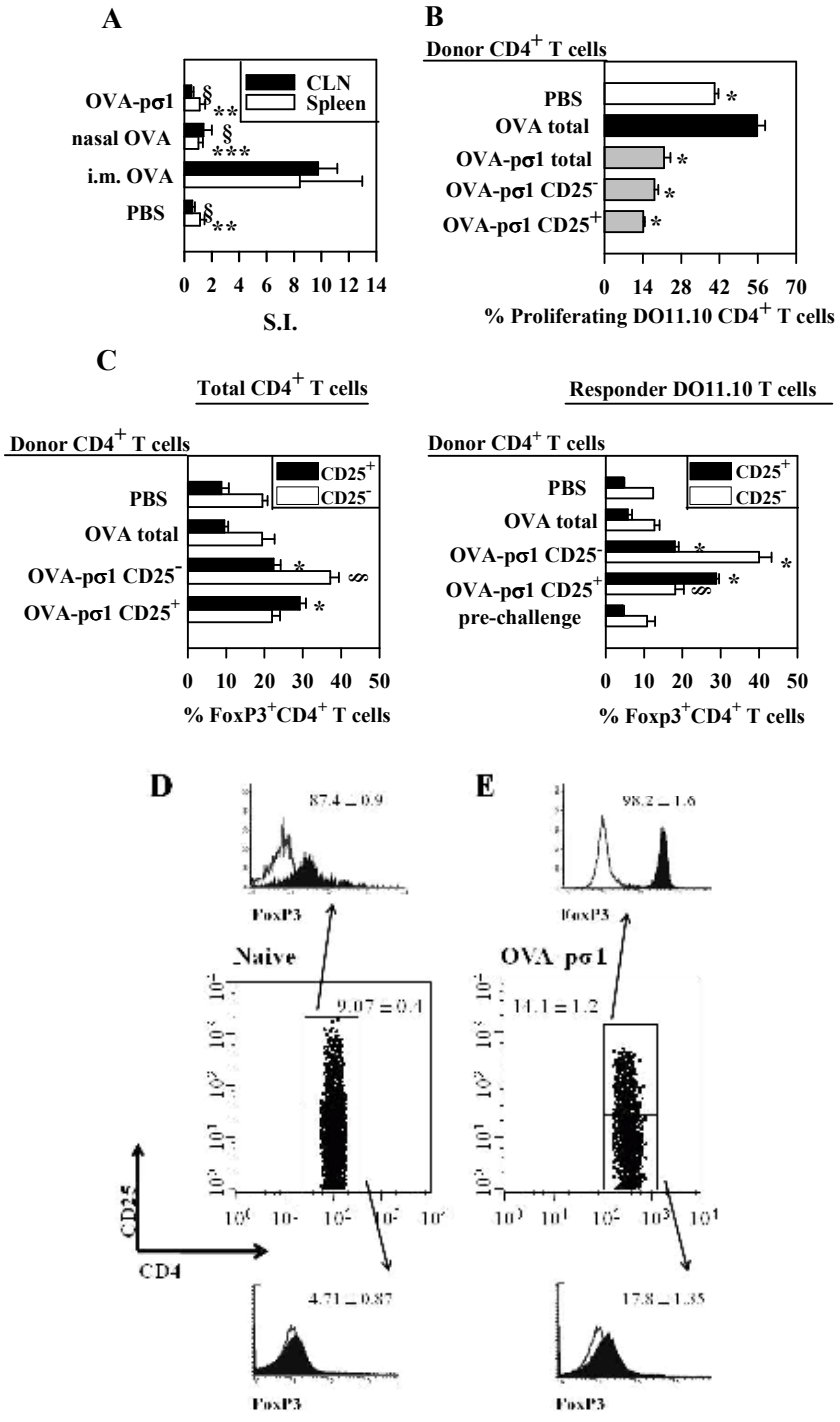


Figure 2.2. OVA-pσ1-induced unresponsiveness is mediated by $CD4^+$ T cells. (*A*) $CD4^+$ T cells isolated from CLNs and spleens of mice adoptively transferred with CLN-derived $CD4^+$ T cells from OVA-pσ1- or OVA-dosed mice were cultured *in vitro* without or with 1 mg of OVA for 5 days. ^{3}H-TdR incorporation was measured and expressed as a stimulation index (SI). For CLN, § $p \leq 0.001$ versus i.m. OVA; for spleen** $p = 0.003$, ***$p = 0.006$ vs. i.m. OVA, calculated by Student's *t* test.

Figure 2.2. – continued. (*B, C*) BALB/c mice were adoptively transferred with Vybrant-labeled DO11.10 Tg $CD4^+$ T (responder) cells and with the designated T cell population (donor $CD4^+$ T cells) isolated from OVA-pσ1- or OVA-dosed mice. (*B*) *In vivo* suppression of proliferation of DO11.10 Tg $CD4^+$ T cells following challenge with OVA was measured by FACS. * $p \leq 0.001$ vs. mice given $CD4^+$ T cells from OVA-dosed mice, calculated by Student's *t* test. (*C*) Lymphocytes pooled from HNLNs, MLNs, and spleens from recipient mice were stained with anti-CD4, anti-CD25, and anti-FoxP3 mAbs and analyzed by FACS. The mean percentage (± SD) of $FoxP3^+$ $CD25^-CD4^+$ T cells and $FoxP3^+$ $CD25^+CD4^+$ T cells as a (left panel) proportion of total $CD4^+$ T cells, or (right panel) DO11.10 $CD4^+$ T cells is depicted. * $p \leq 0.001$ vs. $CD25^+CD4^+$ T cells from mice given $CD4^+$ T cells from OVA-dosed mice; § $p \leq 0.001$, ** $p \leq 0.05$ vs. $CD25^-CD4^+$ T cells from mice given $CD4^+$ T cells from OVA-dosed mice, calculated by Student's *t* test. (*D, E*) FACS analysis was performed on lymphocytes isolated from C57BL/6 mice (*D*) naïve or (*E*) nasally dosed with OVA-pσ1 three times at weekly intervals. Depicted are percentages of unstimulated T_{reg} cells isolated from HNLNs. Percentage of $FoxP3^+$ T_{reg} cells (upper filled histogram) and $FoxP3^+$ $CD25^-CD4^+$ T cells (lower filled histograms) as proportion of $CD4^+$ T cells are plotted versus isotype control for FoxP3 (empty histogram). Results are presented as an average ± SD of 5 animals per tissue.

Dosing with OVA-pσ1
Induces $FoxP3^+$ T_{reg} Cells

To define the possible regulatory T cells induced by tolerization with OVA-pσ1, FACS analysis was performed. Mice given nasal OVA-pσ1 revealed a significant induction of $FoxP3^+$ $CD25^+CD4^+$ T cells (Figure 2.2*E*), even following a peripheral OVA challenge (Tables 2.1 and 2.2). More than a 40 % increase in $FoxP3^+$ $CD25^+CD4^+$ T cells in HNLNs (Figure 2.2*E*) and in MLNs (data not shown) was observed in mice given nasal OVA-pσ1, when compared to naïve mice (Figure 2.2*D* and *E*-upper histograms). In a similar fashion, OVA-pσ1-dosed mice, but not naïve mice, showed > 60 % increase in $FoxP3^+$ $CD25^-CD4^+$ T cells (Figure 2.2*D* and *E* - lower histograms). The effector function of T_{reg} cells is often associated with secretion of regulatory cytokines, such as IL-10 and/or TGF-β1. Subsequent FACS analysis confirmed that increased T_{reg} cells in OVA-pσ1-dosed mice expressed significantly more IL-10, but not TGF-β1, when compared with PBS- dosed mice (Table 2.1). These results further suggest that tolerance

induced by OVA-pσ1 is supported by T_{reg} cells, which may act in an IL-10-dependent manner.

Table 2.1. Nasal immunization with OVA-pσ1 induces IL-10-secreting $CD25^+CD4^+$ T_{reg} cells

Immunization Group[a]	**OVA-pσ1**[b]	**sPBS**[b]
% $CD25^+CD4^+$ T_{reg}	15.4 ± 4.5[c]	9.7 ± 3.1
% IL-10^+ T_{reg}	63.2 ± 6.4[c]	45.1 ± 6.6
% IL-10^- T_{reg}	33.9 ± 4.8[c]	54.7 ± 4.2
% of TGF-β^+ T_{reg}	49.4 ± 3	46 ± 3.2

[a] C57BL/6 mice were given 100 μg of OVA-pσ1 or PBS nasally on days 0, 1, and 2, and were challenged s.c. on day 10 p.i. with OVA in IFA.
[b] FACS analysis was performed on lymphocytes isolated from HNLNs and *in vitro* stimulated for 72 h with 1 mg OVA. Mean ± SEM of 5 mice per group is presented. Mice nasally dosed with OVA-pσ1 showed significant increases in OVA-specific T_{reg} cells that were IL-10^+.
[c] Statistical significance was calculated by Student's *t* test. $p < 0.05$, for the ifferences between OVA-pσ1- versus PBS-dosed mice.

OVA-Specific T_{reg}, as well as
$CD25^-CD4^+$ T Cells, Suppress *In Vivo*
Proliferation of Ag-Specific Effector $CD4^+$ T Cells

The combined evidence from the adoptive transfer study and from the phenotypic analysis suggests that OVA-pσ1 induces Ag-specific T_{reg} cells. To investigate the ability of OVA-pσ1-induced T_{reg} cells to inhibit proliferation of OVA-specific effector $CD4^+$ T cells *in vivo*, naïve BALB/c mice were adoptively transferred with $CD4^+$, $CD25^+CD4^+$, or $CD25^-CD4^+$ T cells isolated from mice given nasal OVA-pσ1 or with $CD4^+$ T cells from mice given only nasal OVA. All recipient mice simultaneously received Vybrant-labeled OVA-specific $CD4^+$ T cells isolated from Tg DO11.10 mice. Recipient mice were s.c.

challenged with OVA in IFA 24 h after adoptive transfer, and 4 days later, spleenic Tg $CD4^+$ T cells were evaluated by flow cytometry for *in vivo* proliferation (Figure 2.2*B*). Proliferation of the Tg $CD4^+$ T cells was significantly reduced in mice receiving OVA-pσ1-specific T_{reg} cells, since only 15 % of these cells underwent cell division (Figure 2.2*B*). In contrast, adoptively transferred $CD4^+$ T cells from OVA-immunized mice, allowed expansion of the Tg $CD4^+$ T cells, as evidenced by > 55 % of them undergoing proliferation (Figure 2.2*B*). Significant suppression of the Tg $CD4^+$ T cell proliferation was also obtained using $CD25^-CD4^+$ and total $CD4^+$ T cells from OVA-pσ1-dosed mice ($p < 0.001$), but to a lesser extent (20 % proliferation). This study shows that OVA-pσ1-derived T_{reg} cells significantly inhibited the proliferation of Tg $CD4^+$ T cells greater than OVA-pσ1-derived $CD25^-$ ($p = 0.009$) or OVA-pσ1-derived total $CD4^+$ T cells ($p = 0.001$). Collectively, these results suggest that OVA-pσ1-induced tolerance is mediated by $CD25^+CD4^+$ T cells and, interestingly, may also be contributed in part by $CD25^-CD4^+$ T cells, which inhibited the proliferation of OVA-specific effector T cells.

In an effort to define the mechanism by which OVA-pσ1-derived $CD4^+$ T cells inhibit proliferation of these Tg $CD4^+$ T cells, we analyzed the phenotypes of the $CD4^+$ T cells isolated from the OVA-challenged recipients. Mice receiving OVA-pσ1-derived $CD25^-CD4^+$ T cells showed significant increases in $FoxP3^+$ $CD25^-CD4^+$ T cells (~ 40 %) and elevated numbers of $FoxP3^+$ T_{reg} cells (~ 20 %; Figure 2.2*C* -left panel). On the other hand, mice adoptively transferred with OVA-pσ1-derived $CD25^+CD4^+$ T cells showed even greater increases in $FoxP3^+$ T_{reg} cells (~ 30 %), but $FoxP3^+$ $CD25^-CD4^+$ T cells in these mice were not elevated and, in fact, were equivalent with control $CD4^+$ T

cells from OVA-immunized or PBS-dosed mice (Figure 2.2*C* -left panel). To determine if adoptively transferred OVA- or OVA-pσ1-derived $CD4^+$ T cells induce $CD4^+$ regulatory T cells among the responder DO11.10 $CD4^+$ T cell subset, the percentages of DO11.10 $FoxP3^+$ $CD25^-CD4^+$ and $FoxP3^+$ $CD25^+CD4^+$ T cells in mice given OVA- or OVA-pσ1-derived $CD4^+$ T cells were evaluated. FACS analysis revealed that prior to the adoptive transfer, the DO11.10 $CD4^+$ T cell population contained ~ 5 % T_{reg} cells and ~ 10 % $FoxP3^+$ $CD25^-CD4^+$ T cells (Figure 2.2*C* - right panel). These $FoxP3^+$ $CD25^+CD4^+$ T cells expanded in mice given OVA-pσ1-derived (both $CD25^+$ and $CD25^-$) $CD4^+$ T cells, although the greatest increase, by nearly 5-fold, was in mice given OVA-pσ1-derived $CD25^+CD4^+$ T cells (Figure 2.2*C* - right panel). In contrast, expansion of DO11.10 $FoxP3^+$ $CD25^+CD4^+$ T cells was not observed in mice dosed with PBS or adoptively transferred with OVA-derived $CD4^+$ T cells. Interestingly, more than a 3-fold increase in Tg $FoxP3^+$ $CD25^-CD4^+$ T cells was observed in mice co-transferred with OVA-pσ1-derived $CD25^-CD4^+$ T cells. By examining the expansion of Tg $FoxP3^+$ $CD25^-CD4^+$ T cells in mice given OVA-pσ1-derived $CD25^+CD4^+$ T cells, it was found that these $FoxP3^+$ $CD25^-CD4^+$ T cells expanded to a lesser extent. No increases in $FoxP3^+$ $CD25^-CD4^+$ T cells were observed in mice given OVA- or PBS-dosed recipients given Tg $CD4^+$ T cells (Figure 2.2*C* - right panel). This evidence suggests that OVA-pσ1-induced $FoxP3^+$ $CD25^-CD4^+$ T cells do contribute to OVA-pσ1-mediated inhibition of effector T cell proliferation, possibly via expansion of either $CD4^+$ T cell subset or via conversion of $FoxP3^+$ T cells (122).

Table 2.2. Nasal immunization with OVA-pσ1 induces FoxP3$^+$ CD25$^+$ and FoxP3$^+$ CD25$^-$ CD4$^+$ T cells

Immunization Group[a]	OVA-pσ1[b]	OVA Oral[b]	OVA Nasal[b]	sPBS[b]
% FoxP3$^+$ CD25$^+$CD4$^+$ T cells				
CLN	19.15 ± 1.13[cd]	11.6 ± 1.07	6.92 ± 0.8	5.92 ± 1.44
HNLN	18.04 ± 1.5[cd]	11.98 ± 1.71	7.29 ± 1.4	7.5 ± 1.03
MLN	17.46 ± 2.8[d]	17.45 ± 0.5	8.07 ± 1.01	7.3 ± 1.4
PP	18.35 ± 1.48[cd]	14.45 ± 1.4	6.91 ± 0.9	6.7 ± 1.84
Spleen	15.95 ± 2.69[cd]	9.49 ± 2.57	5.56 ± 1.16	7.33 ± 2.08
% FoxP3$^+$ CD25$^-$CD4$^+$ T cells				
CLN	22 ± 1.42[cd]	16.7 ± 2.4	14 ± 2.55	14.5 ± 3.39
HNLN	21.88 ± 2.71[cd]	15.6 ± 1.5	14.13 ± 1.05	10.5 ± 1.14
MLN	19.83 ± 2.4[cd]	10.02 ± 1.7	13 ± 0.95	12.9 ± 1
PP	19.93 ± 2.16[cd]	12.03 ± 3.36	12.05 ± 1.64	12.25 ± 2.19
Spleen	21.73 ± 2.81[cd]	10.5 ± 1.42	10.2 ± 3.01	9.62 ± 3.51

[a] C57BL/6 mice were nasally dosed with 100 μg OVA-pσ1, OVA or PBS, or orally with 25 mg OVA and challenged, as described in Table 2.1.

[b] FACS analysis was performed on lymphocytes isolated from CLNs, HNLNs, MLNs, PPs, and spleens and *in vitro* stimulated for 72 h with 1 mg of OVA. Mean ± SD of 5 mice per group is presented. Mice nasally dosed with OVA-pσ1 showed significant increases in OVA-specific FoxP3$^+$ CD25$^+$CD4$^+$ T cells and FoxP3$^+$ CD25$^-$CD4$^+$ T cells.

[c, d] Statistical significance was calculated by Student's *t* test.

[c] $p < 0.04$, differences between OVA-pσ1- versus OVA oral-dosed mice.

[d] $p < 0.005$, differences between OVA-pσ1- versus nasal OVA- and PBS-dosed mice.

OVA-pσ1-Induced Tolerance Is Mediated by Increased Regulatory Cytokine Production

To investigate the specific mechanisms of the observed tolerance, cytokine secretion by $CD4^+$ T cells was measured. Total $CD4^+$ T cells were isolated from recipient HNLNs and spleens given Tg $CD4^+$ T cells and various $CD4^+$ T cell subsets, as described in the adoptive transfer studies in Figures 2.2*B* and *C*. After *in vitro* restimulation with $OVA_{323-339}$ peptide, the cytokine profiles of the responding OVA-specific $CD4^+$ T cells were analyzed. Unlike controls, mice receiving OVA-pσ1-derived ($CD25^+$, $CD25^-$, and total $CD4^+$) T cells failed to induce IL-17-secreting $CD4^+$ T cells and showed no or reduced IFN-γ-secreting $CD4^+$ T cells in all tested lymphoid tissues (Figure 2.3*A* and *B*). Instead, these mice showed greater anti-inflammatory responses, as evidenced by significant increases in the numbers of IL-10- and IL-4-producing $CD4^+$ T cells. Adoptive transfer of T_{reg} cells resulted in a 15-fold enhancement in IL-10-producing $CD4^+$ T cells, whereas recipient mice given $CD25^-CD4^+$ T cells from OVA-pσ1-dosed mice showed a 10-fold enhancement IL-4-producing $CD4^+$ T cells when compared to recipient mice given $CD4^+$ T cells from OVA-vaccinated or PBS-dosed mice (Figure 2.3).

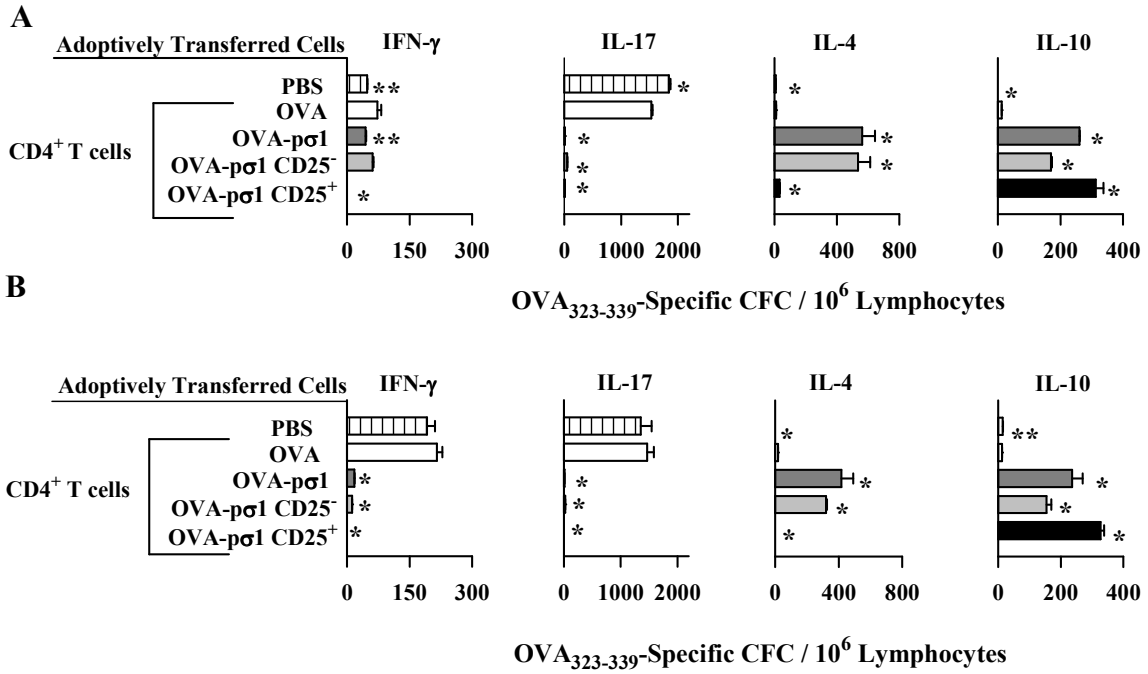


Figure 2.3. OVA-pσ1-induced tolerance is dependent upon increased production of anti-inflammatory/regulatory cytokines and depression of proinflammatory cytokines. $CD4^+$ T cells were isolated from (*A*) HNLNs and (*B*) spleens of recipient mice given the various $CD4^+$ T cell subsets plus responding Tg $CD4^+$ T cells in Figure 2*B* and *C*. To evaluate differences in cytokine production by the responder (DO11.10) $CD4^+$ T cells influenced to become tolerogenic or immunogenic, $CD4^+$ T cells, $CD4^+$ T cells were isolated from recipient mice and subjected to T cell ELISPOT assay after *in vitro* re-stimulation with $OVA_{323-339}$ peptide. Results are depicted as cytokine-forming cells (CFC) per 10^6 $CD4^+$ T cells corrected for unstimulated responses. Adoptive transfer of OVA-pσ1-specific $CD25^+CD4^+$ T cells or $CD25^-$ $CD4^+$ T cells significantly enhanced numbers of IL-10- and IL-4-producing $CD4^+$ T cells, respectively, in recipient mice with concomitant reductions in IFN-γ and IL-17 CFC responses. Adoptive transfer of OVA-stimulated $CD4^+$ T cells showed enhanced IFN-γ and IL-17 CFCs with little to no IL-4 or IL-10 CFCs. * $p < 0.001$, ** $p \leq 0.05$ vs. mice given $CD4^+$ T cells from OVA-dosed mice, calculated by Student's *t* test.

To evaluate the specific cytokine profiles for $CD25^+$ and $CD25^-$ T cell subsets from OVA-pσ1-dosed mice, BALB/c mice adoptively transferred with DO11.10 Tg $CD4^+$ T cells were given a single nasal dose of OVA-pσ1, OVA, or PBS. Recipient mice were then challenged with OVA/IFA on day 3 post-immunization (p.i.) and sacrificed on day 7 p.i. Induction of tolerance was confirmed in OVA-pσ1-dosed mice by the absence of an OVA-specific DTH response (data not shown). $CD25^+CD4^+$ and $CD25^-CD4^+$ T

cells from pooled HNLNs, MLNs, and spleens were stimulated with plate-bound anti-CD3 and soluble anti-CD28 mAbs. Analysis of cytokines produced by cultured cells revealed that the majority of IL-10 from OVA-pσ1-dosed mice was derived from T_{reg} cells (Figure 2.4*D*), whereas almost all of the IL-4 was produced by $CD25^-CD4^+$ T cells (Figure 2.4*C*). These data further confirm that OVA-pσ1-induced tolerance depends largely upon IL-10-producing T_{reg} cells and is further supported Th2 cells. Although TGF-β1 was secreted by both $CD25^+$ and $CD25^-CD4^+$ T cells, this cytokine was not strikingly elevated in OVA-pσ1-dosed mice when compared to OVA- and PBS-dosed mice (Figure 2.4*E*), further implying that TGF-β1 may have a lesser role for OVA-pσ1-induced tolerance. Contrary to control mice, production of proinflammatory cytokines, IFN-γ (Figure 2.4*A*) and IL-17 (Figure 2.4*B*), was significantly diminished by both T_{reg} and $CD25^-CD4^+$ T cell subsets.

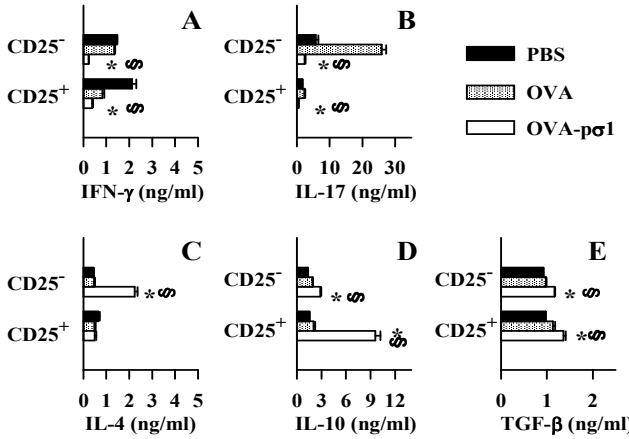


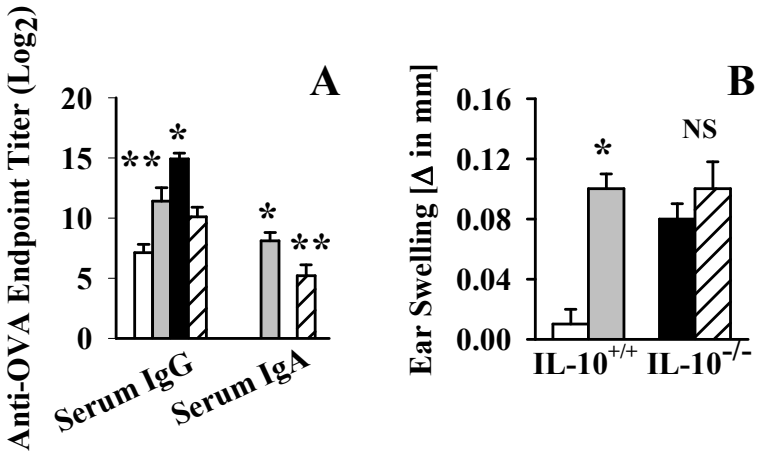
Figure 2.4. OVA-pσ1-induced tolerance is mediated by IL-10-producing $CD25^+CD4^+$ T cells and supported by IL-4-producing $CD25^-CD4^+$ T cells. BALB/c mice were adoptively transferred with 1 x 10^7 DO11.10 Tg $CD4^+$ T cells and 24 h later nasally dosed with 50 μg OVA-pσ1 or OVA only. Mice were challenged s.c. on day 3 after transfer and sacrificed 4 days later. $CD25^+CD4^+$ T cells and $CD25^-CD4^+$ T cells were isolated from HNLNs, MLNs, and spleens of these mice and cultured *in vitro*. Presence of (*A, B*) proinflammatory and (*C, D, E*) regulatory cytokines in cultured supernatants was measured by ELISA. $CD25^+CD4^+$ T cells from OVA-pσ1-dosed mice produced significantly more (*D*) IL-10, whereas the majority of (*C*) IL-4 was secreted by $CD25^-CD4^+$ T cells. Mean ± SEM of 5 mice per group is shown.

Figure 2.4. – continued. Statistical significance for cytokine -producing $CD25^-$ or $CD25^+$ $CD4^+$ T cells was calculated by one-way ANOVA followed by a post hoc Tukey test. § $p < 0.05$ for OVA-pσ1- versus PBS-dosed mice, * $p < 0.05$ for OVA-pσ1- versus OVA-dosed mice.

IL-10 Is Necessary for Induction of OVA-pσ1-Mediated Tolerance

Results thus far have shown that mucosal administration of OVA-pσ1 induces secretion of regulatory cytokines, IL-4, IL-10, and TGF-β, although the most pronounced increase was observed in the production of IL-10. Since past studies have shown that IL-10 is not responsible for tolerance induction (359-361), we queried whether tolerance could be induced in IL-$10^{-/-}$ mice given nasal OVA-pσ1. C57BL/6 mice given nasal OVA-pσ1 were unresponsive to OVA, as evidenced by the low plasma IgG and mucosal IgA anti-OVA Ab titers and a lack of DTH responses, when compared with PBS-primed mice (Figure 2.5*A* and *B*). In contrast, OVA-pσ1-dosed IL-$10^{-/-}$ mice became immune to OVA after peripheral challenge, as did PBS-primed IL-$10^{-/-}$ mice (Figure 2.5*A* and *B*). $CD4^+$ T cells isolated from HNLNs, MLNs, and spleens of IL-$10^{-/-}$ mice dosed with OVA-pσ1 showed significant increases in OVA-specific T cell proliferation when compared to the same cells obtained from tolerized C57BL/6 mice. As evidenced in OVA-pσ1-dosed C57BL/6 mice, these had at least three-fold less $CD4^+$ T cell proliferative responses to OVA in HNLNs and spleens than their PBS-dosed litter mates (Figure 2.5*C*). Interestingly, FACS analysis of $FoxP3^+$ T_{reg} cells revealed no differences in T_{reg} cell numbers between PBS- and OVA-pσ1-dosed IL-$10^{-/-}$ mice (Figure 2.5*D*), and, in fact, they resembled baseline levels of $CD25^+CD4^+$ T cells in these mice (Figure 2.5*E*). In contrast, the HNLNs and spleens of OVA-pσ1-dosed C57BL/6 mice contained

significantly more OVA-specific T_{reg} cells than the HNLNs and spleens of both PBS-dosed C57BL/6 mice and OVA-pσ1-dosed IL-$10^{-/-}$ mice, the latter result suggesting that T_{reg} cells in IL-$10^{-/-}$ mice were simply not induced (Figure 2.5*D*). Additionally, almost 50 % of the FoxP3^{+} T_{reg} cells in HNLNs and spleens of OVA-pσ1-dosed C57BL/6 mice showed intracellular expression of IL-10 (Figure 2.5*F*), confirming the supportive role of IL-10 in OVA-pσ1-induced tolerance. Upon OVA restimulation, analysis of cytokine-secreting CD4^{+} T cells from C57BL/6 (Figure 2.6*A* and *C*) and IL-$10^{-/-}$ mice (Figure 2.6*B* and *D*) was performed. As expected, OVA-pσ1-dosed C57BL/6 mice showed a significant increase in CD4^{+} T cells secreting regulatory cytokines IL-10 and TGF-β1 in HNLNs and MLNs, and IL-4-producing cells in all tested tissues when compared with PBS-dosed mice (Figure 2.6*C*). Numbers of CD4^{+} T cells producing IL-13 were not significantly different between immunized and control mice (Figure 2.6*C*).



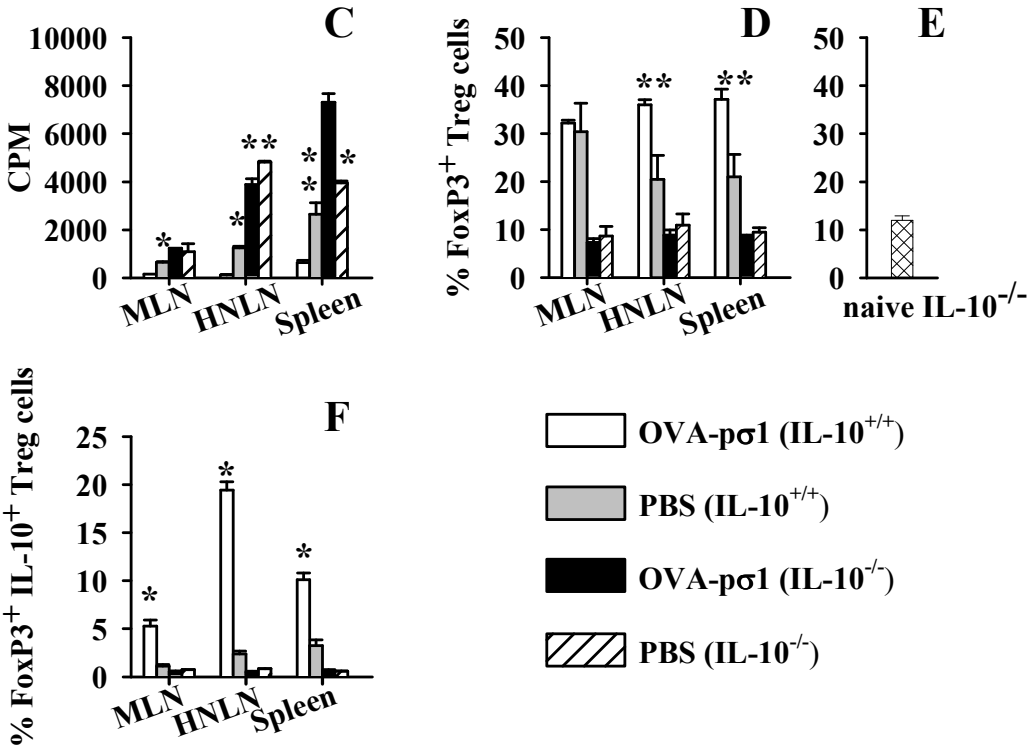


Figure 2.5. IL-10 is required for induction and maintenance of OVA-pσ1-mediated tolerance. IL-$10^{-/-}$ and C57BL/6 (IL-$10^{+/+}$) (B6) mice were given 100 μg of OVA-pσ1 or PBS nasally on days 0, 1, and 2 and were then challenged s.c. on day 10 p.i. with OVA in IFA. (*A*) OVA-specific ELISA was performed on serum samples collected from OVA-pσ1- and PBS-dosed C57BL/6 and IL-$10^{-/-}$ mice on day 17 p.i. OVA-pσ1-dosed IL-$10^{-/-}$ mice elicited a serum IgG anti-OVA response. (*B*) OVA-specific DTH responses measured on day 15 p.i. OVA-pσ1-dosed IL-$10^{-/-}$ mice were responsive to OVA. (*C*) $CD4^+$ T cells isolated from HNLNs, MLNs, and spleens of OVA-pσ1- or PBS-dosed C57BL/6 (IL-$10^{+/+}$) and IL-$10^{-/-}$ mice were measured for their proliferative responses after *in vitro* stimulation with OVA. $CD4^+$ T cells from IL-$10^{-/-}$ mice dosed with OVA-pσ1 or PBS, and PBS-dosed C57BL/6 mice proliferated in response to OVA. Depicted values are corrected for ^{3}H-TdR uptake by unstimulated cells. (*D*) Percentages of $FoxP3^+$ $CD25^+CD4^+$ T cells were measured by FACS after *in vitro* stimulation with OVA, and depicted are values corrected for unstimulated cells. IL-$10^{-/-}$ mice failed to show induction of T_{reg} cells independent of treatment. The mean of two experiments ± SEM per group is shown. * $p < 0.001$, ** $p < 0.05$ for OVA-pσ1- vs. PBS-dosed C57BL/6 or IL-$10^{-/-}$ mice; NS, not significant, calculated by Student's *t* test. (*E*) Depicted is the percentage of $CD25^+CD4^+$ T cells for the combined MLNs, PPs, and spleens from 5 naïve IL-$10^{-/-}$ mice. Results determined by FACS show the mean ± SD. (*F*) Percentages of $FoxP3^+$IL-10^+ T_{reg} cells, as a proportion of $CD25^+CD4^+$ T cells, were measured by FACS. T_{reg} cells induced in C57BL/6 mice given nasally OVA-pσ1 expressed IL-10, whereas significantly less IL-10 was expressed by PBS-dosed C57BL/6 mice. Average ± SEM of 4 mice is shown. * $p < 0.001$ for OVA-pσ1-versus PBS dosed C57BL/6 mice, calculated by Student's *t* test; NS = not significant.

Production of proinflammatory cytokines, IFN-γ, IL-2, and IL-17 (Figure 2.6*A*), was significantly inhibited in all tested tissues of OVA-pσ1-dosed C57BL/6 mice. IL-10$^{-/-}$ mice revealed that, although the IL-4 and IL-13 responses were significantly more elevated in OVA-pσ1- than PBS-dosed mice (Figure 2.6*D*), TGF-β-secreting CD4^{+} T cells were significantly depressed, except in the spleen (Figure 2.6*D*). No significant differences in numbers of IL-2-producing CD4^{+} T cells were observed between OVA-pσ1- and PBS-dosed IL-10$^{-/-}$ mice. Consistent with an antagonistic relationship between IFN-γ and IL-17 (362), there was a significant decrease in IFN-γ and a concomitant increase in the IL-17 in OVA-pσ1-dosed IL-10$^{-/-}$ mice when compared to PBS-dosed mice (Figure 2.6*B*).

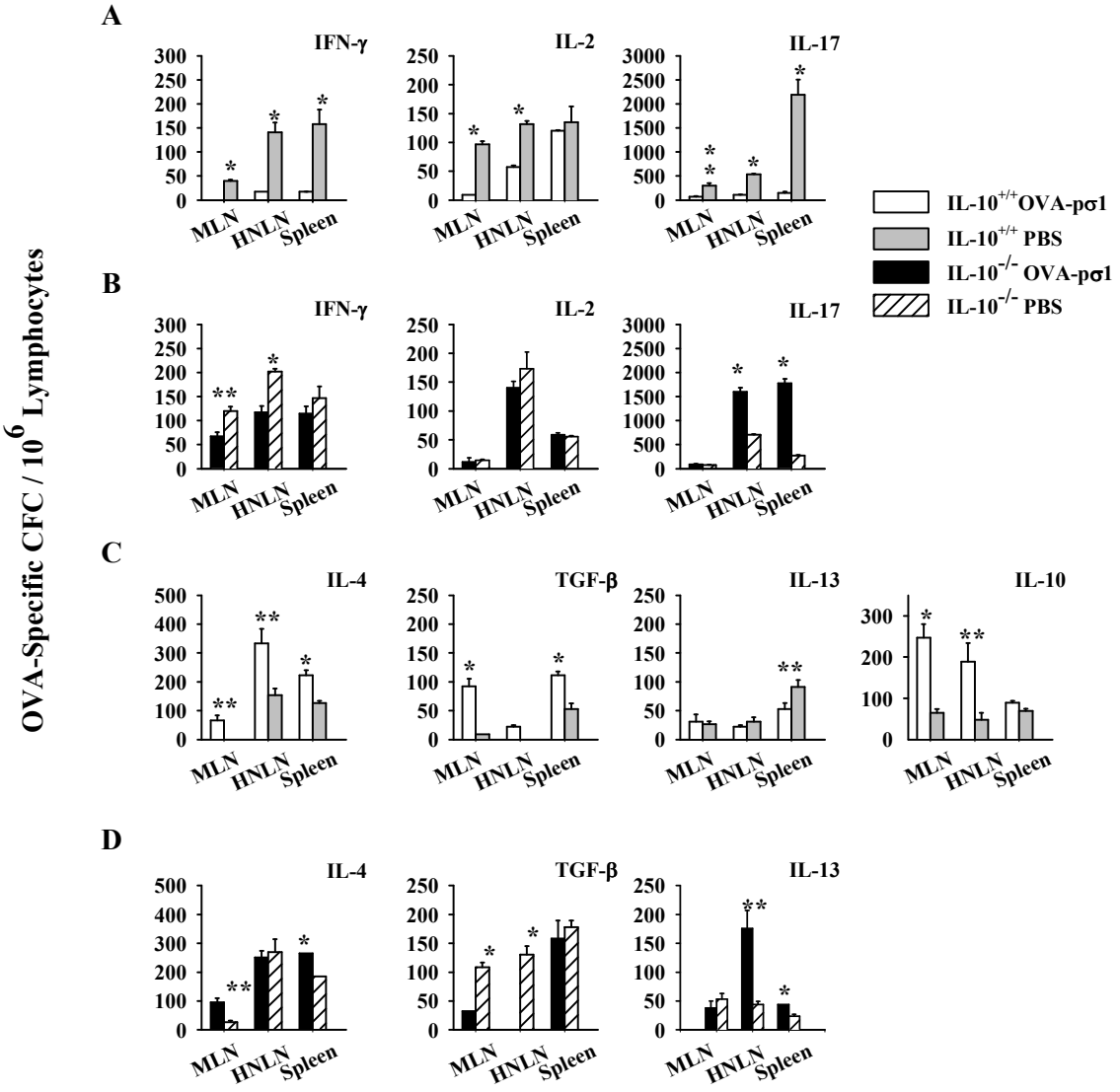


Figure 2.6. Immunization of IL-10$^{-/-}$ mice with OVA-pσ1 increases IL-17- and IFN-γ-producing CD4^{+} T cells with concomitant increases in anti-inflammatory cytokines, (IL-4 and IL-13) responses when compared to similarly treated C57BL/6 mice. C57BL/6 (IL-10$^{+/+}$) (B6) and IL-10$^{-/-}$ mice were immunized and challenged, as described in Figure 2.5 legend. CD4^{+} T cells isolated from HNLNs, MLNs, and spleens were restimulated with OVA, and cytokine-specific T cell ELISPOT was performed. CFC / 10^{6} CD4^{+} T cells in (*A, C*) C57BL/6 and (*B, D*) IL-10$^{-/-}$ mice are depicted. For OVA-pσ1-dosed C57BL/6 mice, CD4^{+} T cells producing anti-inflammatory cytokines in MLNs and HNLNs were significantly elevated when compared with (*C*) PBS-dosed mice. The numbers of CD4^{+} T cells producing (*A*) proinflammatory cytokines were significantly reduced in OVA-pσ1-dosed mice versus PBS-dosed C57BL/6 mice. OVA-pσ1-dosed IL-10$^{-/-}$ mice showed significant increases when compared with PBS-dosed mice in (*B, D*) IL-4, IL-13-, and IL-17-producing CD4^{+} T cells, but not TGF-β-producing CD4^{+} T cells.

Figure 2.6. –continued. IFN-γ- and IL-17-producing $CD4^+$ T cells were significantly elevated in IL-$10^{-/-}$ mice given OVA-pσ1 when compared to OVA-pσ1-dosed C57BL/6 mice (*A, B*). Depicted is the mean of two experiments ± SEM. * $p < 0.001$, ** $p < 0.05$ for OVA-pσ1- versus PBS-dosed C57BL/6 or IL-$10^{-/-}$ mice, calculated by Student's *t* test.

OVA-pσ1- Mediated Tolerance Can Be Induced after a Single Dose

Since the pσ1-based approach offers the possibility of inducing tolerance while using significantly less Ag, the persistence of OVA-pσ1-induced tolerance was investigated after single dose. C57BL/6 mice were nasally given either 100 μg per dose of OVA, OVA-pσ1, or sterile PBS for three consecutive days, or a single 50 μg dose of OVA-pσ1 or OVA alone. Additionally, a group of C57BL/6 mice was given 0.5 mg of OVA nasally for 5 consecutive days. Ten days p.i., all mice were challenged with OVA by the s.c. route. One day prior to challenge, no OVA-specific serum IgG Ab responses in either immunization group were detected (data not shown). By day 18 post-challenge (day 28 p.i.), mice given OVA or PBS revealed significantly elevated OVA-specific Ab titers in both mucosal (not shown) and systemic immune compartments, and these responses were sustained (Figure 2.7*A*). Moreover, we were unable to induce tolerance to OVA, even in mice dosed nasally with as much as 2.5 mg of OVA alone. In contrast, virtually no OVA-specific serum-IgG Abs (Figure 2.7*A*) or DTH responses (Figure 2.7*B*) were observed in OVA-challenged mice given a single or three nasal doses of OVA-pσ1. These data show that mice given OVA-pσ1 nasally are tolerized to OVA, and that this tolerance can be induced by substantially lower doses of pσ1-delivered Ag.

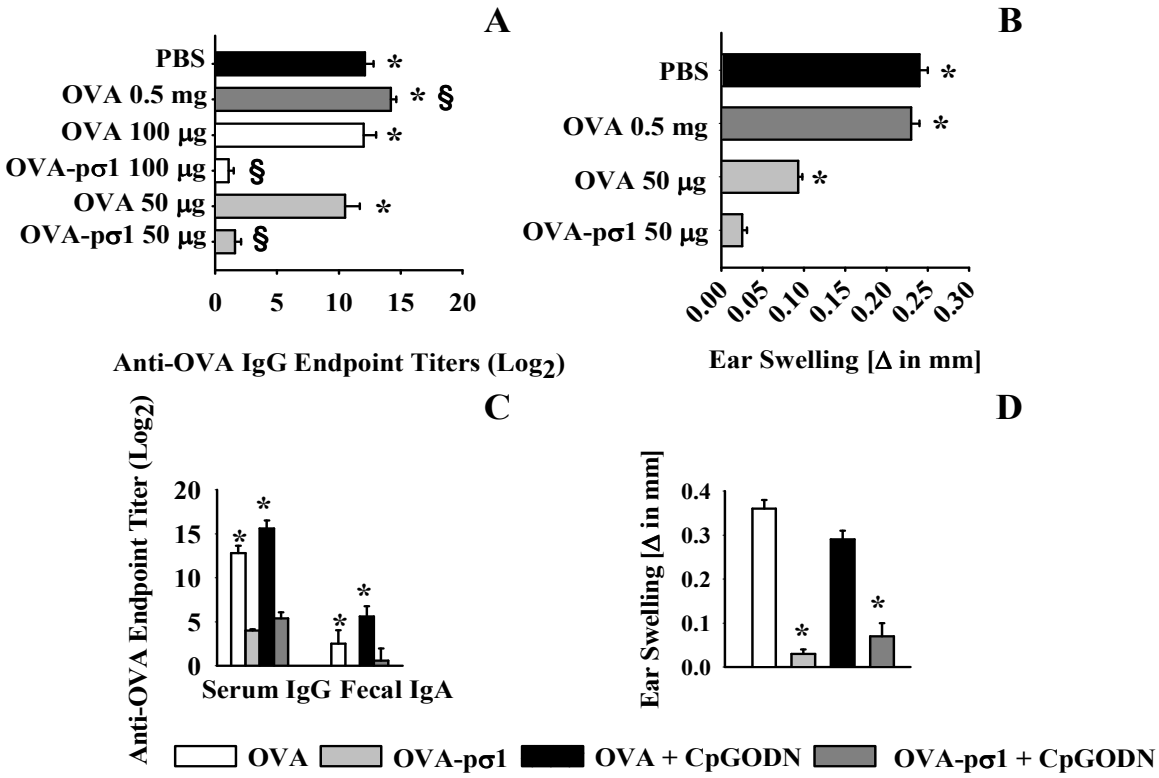


Figure 2.7. A single nasal dose of OVA-pσ1 is sufficient to induce tolerance to OVA. (*A, B*) C57BL/6 mice were given OVA-pσ1, OVA, or sterile PBS and were subsequently challenged s.c. with OVA/IFA. (*A*) Data depict serum IgG anti-OVA Ab titers at 18 days post-challenge (28 d p.i.). Statistical significance was calculated by one-way ANOVA followed by post hoc Tukey test. * $p < 0.05$ versus mice nasally given 50 µg of OVA-pσ1, § $p < 0.05$ versus. mice given nasally 50 µg of OVA. (*B*) OVA-specific DTH responses were measured at day 15 p.i. (day 5 post-challenge). A single 50 µg dose of OVA-pσ1, but not OVA alone, was sufficient for tolerance induction. * $p < 0.001$ for OVA-pσ1-dosed vs. control mice, calculated by Student's *t* test. Data are representative of three separate experiments. (*C, D*) BALB/c mice were nasally given 100 µg of OVA-pσ1 or OVA, with or without 25 µg of CpG. Ten days p.i., mice were challenged s.c. with OVA/IFA. Data depict (*C*) plasma IgG and fecal IgA anti-OVA Ab titers 18 days post-challenge (28 d p.i.), and (*D*) OVA-specific DTH responses at day 15 p.i. (day 5 post-challenge). Statistical significance * $p < 0.001$ for OVA-pσ1 versus OVA-dosed mice, or for OVA-pσ1 + CpG versus. OVA + CpG -dosed mice, calculated by Student's *t* test.

While OVA-pσ1 can induce tolerance to OVA, even in the presence of CT, chemically modified pσ1 is found to be immunogenic. Mucosal delivery of DNA complexed to poly-L-lysine-modified pσ1 results in enhanced transgene-specific immune responses (82). To determine whether there is an adjuvant effect by the plasmid cDNA

that can overcome the OVA-pσ1-induced tolerance, BALB/c mice were given a single nasal dose of OVA or OVA-pσ1 alone, or co-administered with CpG-ODN. In contrast to mice dosed with OVA or OVA + ODN, mice dosed with OVA-pσ1 alone or OVA-pσ1 + ODN showed significantly reduced OVA-specific serum and mucosal Ab (Figure 2.7*C*) and DTH responses (Figure 2.7*D*). Therefore, the tolerogenic effect of this pσ1-based delivery system cannot be overridden by co-administration of the immune-modulatory molecule CpG-ODN.

Even though co-administration of CpG-ODN did not interfere with generation of OVA-pσ1-mediated tolerance to OVA, interesting differences have been revealed by cytokines produced between mice receiving OVA-pσ1 and OVA-pσ1 + ODN. Lymphocytes obtained from HNLNs, MLNs, and spleens of mice nasally dosed with OVA, OVA-pσ1, OVA + ODN, and OVA-pσ1 + ODN were analyzed for cytokine production after *in vitro* re-stimulation with OVA. In contrast to mice given OVA-pσ1, at least 2-fold reduction in IL-4, significant reduction in IL-10, and concomitant enhancement in secretion of IL-13 and TGF-β was observed in lymphocytes obtained from OVA-pσ1 + ODN-dosed mice (Figure 2.8*A*). Unlike cells isolated from OVA-pσ1-dosed mice that produced very little to no proinflammatory cytokines, significant increase was observed in secretion of IL-6 and IFN-γ in LN, IL-21 in MLNs, IL-17 and IL-27 in MLNs and spleens by lymphocytes acquired from OVA-pσ1 + ODN-dosed mice (Figure 2.8*B*). Mice that received OVA or OVA + ODN showed virtual absence of IL-4 and IL-10 production in all tested tissues (Figure 2.8*A*). In partial agreement with OVA-pσ1 + ODN-dosed mice, mice that received OVA + ODN produced significantly more of IL-13,

though noticeably less of TGF-β than OVA-dosed mice. In fact, TGF-β was predominantly produced by lymphocytes obtained from OVA-pσ1 + ODN-dosed mice, and progressively less of TGF-β was produced by cells from OVA-pσ1-, OVA-, and OVA + ODN-immunized mice. Lymphocytes isolated from mice nasally given OVA produced significantly more of proinflammatory cytokines including IL-6, IL-17, IL-21, IL-27, and IFN-γ than mice nasally given OVA-pσ1. Moreover, production of these proinflammatory cytokines was almost always considerably higher in OVA + ODN-dosed mice than in OVA-dosed mice (Figure 2.8*B*). Cells obtained from OVA-pσ1 + ODN-dosed mice showed significant decrease in production of IFN-γ, IL-27, IL-21, and IL-17 in all tested tissues, as well as IL-6 in MLNs and spleens when compared to OVA + ODN-dosed mice (Figure 2.8*B*). In contrast to proinflammatory cytokines, lymphocytes obtained from OVA-pσ1 + ODN-dosed mice exhibited significant increase in levels of TGF-β, IL-10, and IL-4 and elevated IL-13 in relation to cells isolated from OVA + ODN-dosed mice (Figure 2.8*A*).

In summary, these studies show that OVA-pσ1 can readily accomplish tolerance with a single dose and stimulate the production of $FoxP3^+$ T_{reg} cells producing IL-10, and tolerance induction is IL-10 -dependent.

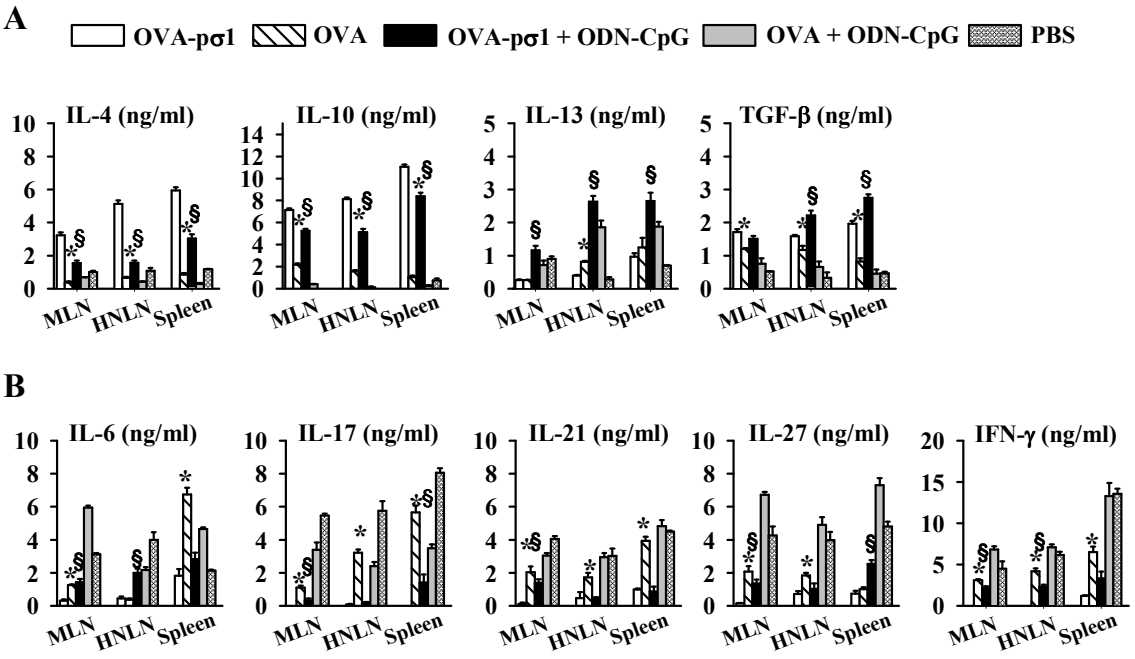


Figure 2.8. Induction of OVA-specific tolerance by nasal administration of OVA-pσ1 + CpG-ODN is associated with an increase in IL-13 and TGF-β and concomitant reduction in IL-10 and IL-4. BALB/c mice were immunized and challenged as described in Figure 2.7*B* and *C* legend. Whole lymphocytes isolated from their HNLNs, MLNs, and spleens were *in vitro* stimulated with 1 mg/ml of OVA for 72 h. Concentrations of (*A*) proinflammatory and (*B*) anti-inflammatory cytokines in cultured supernatants were determined by ELISA, and cytokine production by unstimulated cells was set as a background concentration and subtracted from the results. Mice dosed with OVA-pσ1 + ODN-CpG produced significantly more of IL-13 and TGF-β, but less IL-10 and IL-4 when compared to OVA-pσ1-dosed mice. OVA and OVA + ODN-CpG-dosed mice produced more proinflammatory cytokies than OVA-pσ1- and OVA-pσ1 + ODN-CpG-dosed mice, respectively. Statistical significance * $p < 0.05$ for OVA + ODN-CpG vs. OVA-pσ1 + ODN-CpG and OVA vs. OVA-pσ1; §, $p < 0.05$ for OVA-pσ1 vs. OVA-pσ1 + ODN-CpG.

Mucosal Administration of OVA-pσ1 Is Necessary for Induction of Tolerance

We have shown here that mucosal administration of OVA-pσ1 induces a low-dose tolerance to OVA. In an effort to determine if the pσ1-mediated tolerance can be induced via a non-mucosal route, mice were i.m. immunized on days 0, 7, and 14 with OVA-pσ1 without or with CT and compared to mice similarly immunized with OVA (Figure 2.9*A* and *B*). On day 21 (one week after the last immunization), mice were

evaluated for serum IgG anti-OVA Ab titers by ELISA. As expected, OVA-immunized responded well, but naïve and OVA-pσ1-immunized mice remained OVA unresponsive (Figure 2.9*A* and *B* - black bars). All mice were subsequently challenged on day 28 with OVA in IFA, and one week later, serum from individual mice was collected and measured for IgG anti-OVA titers. Even though, mice dosed with OVA-pσ1 + CT (Figure 2.9*B*) showed reduced Ab titers relative to OVA + CT-dosed mice, these anti-OVA Ab titers were greater than those obtained in the OVA-challenged, naïve mice (Figure 2.9*A*). Thus, these results show that induction of the optimal tolerance to OVA via the use of pσ1 requires mucosal, not parenteral, administration of OVA-pσ1 (Figure 2.9*A* and *B* - open bars).

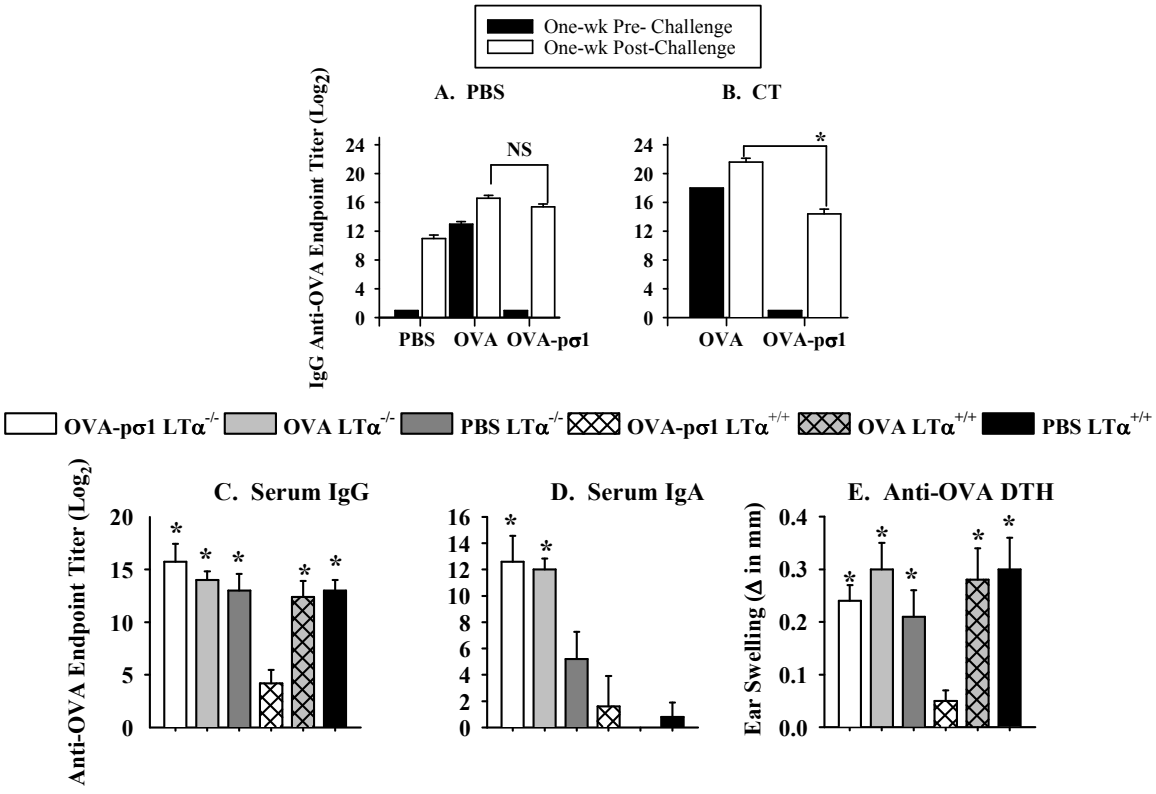


Figure 2.9. Mucosal administration of OVA-pσ1 and presence of mucosal inductive tissues are required for induction of OVA-pσ1-mediated tolerance to OVA.

Figure 2.9. – continued. (*A, B*) C57BL/6 mice were i.m. immunized with (*A*) PBS, 50 μg OVA, or OVA-pσ1, or (*B*) with 50 μg OVA or OVA-pσ1 p + 1 μg CT, on days 0, 7, and 14. On day 21, sera were collected and analyzed for the presence of anti-OVA IgG Abs by ELISA (black bars), and showed unresponsiveness by PBS-, OVA-pσ1-, and OVA-pσ1 + CT-dosed mice. On day 28, mice were s.c. challenged with OVA in IFA. Sera collected from mice one week post-challenge (day 35) revealed the presence of IgG anti-OVA Abs (open bars) in all tested groups, although the anti-OVA response was higher in mice dosed with OVA + CT compared to OVA-pσ1 + CT-dosed mice. NS = not significant; * $p < 0.001$ for OVA vs. OVA-pσ1 and OVA + CT vs. OVA-pσ1 + CT -dosed mice, calculated by Student's *t* test. (*C, D, E*) C57BL/6 and LTα$^{-/-}$ mice were fed with 100 μg of OVA-pσ1, OVA or with PBS on day 0, and were s.c. challenged with OVA in IFA on day 10 p.i. Presence of OVA-specific IgG (*C*) and IgA (*D*) Abs in serum was assessed by ELISA 7 d p.ch. OVA-pσ1-fed C57BL/6 mice showed significant reduction in OVA-specific IgG and IgA Abs in serum. OVA- and PBS-dosed and challenged C57BL/6 and LTα$^{-/-}$ mice developed pronounced OVA-specific IgG Abs in serum. All LTα$^{-/-}$ mice exhibited enhanced OVA-specific IgA Ab titers when compared to similarly treated C57BL/6 mice. (*E*) OVA-specific DTH response measured 5 d p.ch revealed induction of OVA-specific Th1 cells in all experimental groups except OVA-pσ1-fed C57BL/6 mice which exhibited significant reduction of OVA-specific DTH response. Average ± SD of four to five mice per group is depicted. *, $p < 0.05$ vs. OVA-pσ1-dosed C57BL/6 mice.

LTα$^{-/-}$ Mice Are Responsive to OVA after Oral Administration of OVA-pσ1

Lymphotoxin-α (LTα)-deficient mice lack LNs and PPs, and due to the abnormal splenic architecture they also fail to form germinal centers within the splenic while pulps (363). To determine if M cells are required for OVA-pσ1-mediated tolerance, LTα$^{-/-}$ and C57BL/6 mice were orally given a single 100 μg dose of OVA-pσ1, OVA or with PBS, and were challenged with OVA in IFA. OVA-specific DTH response was measured in mice 5 days after challenge with OVA in IFA, and presence of OVA-specific Ab titers in serum and mucosal secretions were assessed by ELISA 7 days after challenge. No OVA-specific Abs were detected in mucosal secretion of C57BL/6 or LTα$^{-/-}$ mice (data not shown). C57BL/6 mice given oral OVA-pσ1 become tolerant to OVA, as evidenced by significantly reduced OVA-specific IgG and virtual lack of OVA-specific IgA Abs in serum when compared to OVA- or PBS-dosed and challenged litter mates (Figure 2.9*C*, and *D*). Additionally, OVA-specific DTH response was significantly depressed in these

OVA-pσ1-dosed C57BL/6 mice when compared to any other experimental group. In contrast to these mice, C57BL/6 and LTα$^{-/-}$ mice dosed with OVA or PBS and s.c. challenged with OVA exhibited pronounced OVA-specific IgG Abs in serum and OVA-specific DTH responses (Figure 2.9*C*, and *E*). Interestingly, LTα$^{-/-}$ mice given oral OVA-pσ1 or OVA, and to a lesser extent also PBS, developed enhanced OVA-specific IgA Abs in serum after OVA challenge, whereas C57BL/6 mice produced little to no OVA-specific IgA in serum independently on the oral treatment (Figure 2.9*D*). When compared to OVA-pσ1-fed C57BL/6 mice, LTα$^{-/-}$ mice dosed with OVA-pσ1 developed greatly enhanced OVA-specific IgG and IgA titers, and OVA-specific DTH response (Figure 2.9*C*, *D*, and *E*). These results indicate that pσ1-delivered oral tolerance cannot be established in the absence of PP-associated M cells, suggesting that the presence of M cells is essential for OVA-pσ1-induced tolerance to OVA.

OVA-pσ1 Induces Apoptosis of OVA-Tg CD4$^+$ T Cells *In Vitro*

Mucosal delivery of OVA-pσ1 triggers the regulatory T cell response and secretion of anti-inflammatory cytokines. In contrast, parenteral administration of OVA-pσ1 induces anti-OVA Ab response after s.c. challenge with OVA. To evaluate if pσ1 has a direct impact on CD4$^+$ T cell populations, or if the induction of tolerance relies solely on the pσ1's interaction with the mucosal epithelium, an *in vitro* study was performed. Lymphocytes isolated from naïve DO11.10 Tg mice were cultured with high- (1 mg/ml) or low-dose (0.05 mg/ml) of OVA-pσ1 or pσ1 and compared to *in vitro* cultures stimulated with OVA or OVA + pσ1. After 24 or 72 h, cells and culture

supernatants were harvested and evaluated for $CD4^+$ T cell apoptosis by FACS, and cytokine production by ELISA, respectively. Only about 13 % of Tg $CD4^+$ T cells underwent apoptosis in response to *in vitro* stimulation with OVA for up to 72 h. In contrast, stimulation with pσ1, OVA-pσ1, or OVA + pσ1 induced much greater apoptosis of Tg $CD4^+$ T cells after 24 and 72 h (Table 2.3), and the extent of this apoptosis was time- and dose- dependent. Less than 31 % of these cells died after 24 h of culture with a low-dose (0.05 mg/ml) stimulation, whereas 1 mg/ml of OVA-pσ1, pσ1 or OVA + pσ1 doubled the number of apoptotic cells at this time point (Table 2.3). After 72 h, low-dose pσ1 exposure resulted in ~ 54 - 62 % of Tg $CD4^+$ T cell undergoing apoptosis unlike high-dose treatment that resulted in ~ 80 % apoptosis (Table 2.3). Concomitant cytokine analysis of cultured supernatants revealed increased production of IL-4 and IL-10 by the cells stimulated for 24 h with 1 mg/ml OVA-pσ1 or 72 h with 0.05 mg/ml OVA-pσ1 (Table 2.4). Consistently, these OVA-pσ1-stimulated cells showed significant reduction in inflammatory cytokines IFN-γ and IL-17 at both time points, when compared to OVA-stimulated cells. Cells cultured with a high-dose of OVA-pσ1 for 72 h failed to produce any cytokines, presumably due to apoptosis of 80 % of these cells (Table 2.3). Interestingly, cells incubated with unconjugated pσ1 and OVA mimicked the inflammatory response of OVA-stimulated cells, showing elevated IL-17 and IFN-γ secretion after 24 h, and even after 72 h, the production of IL-10 and IL-4 by these cells was significantly reduced in relation to OVA-stimulated cells (Table 2.4). These results offer an alternative mechanism in which pσ1 may induce apoptosis of OVA-responsive $CD4^+$ T cells if intact pσ1 survives delivery to mucosal inductive sites.

Table 2.3. Apoptosis of Tg DO11.10 $CD4^+$ T cells

Treatment[a]		% Apoptosis of DO11.10-Tg $CD4^+$ T Cells[b]			
Ag	**Dose, in mg/ml**	**24 h[c]**	***P*$_1$ [d]**	**72 h[e]**	***P*$_2$ [f]**
Media		7.06 ± 0.6		10.37 ± 0.92	
OVA	1	10.96 ± 2.28		12.73 ± 6.53	
OVA-pσ1	1	67.35 ± 7.97	< 0.001	83.80 ± 7.10	< 0.001
OVA-pσ1	0.05	30.99 ± 3.98	< 0.001	54.41 ± 11.6	0.003
pσ1	1	77.82 ± 4.82	< 0.001	86.52 ± 11.8	< 0.001
pσ1	0.05	32.43 ± 7.26	0.005	62.58 ± 10.6	< 0.001
OVA + pσ1	1, 1	77.56 ± 8.42	< 0.001	80.34 ± 8.44	< 0.001
OVA + pσ1	1, 0.05	29.83 ± 9.86	0.025	62.29 ± 10.13	< 0.001

[a] Lymphocytes were isolated from spleens of naïve DO11.10 Tg mice. Cells were stimulated *in vitro* with designated concentrations of Ags and analyzed by FACS.
[b] Apoptosis was determined by co-staining with Annexin-V and 7-AAD.
[c] Mean ± SD of three to four replicates are presented after 24 h of culture.
[d] Values of *p* for DO11.10 $CD4^+$ T cells stimulated with OVA vs OVA-pσ1, pσ1, or OVA + pσ1 for 24 h.
[e] Mean ± SD of three to four replicates are presented after 72 h of culture
[f] Values of *p* for DO11.10 $CD4^+$ T cells stimulated with OVA vs OVA-pσ1, pσ1, or OVA + pσ1 for 72 h.

Table 2.4. Cytokine production by *in vitro* cultured DO11.10 lymphocytes

Treatment[a]					
Ag	**Dose, in mg/ml**	**IL-4 (ng/ml)[c]**	**IL-10 (ng/ml)[c]**	**IL-17 (ng/ml)[c]**	**IFN-γ (ng/ml)[c]**
			Cytokine Secretion (24 h[d])		
OVA	1	0.58 ± 0.03	0.66 ± 0.04	5.59 ± 0.54	4.06 ± 0.52
OVA-pσ1	1	1.20 ± 0.1 §	2.76 ± 0.14 *	0.37 ± 0.11 *	0.31 ± 0.08 §
OVA-pσ1	0.05	1.67 ± 0.2 §	4.73 ± 0.29 *	0 ± 0 *	0 ± 0 *
pσ1	1	0 ± 0 *	0.26 ± 0.04 §	0.63 ± 0.17 *	0.22 ± 0.07 §
pσ1	0.05	0 ± 0 *	0 ± 0 *	0.58 ± 0.14 *	0 ± 0 *
OVA + pσ1	1, 1	0 ± 0 *	0.56 ± 0.02	4.17 ± 0.6	2.6 ± 0.45
OVA + pσ1	1, 0.05	0 ± 0 *	0.42 ± 0.17	1 ± 0.14 §	0.97 ± 0.08 §
			Cytokine Secretion (72 h[e])		
OVA	1	2.21 ± 0.13	1.82 ± 0.12	6.22 ± 0.46	10.1 ± 0.98
OVA-pσ1	1	0 ± 0 *	0 ± 0 *	0 ± 0 *	0 ± 0 *
OVA-pσ1	0.05	3.54 ± 0.15 §	2.2 ± 0.03 §	0.52 ± 0.13 *	0.38 ± 0.03 *
pσ1	1	0 ± 0 *	0 ± 0 *	0.58 ± 0.14 *	0.72 ± 0.05 *
pσ1	0.05	0 ± 0 *	0.29 ± 0.02 *	0 ± 0 *	0.94 ± 0.09 *
OVA + pσ1	1, 1	0 ± 0 *	0.37 ± 0.19 §	0.72 ± 0.18 *	0.77 ± 0.2 *
OVA + pσ1	1, 0.05	1.22 ± 0.18 *	0.46 ± 0.16 §	2.87 ± 0.47 §	2.3 ± 0.29 §

[a] Statistical significance was calculated by Student's t test; *, $p \leq 0.001$, §, $p < 0.05$ for the differences between cells stimulated with OVA vs OVA-pσ1, pσ1, or OVA + σ1-stimulated cells, for each time point. [b] Lymphocytes were isolated from spleens of naïve DO11.10-Tg mice. Cultured supernatants from cells stimulated *in vitro* with designated concentrations of Ags were analyzed by cytokine ELISA.
[c] Cytokine concentration was measured in triplicates and is depicted as an average ± SEM. Presented cytokine concentration is corrected over the cytokine production by unstimulated cells. [d]Cell were cultured with or without Ag for 24 h. [e] Cell were cultured with or without Ag fir 72 h.

Discussion

We previously showed that the reovirus adhesin, pσ1, can enhance the efficacy of mucosal DNA vaccination (82). In this work, we investigated if a genetic fusion between pσ1 and a model Ag (OVA) would have the same effect. Unlike the previous findings, it was learned that when pσ1 was fused to a protein Ag, tolerance was induced. In fact, tolerance could be accomplished even with a single dose of OVA-pσ1 delivered mucosally. In contrast, parenteral delivery of OVA-pσ1 failed to induce tolerance to OVA. This pσ1-based mucosal tolerance even resisted co-treatment with potent mucosal adjuvants (CT and ODN), and tolerance was not broken after peripheral challenge with OVA. In contrast to OVA-pσ1, nasal administration of OVA alone, which is known to be a poor mucosal immunogen (364), elicited greater Ag-specific Ab responses than did OVA-pσ1. Moreover, the mechanism for this pσ1-induced tolerance was $CD4^+$ T cell-dependent and could be adoptively transferred into naïve mice. Adoptive transfer of CLN-derived $CD4^+$ T cells from mice tolerized with OVA-pσ1 significantly inhibited OVA-specific proliferation of $CD4^+$ T cells *in vitro*. In subsequent analysis, it was revealed that tolerization via OVA-pσ1 induced significant increases in $FoxP3^+$ $CD25^+CD4^+$ T cells, as well as an elevation in $FoxP3^+$ $CD25^-CD4^+$ T cells. Adoptive transfer of OVA-pσ1-primed $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells significantly inhibited Ag-specific proliferation of OVA-Tg $CD4^+$ T cells *in vivo*. This suppression was due to increased production of IL-10 by OVA-pσ1-induced T_{reg} cells, as evidenced by cytokine and FACS analyses, as well as by the lack of OVA-specific tolerance in OVA-pσ1-dosed

IL-10$^{-/-}$ mice. Additionally, it was learned from the *in vitro* studies that OVA-pσ1 induces apoptosis of OVA-responsive CD4^{+} T cells in a time- and a dose- dependent manner, offering an additional potential mechanism for pσ1's action if it can survive delivery beyond the initial cell binding to the mucosal epithelium or M cells.

Induction of peripheral tolerance to a number of mucosally delivered Ags has been demonstrated previously using both high- and low-dose tolerization regimen (24, 29, 46, 81, 87); however, we propose that fusion of pσ1 to a protein Ag can significantly improve both mucosal and systemic unresponsiveness to this Ag. Previously, we have shown that chemically modified pσ1 with poly-L-lysine significantly enhances immunity to delivered DNA (82), and because of this modification, Abs to pσ1 are induced, but not to the unmodified pσ1. Thus, genetic fusion of an Ag to pσ1 is instrumental to stimulate tolerance. When OVA-pσ1 was co-administered with potent mucosal adjuvants, CT or CpG-ODN, the results provided further evidence that immunity to OVA was impaired. This finding was surprising since it is well established that CT given mucosally always induces Ag-specific Abs (152). Although the mechanism by which OVA-pσ1-induced tolerance overrides the immunostimulatory effects of CT is unclear, it is plausible that OVA-pσ1 resists co-treatment with CT due to the pσ1 adhesive properties which, in turn, may act on different receptors than CT-B. Nonetheless, pσ1-induced tolerance to OVA remained effective. Another mucosal immune modulator, the unmethylated CG dinucleotides CpG-containing motifs (CpG-ODN), was also shown to enhance immunity to OVA after oral administration (358). Here it was shown nasal co-administration of OVA-pσ1 plus CpG-ODN also resulted in tolerance. In contrast to mice given OVA plus

CpG-ODN, OVA-pσ1 plus CpG-ODN-dosed mice lacked OVA-specific DTH responses and showed limited OVA-specific systemic and mucosal Ab titers, suggesting that the presence of a plasmid cDNA does not affect tolerogenic properties of genetically modified pσ1. Interestingly, significant enhancement in IL-13 and TGF-β, but concomitant reduction in IL-10 and IL-4 was observed in mice dosed with OVA-pσ1 plus CpG-ODN when compared to mice that received OVA-pσ1. OVA-pσ1 plus CpG-ODN-dosed mice revealed moderate, though significant augmentation in the levels of IL-6, IL-21, IL-27 and IFN-γ, when compared to OVA-pσ1-dosed mice. Importantly, in contrast to OVA- and OVA plus CpG-ODN-dosed mice, the levels of proinflammatory cytokines were greatly depressed in OVA-pσ1 plus CpG-ODN-dosed mice. Consistently with the tolerogenic properties of OVA-pσ1 and OVA-pσ1 plus CpG-ODN, IL-17 production was significantly reduced in these mice but not in OVA or OVA plus CpG-ODN-dosed mice. It is well established that CpG-ODN induces strong Ag-specific Ab and Th1-type immune responses when mucosally administered to mice (141). In agreement with this finding, mice dosed with OVA plus CpG-ODN developed strong OVA-specific Ab and DTH responses. Also, these OVA plus CpG-ODN-dosed mice produced significantly more Th1-type cytokines including IFN-γ, IL-27 than OVA-dosed mice. In contrast to OVA plus CpG-ODN-dosed mice, mice given nasally OVA-pσ1 plus CpG-ODN produced predominantly anti-inflammatory cytokines IL-4, IL-10, IL-13, and TGF-β, and exhibited reduced OVA-specific IgG Ab titers in serum and OVA-specific DTH response. The suppression of IL-4, and IL-10, and concomitant raise in IL-13 and TGF-β observed in mice nasally dosed with OVA-pσ1 plus CpG-ODN when

compared to OVA-pσ1-tolerized mice, implicates supportive role for TGF-β and IL-13 in establishment of OVA-pσ1-mediated tolerance to OVA in the presence of CpG-ODN.

Despite the reported feasibility to induce low-dose nasal tolerance with OVA (87, 88), a sustainable tolerance by a variety of nasal OVA doses (0.05 - 2.5 mg) could not be induced, but a single oral 25 mg dose of OVA was found effective. In contrast, as little as a single 50 μg nasal dose of OVA-pσ1 was sufficient to induce OVA-specific tolerance that was long-lasting and resisted peripheral challenge with OVA in IFA, showing that OVA-pσ1 is at least a 1000-fold more efficient on a molar basis.

Previous studies have shown that the CT-B subunit could be adapted for mucosal tolerance induction (67, 69, 150, 151). In those reports, efficiency of Ag-specific tolerance was improved by its conjugation to CT-B, as evidenced by reduced Ag-specific Ab and DTH responses following mucosal delivery. Many of these studies depended upon chemically coupling the tolerogen, which produces a heterogeneous population of carrier-conjugates and may limit only a fraction of these to bind the intended cell surface receptor (35). Additionally, CT-B is also commonly used as a mucosal adjuvant (365, 366) because it lacks the toxic moieties associated with native CT (64). To circumvent these potential barriers for using CT-B to induce tolerance, a genetic fusion between CT-B and proteolipid protein (PLP) immunodominant peptide was done to ameliorate the clinical manifestations of EAE (35). Although multiple doses were required, nasal administration of this CT-B-PLP peptide fusion protein was effective for inducing tolerance.

T_{reg} cells consist of a phenotypically diverse group bearing a variety of cell surface receptors, but they commonly share their ability to suppress T cell function (60, 112, 367). Induction and maintenance of low-dose mucosal tolerance has been associated with activation of T_{reg} cells acting in an Ag-specific fashion (24, 25, 44, 54, 124). Evidence provided in this study shows that OVA-pσ1-induced tolerance is mediated by Ag-specific $CD4^+$ T cells, since adoptive transfer of these OVA-pσ1-primed $CD4^+$ T cells conferred unresponsiveness in naïve recipients to *in vivo* peripheral OVA challenge and inhibited OVA-specific $CD4^+$ T cell proliferation. Studies have also found that low-dose mucosal tolerance is mediated by T_{reg} cells that express the FoxP3 transcription factor (3, 37, 60, 109, 368). Consistent with these findings, nasal administration of OVA-pσ1 significantly increased the numbers of T_{reg} cells, in which > 97 % were $FoxP3^+$. Along these lines, expression of FoxP3 was also induced in $CD25^-CD4^+$ T cells following tolerization with OVA-pσ1, although the intensity of FoxP3 staining was ~50-fold less than that observed for the $CD25^+CD4^+$ T cells. A low level intensity of FoxP3 and its transient expression on activated human $CD25^-$ T cells has been previously reported (369). These FoxP3-expressing $CD25^-$ T cells are not capable of inhibiting cytokine production nor proliferation, and expression of FoxP3 on these cells is not correlated with the induction of a T_{reg} cell phenotype (369). In contrast to that study, adoptive transfer of OVA-pσ1-primed $CD25^+$ or $CD25^-$ T cells significantly inhibited *in vivo* proliferation of OVA-Tg $CD4^+$ T cells. Additionally, FACS analysis of lymphocytes isolated from mice adoptively transferred with OVA-pσ1-derived $CD25^-$ T cells showed significant enrichment in $FoxP3^+CD25^-CD4^+$ T cells. A significant increase in FoxP3 expression in

DO11.10 responder $CD4^+$ T cells (both $CD25^+$ and $CD25^-$) was observed in mice adoptively transferred with OVA-pσ1-derived $CD25^+$ or $CD25^-$ $CD4^+$ T cells, suggesting that OVA-pσ1-derived $CD4^+$ T cells induce $FoxP3^+$ expression by the responder $CD4^+$ T cells. This finding is not surprising, since tolerance induction can occur via conversion of $CD25^-$ to $FoxP3^+$ $CD25^+CD4^+$ T cells, as reported by others (122, 220), and these FoxP3-expressing $CD25^-CD4^+$ T cells, as well as converted $CD25^+CD4^+$ T cells, are potent inhibitors of $CD4^+$ T cell expansion *in vivo* (122). Thus, the OVA-pσ1-induced $CD25^-$ T cells also have regulatory properties, as evident by being able to suppress proliferation of Tg $CD4^+$ T cells.

Examination of $OVA_{323-339}$-specific $CD4^+$ T cell cytokine production in recipient mice adoptively transferred with either total $CD25^-$ or $CD25^+$ $CD4^+$ T cells from OVA-pσ1-primed mice revealed greatly reduced numbers of $CD4^+$ T cells secreting the proinflammatory cytokines, IFN-γ and IL-17, and instead produced IL-4 and IL-10. These primarily segregated with IL-4 coming from recipients given OVA-pσ1-primed $CD25^-CD4^+$ T cells and with IL-10 from recipients given OVA-pσ1-primed T_{reg} cells. The increased production of IL-4 may play a beneficial role in OVA-pσ1-mediated tolerance, perhaps, via the induction of a Th2-type immune bias, which was previously shown to support mucosal tolerance (76, 117). Additionally, IL-4 can also facilitate induction of T_{reg} cells from naïve, peripheral $CD25^-CD4^+$ T cells, as recently demonstrated *in vitro* by others (117). The role for IL-10 in maintenance of tolerance is well established (359-361), even though its role in the induction of both mucosal and peripheral unresponsiveness remains controversial (370-372). One report has shown that

the early production of IL-10 by $CD4^+$ T cells is important for induction of high- dose oral tolerance (370). Here, we demonstrated that IL-10 is also necessary for induction of a low-dose nasal tolerance mediated by pσ1, since OVA-pσ1-mediated tolerance could not be established in IL-$10^{-/-}$ mice. IL-$10^{-/-}$ mice given OVA-pσ1 nasally developed elevated serum IgG anti-OVA Ab and DTH responses. In contrast to C57BL/6 mice, immunization of IL-$10^{-/-}$ mice with OVA-pσ1 failed to induce Ag-specific T_{reg} cells and failed to inhibit Ag-specific proliferation of $CD4^+$ T cells. Therefore, we conclude that IL-10 is essential for induction and maintenance of pσ1-induced tolerance, and IL-10-producing T_{reg} cells, as well as possibly $CD25^-CD4^+$ T cells, mediate the observed pσ1-induced tolerance.

The role of TGF-β1 appears to be less essential for OVA-pσ1-induced tolerance. TGF-β1 was only modestly induced in OVA-pσ1 tolerized BALB/c mice; however, TGF-β1 was induced in OVA-pσ1 tolerized C57BL/6 mice and greatly reduced in OVA-pσ1-dosed IL-$10^{-/-}$ mice. The PBS-dosed, OVA-challenged IL-$10^{-/-}$ mice also showed elevated numbers of TGF-β1-producing $CD4^+$ T cells. This suggests that TGF-β1 may play a supportive role in OVA-pσ1-induced tolerance, and secretion of TGF-β1 in OVA-pσ1-tolerized mice depends upon the presence of IL-10, as previously suggested (373). Alternatively, the production of TGF-β1 may contribute to the conversion of $CD25^-CD4^+$ to $CD25^+CD4^+$ T cells in tolerized mice, as implicated by recent studies demonstrating that TGF-β1 induces conversion of naïve peripheral $CD25^-CD4^+$ T cells into T_{reg} cells by enhancing FoxP3 expression (81, 122, 220). Further evidence for the supportive role of

TGF-β1 in OVA-pσ1-mediated tolerance is suggested by the depressed levels of TGF-$\beta1^+$ $CD4^+$ T cells in the OVA-pσ1-dosed IL-$10^{-/-}$ mice.

We have demonstrated, here and elsewhere (82, 154), that reovirus adhesin, pσ1, can be engineered to induce either immunity or tolerance, although the exact mechanism of pσ1-mediated modulation of an immune response remains undefined. Pσ1, aside from binding M cells in murine NALT (82), interacts with a variety of rodent and human cell types, including murine L929 (L) cells, RFL-6 cells, and Caco-2 cells (154). Additionally, pσ1 binds to mammalian erythrocytes (153), as well as to intestinal epithelial cells (374). It is known that pσ1 has two distinct binding domains (374, 375). One domain, located in pσ1's head structure, has been shown to interact with cells expressing the junctional adhesion molecule 1 (JAM1), whereas a second binding domain in pσ1's tail is thought to be responsible for binding to ubiquitously expressed sialic acid (374). Interaction of pσ1 with host cells may be JAM1-mediated, since some of them, including primary human DCs and epithelial cells, express this molecule (374). A possible mechanism for pσ1-induced modulation of an immune response could be a well-described ability of pσ1 to induce apoptosis (375, 376). Interestingly, the efficiency of pσ1-mediated apoptosis has been linked to the presence of sialic acid binding domain, since pσ1 mutants, unable to bind sialic acid, are insufficient inducers of apoptosis (375). Given these findings, we showed here that OVA-pσ1 and pσ1 trigger apoptosis of OVA-Tg $CD4^+$ T cells *in vitro*. However, in contrast to pσ1 alone, or unconjugated pσ1 + OVA, only OVA-pσ1 was capable of stimulating these cells to produce increased levels

of regulatory cytokines. The means by which pσ1 induces tolerance are still being investigated, and our results suggested that more than one mechanism may be associated with this process. Nonetheless, mucosal delivery of OVA-pσ1 is obviously crucial for the induction of OVA-specific unresponsiveness. It is known that stimulation of mucosal tolerance is dose-dependent, resulting from active suppression, induction of anergy, or clonal deletion of effector cells (29, 37). Results presented here suggest that a low dose of OVA-pσ1 delivered mucosally induces active suppression of anti-OVA immune responses by regulatory $CD4^+$ T cells. Although the *in vitro* studies suggested possible clonal deletion because of the accompanied pσ1-induced apoptosis of OVA-specific effector $CD4^+$ T cells, it remains to be determined if such events occur *in vivo* following mucosal delivery.

Interestingly, pσ1-delivered oral tolerance to OVA could not be established in $LT\alpha^{-/-}$ mice lacking PPs and LNs (43, 356, 363). The requirement for PPs in induction of oral tolerance has been demonstrated (43, 44, 347). In agreement with our data, it has been shown that PPs-null mice generated by treatment with LTβR-IgG fusion protein *in utero*, as adults were unable to develop oral tolerance upon feeding with large doses of OVA (43). Others have reported that $LT\alpha^{-/-}$ mice immunized with the low-doses of NP-OVA in alum developed impaired NP-specific IgG Abs, but immunization with a high doses of these Ags induced NP-specific IgG Ab response in these mice (363). Induction of high Ag-specific IgG Ab titers and DTH response were also demonstrated in $LT\alpha^{-/-}$ mice immunized with type II collagen (356). Additionally, these collagen-dosed $LT\alpha^{-/-}$ developed more severe collagen-induced arthritis (CIA) than C57BL/6 mice. In our

study unlike OVA-pσ1-fed C57BL/6 mice that remained unresponsive to OVA-challenge, LTα$^{-/-}$ mice fed with low doses of OVA-pσ1 or OVA and systemically challenged with OVA developed very evident OVA-specific Ab titers in serum and OVA-specific DTH response, suggesting that presence of PPs is required for induction of OVA-pσ1-mediated tolerance to OVA.

In summary, we showed that pσ1-mediated tolerance to OVA can be established with a minimal amount of tolerogen when genetically fused to pσ1 and when applied mucosally. In some instances, a single nasal administration using ~1000-fold less OVA was sufficient to induce tolerance to OVA. Tolerance induced by OVA-pσ1 resisted co-treatment with CT, as well as peripheral challenge with OVA and IFA. Although IL-4 and TGF-β seemed to have supportive roles, pσ1-delivered tolerance relied largely on activation of specific FoxP3$^+$ T_{reg} cells that acted in an IL-10-dependent manner. The induced tolerance was IL-10-dependent since IL-10$^{-/-}$ mice were unable to undergo tolerance by OVA-pσ1 presumably via the failure to produce T_{reg} cells. The impact by the OVA-pσ1-induced T_{reg} cells was enhanced by regulatory CD25$^-$CD4$^+$ T cells since a subset of these was FoxP3$^+$ and produced predominantly IL-4 in addition to IL-10. These CD25$^-$CD4$^+$ T cells could also inhibit the proliferation of OVA-Tg CD4$^+$ T cells. TGF-β1 appeared to have a supportive role in tolerance induction by OVA-pσ1. Depression of TGF-β1-producing CD4$^+$ T cells in OVA-pσ1-dosed IL-10$^{-/-}$ mice was observed suggesting that the production of this cytokine may be triggered by IL-10. These collective data show that IL-10 is pivotal in OVA-pσ1-induced tolerance. This finding is relevant because of the low-dose of Ag required for tolerance induction and its potential

applicability to treat or prevent a variety of autoimmune diseases and allergies. The opportunity to deliver pσ1-based tolerogens via the nasal route offers a safer, easier, and more cost effective alternative for tolerization.

NOTE: Dr. Massimo Maddaloni originally cloned and expressed OVA-pσ1 in *P. pastoris* yeast. Experiments presented in Figure 2.1*C* were performed by Carol Riccardi. Experiment described in Figure 2.1*D* was performed in collaboration with Tery Hoyt. Experiment performed in Figure 2.2*A* was performed by Dr. Dagmara Mierzejewska. Experiments described in Figure 2.7*A* and *B* were performed in collaboration with Dr. Beata Barszczewska

SINGLE-DOSE NASAL THERAPY FOR EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) USING PROTEIN σ1-DELIVERED PROTEOLIPID PROTEIN ($PLP_{139-151}$)

Introduction

Multiple Sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) and one of the most common human autoimmune disorders (377). Experimental autoimmune encephalomyelitis (EAE) resembles the clinical manifestation and neuropathology of MS (33, 78, 336, 377, 378) and like MS, is characterized by a chronic inflammatory response against myelin components (33, 77, 78, 172). EAE is typified by the specific recognition of myelin epitopes by autoreactive T lymphocytes (379) and can be induced in rodents either by the active immunization with myelin antigens (Ags), including myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG), or by adoptive transfer of activated encephalitogenic $CD4^+$ T cells (56, 380) or $CD8^+$ T cells (377, 381). It is now well-established that Th17 cells play a pivotal role in disease pathogenesis (168, 169), whereas various subsets of regulatory cells (22-24, 37, 54, 56), and/or Th2 cells (33, 34, 78) have been shown to prevent, ameliorate, and/or treat EAE in mice. Passive transfer of myelin-specific Th2 cells (37, 177), induction of Ag-unspecific Th2 cell bias (33, 78), and immunization with altered peptide ligand (APL) have been shown to significantly down regulate the proliferation of proinflammatory cells, and protect against EAE (382).

$CD25^+CD4^+$ T_{reg} cells originally described in neonatally thymectomized mice (383) have been widely implicated in maintenance of peripheral tolerance and protection against EAE (384, 385), colitis (81), and arthritis (386). The suppressive function of

adaptive $CD25^+CD4^+$ T_{reg} cells has been linked with expression of forkhead box (FoxP3) transcription factor (114, 116, 117), and secretion of regulatory cytokines IL-10 (3, 56, 89, 105, 113, 114), and/or TGF-β (3, 33, 34, 56, 81).

Induction of mucosal tolerance has been successfully applied to treat or prevent autoimmunity in animal models (3, 24-26). Though oral administration of encephalitogenic peptides has been shown to protect against EAE (56, 387), feeding with bovine myelin preparations has not given satisfactory results in MS clinical trials (22). To improve the efficiency of mucosal tolerance induction, oral administration of Ags delivered by liposomes (149), emulsified in adjuvants (22, 148), or coupled to mucosa binding molecules (35, 150) has been performed. Though most of these strategies significantly ameliorate clinical EAE, they require multiple administrations of Ags, which raises the risk of allergic responses.

Recently, we have demonstrated induction of a long-lasting, sustainable tolerance to chicken ovalbumin (OVA) genetically fused to recombinant reovirus protein sigma 1 (OVA-pσ1) (89). A single low dose of OVA-pσ1 delivered nasally was sufficient to induce vaccine-specific IL-10^+CD25^+ and IL-4^+CD25^- regulatory $CD4^+$ T cells which are able to suppress the immune response induced by systemic challenge with OVA (89). Additionally, OVA-pσ1-induced tolerance was to OVA and to pσ1, and was maintained even after co-administration of mucosal adjuvants.

Here, we question whether pσ1-delivered mucosal tolerance can be applied to prevent and/or treat autoimmune diseases. To test this hypothesis, a genetic fusion between two copies of proteolipid protein (PLP) immunodominant encephalitogenic

peptide ($PLP_{130-151}$) and OVA-pσ1, termed PLP:OVA-pσ1, was built and tested in susceptible SJL mice. PLP:OVA-pσ1, given prior to EAE induction or in the pre-clinical phase of the disease, significantly ameliorated clinical onset and neuropathology of $PLP_{139-151}$-induced EAE. A single, intranasal, low-dose of PLP:OVA-pσ1 greatly enhances IL-10-producing regulatory T cells, and IL-4-secreting $FoxP3^+$ Th2 cells in mice. We have determined that PLP:OVA-pσ1-induced regulatory T cells are essential for protection against EAE, as functional inactivation of PLP:OVA-pσ1-primed $CD25^+$ T_{reg} cells by administration of anti-CD25 mAb to mice rendered them susceptible to very aggressive EAE. Adoptive transfer of PLP:OVA-pσ1-derived T_{reg} cells entirely protected recipient mice from development of EAE upon challenge, while PLP:OVA-pσ1-derived $CD25^-$ Th2 cells also significantly ameliorated the clinical manifestation of the disease.

Materials and Methods

Preparation of PLP:OVA-pσ1

PLP:OVA-pσ1 was constructed based on OVA-pσ1 described previously (89). Two copies of PLP peptide ($PLP_{130-151}$; QAHSLERVC HCLGKWLGHPDKF) separated by the flexible linker (RHRHVDCSGRNLTTLPPGLQE) were synthesized as a single cDNA fragment containing restriction enzyme sites at the 5' and 3' termini (GenScript Corp. Piscataway, NJ). The synthetic cDNA fragment was amplified by PCR and cloned into pUC19. The 5' and 3' primers encoded *EcoR*I sites and 5' primer encoded an ATG initiation codon embedded into an optimal Kozak's sequence. PCR amplified PLP

peptides were ligated with the 5' terminus of OVA-pσ1 in a pPICZ B vector (Invitrogen Corp., Carlsbad, CA) bearing an his-tag carboxy terminus for protein purification (Invitrogen), referred to as pσ1. The junction between the $PLP_{130-151}$ epitopes and the OVA-pσ1 featured a flexible linker (Gly-Arg-Pro) to minimize steric hindrance between the components. The resulting construct was sequenced and expressed in the yeast *P. pastoris*, according to the manufacturer's directions (Invitrogen Corp.). Recombinant proteins were extracted from yeast cells by a bead-beater (Biospec Products, Bertlesville, OK) and purified on a Talon metal affinity resin (BD Biosciences, Palo Alto, CA), according to manufacturer's instructions. Proteins were assessed for purity and quality by Coomassie-stained polyacrylamide gels and by Western blot analysis using a polyclonal rabbit anti-pσ1 (produced in-house) or a polyclonal rabbit anti-OVA Ab (Sigma-Aldrich, St. Louis, MO). All recombinant proteins migrated as a single band with the expected MW.

Mice

Female six week old SJL and C57BL/6N mice were obtained from Frederick Cancer Research Facility, National Cancer Institute (Frederick, MD), and The Jackson Laboratories (Bar Harbor, ME). All mice were maintained at Montana State University Animal Resources Center under pathogen-free conditions in individual ventilated cages under HEPA-filtered barrier conditions and were fed sterile food and water *ad libitum*. The mice were free of bacterial and viral pathogens, as determined by antibody screening and histopathological analysis of major organs and tissues. All animal care and

procedures were in accordance with institutional policies for animal health and well-being.

Tolerance Induction,
PLP:OVA-pσ1 Treatment, and OVA Challenge

For tolerance induction, mice (5 mice/group) were nasally dosed up to three times before EAE challenge, as described in the text, with 50 - 100 μg of PLP:OVA-pσ1 or OVA-pσ1.

For PLP:OVA-pσ1 treatment, mice were nasally dosed with 50 - 100 μg of PLP:OVA-pσ1 six days after EAE induction. Control groups were treated with PBS or OVA-pσ1. PLP:OVA-pσ1 or OVA-pσ1 was administered nasally in a volume of no more than 20 μl / dose up to four times a day, with no less than 2.5 hour intervals between each administration (89).

To assess induction of OVA-specific tolerance, groups of mice were nasally dosed with OVA or PLP:OVA-pσ1 followed by s.c. challenge 4 days later with OVA in IFA, as described previously (89).

EAE Induction

For EAE induction mice were challenged s.c. with 200 μg of the encephalitogenic PLP peptide ($PLP_{139-151}$; HSLGKWLGHPDKF; Global Peptide Services, LLC, Ft. Collins, CO; HPLC-purified to > 90 %) in 200 μl (33). On days 0 and 2 post-challenge, mice received i.p. 200 ng of *Bordetella pertussis* toxin (PT; List Biological Laboratories, Campbell, CA). Mice were monitored and scored daily for disease progression (78): 0,

normal; 1, a limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, quadriplegia; 5, death.

Ab ELISA

Serum and fecal samples collection were performed, as previously described (89). OVA-specific endpoint Ab titers were measured by ELISA, as previously described (46), using purified OVA (Grade V) as coating Ag. Specific reactivity to OVA was determined using HRP conjugates of goat anti-mouse IgG- and IgA-specific Abs (1.0 μg/ml; Southern Biotechnology Associates, Birmingham, AL) and ABTS (Moss Inc., Pasadena, CA) enzyme substrate. The absorbences were measured at 415 nm on a Kinetics Reader model ELx808 (Bio-Tek Instruments, Winooski, VT). Endpoint titers were expressed as the reciprocal dilution of the last sample dilution, giving an absorbence of 0.1 OD units above the OD_{415} of negative controls after 1 h incubation (78, 357).

Measurement of Delayed-Type
Hypersensitivity (DTH) Responses

To measure OVA- or $PLP_{139-151}$- specific DTH responses *in vivo* (46), 10 μg of an Ag (OVA or $PLP_{139-151}$) were injected into the left ear pinna, and PBS alone (20 μl) was administered to the right ear pinna as a control. Ear swelling was measured 24 h later with an electronic digital caliper (World Precision Instruments, Sarasota, FL). The DTH response was calculated as the increase in ear swelling after Ag injection following subtraction of swelling in the control site injected with PBS.

Histological Evaluation of Spinal Cords

For histological evaluation of tissue pathology, spinal cords were removed 14 days after challenge and fixed with neutral buffered formalin (VWR International, West Chester, PA), embedded into paraffin, and sectioned at 5 μm. Cross sections of spinal cords were stained with H&E for pathological changes and inflammatory cell infiltration. Adjacent sections were stained with luxol fast blue (LFB) and examined for loss of myelin. Pathological manifestations were scored separately for cell infiltrates and demyelination. Each H&E section was scored from 0 to 4: 0, normal; 1, cell infiltrate into the meninges; 2, one to four small focal perivascular infiltrates; 3, five or more small focal perivascular infiltrates and/or one or more large infiltrates invading the parenchyma; 4, extensive cell infiltrates involving 20 % or more of the white matter (33, 78). In each LFB stained section, myelin was also scored from 0 to 4: 0, normal; 1, one small focal area of demyelination; 2, two or three small focal areas of demyelination; 3, one to two large areas of demyelination; 4, extensive demyelination involving 20 % or more of white matter (33, 78).

Cytokine ELISA

Spleens, mesenteric lymph nodes (MLNs), and head and neck LNs (HNLNs) were aseptically removed 14 days after EAE induction from PBS-, PLP:OVA-pσ1- and OVA-pσ1- dosed mice. Lymphocytes were prepared, as previously described (33), and resuspended in complete medium (CM) (34). Lymphocytes were cultured in 24-well tissue plates at 5 x 10^6 cells/ml in CM alone or in the presence of OVA (Sigma-Aldrich; 1 mg/ml or 10 μg/ml), PLP:OVA-pσ1 (50 μg/ml), or $PLP_{139-151}$ peptide (30 μg/ml) in a

total volume of 1 ml for three to five days at 37º C. The supernatants were collected by centrifugation and stored at -80º C. Capture ELISA was employed to quantify, on triplicate sets of samples, the levels of IFN-γ, IL-4, IL-10, IL-13, IL-17, and TGF-β produced by lymphocytes, as previously described (33). For detection of IL-21 and IL-22, microtiter wells were coated with 2 μg/ml of purified goat anti-mouse IL-21 Ab, or goat anti-mouse IL-22 Ab, respectively, (R&D Systems, Minneapolis, MN). After blocking with PBS + 1% BSA for 2h at 37º C, washed wells were incubated with cell culture supernatants at 4º C for 24 h. After washing, 0.5 μg/ml biotinylated rat anti-mouse IL-21 mAb or biotinylated goat anti-mouse IL-22 Ab (R&D Systems) was added, respectively, for 90 min at 37º C. Following washing, 1:500 HRP-goat anti-biotin Ab (Vector Laboratories, Inc., Burlingame, CA) was added for 1h at room temperature (RT). After washing, ABTS peroxidase substrate (Moss, Inc.) was added to develop the reaction.

FACS Analysis

Lymphocytes from the HNLNs, MLNs, and spleens were isolated 14 days after challenge, and single cell suspensions were prepared, as described above (33). To obtain lymphocytes from spinal cords, mice were perfussed through the left ventricle with 20 ml of ice cold sPBS, and spinal cords were removed by flushing the vertebral canal with media and prepared, as previously described (33).

Cells were stained for FACS analysis using conventional methods. Leukocyte gates were set within the forward and side scatter profiles to exclude resting microglia cells from the spinal cord preparations. T cell subsets were analyzed using fluorochrome-

conjugated mAbs for CD4, CD25, TCRβ, CD8, GITR, CCR6, (all from BD Pharmingen) and OX-40 (CD134; clone OX-86) (eBioscience), and biotinylated TGF-β (R&D Systems). Intracellular staining for FoxP3 was accomplished using FITC-, Cy-, or PE-anti-FoxP3 mAb (clone FJK-16s; eBioscience, San Diego, CA), FITC, or PE-anti-IFN-γ Ab, PE, or APC- anti-IL-10 and anti-IL-4, and PE-anti-CTLA-4 (CD152) (all from BD Pharmingen). Bound fluorescence was analyzed with a FACS Canto (BD Biosciences, Mountain View, CA).

Adoptive Transfer Studies

Following PLP:OVA-pσ1 immunization, total $CD4^+$ T cells from spleens, HNLNs, and MLNs were obtained (negative $CD4^+$ T cell isolation kit, Dynal Biotech ASA, Oslo, Norway). $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were isolated from total $CD4^+$ T cells with > 95 % and 99 % purity, respectively, by positive selection using CELLection Biotin Binder Kit (Dynal Biotech; Invitrogen) and biotin-conjugated anti-mouse CD25 (PC61, eBioscience), according to manufacture's instructions. To test PLP:OVA-pσ1-primed T_{reg} cell efficacy, 6 x 10^5 $CD25^-CD4^+$ T cells or $CD25^+CD4^+$ T cells were i.v. injected into naïve recipients. Group of control mice was i.v. injected with sPBS. One day after the adoptive transfer of T cell subsets, mice were challenged with $PLP_{139-151}$ and evaluated for the clinical symptoms.

In Vivo Inactivation of CD25
and Neutralization of TGF-β

Mice were nasally dosed with PLP:OVA-pσ1 or PBS on day -14 and -7 relatively to the EAE induction with $PLP_{139-151}$. To functionally inactivate $CD25^+CD4^+$ T cells, the

same mice were given i.p. 1.0 mg of anti-CD25 mAb (clone PC 61.5.3; ATCC TIB-222) on day -5 and -2 before EAE induction. As a control, groups of PLP:OVA-pσ1- or PBS-dosed mice received 1.0 mg of purified rat IgG on the same days before EAE challenge. All mice were monitored daily for development of EAE.

To neutralize TGF-β *in vivo*, mice dosed with PLP:OVA-pσ1 or PBS and treated with anti-CD25 mAbs or rat IgG (see above) were also i.p. injected with 0.5 mg of anti-TGF-β mAbs (clone 1D11.16.8; ATCC) on day -1 and +5 relatively to the day of EAE induction.

In Vitro Suppression of IL-10 and IL-4
and *In Vivo* Neutralization of IL-4

Mice were induced with EAE and treated with a single 100 μg dose of PLP:OVA-pσ1 six days later. Control group for EAE induction received nasally PBS on day 6. At the peak of the disease (day 14 after challenge), mice were sacrificed, and $CD4^+$ T cells were isolated from HNLNs, MLNs, and spleens. 5 x 10^6 $CD4^+$ T cells / ml were cultured with a 1:1 ration of irradiated splenic feeder cells (T cell depleted) alone or with $PLP_{139-151}$ (30 μg/ml) stimulation for 72 h (37° C, 5 % CO_2). To determine an effect of *in vitro* IL-10 suppression on proliferation of these $CD4^+$ T cells and their cytokine profiles, PLP-stimulated cells were also cultured with purified mouse anti-IL-10 mAb (clone JES5-16E3; BD Pharmingen), purified mouse anti-IL-4 mAb (clone 30340.11; R&D Systems) or with purified rat IgG as a control.

To block IL-4 *in vivo*, mice dosed with PLP:OVA-pσ1 on day -14 and -7 before EAE challenge were given i.p. 1.0 mg of anti-IL-4 mAb (clone 11B11; ATCC,

Manassas, VA) on day -1 before challenge, and on day +5 after EAE challenge with $PLP_{139-151}$ (145). Control mice received i.p. injection of 1.0 mg purified rat IgG Ab (AbD Serotec, Oxford, UK).

In Vitro Suppression of TGF-β, IL-13 and/or Blocking of CD25

OVA (transgenic) $CD4^+$ T cells isolated from DO11.10 mice were *in vitro* cultured for a total of 66 h with PLP:OVA-pσ1 (50 μg/ml), OVA (1 mg/ml) or with $OVA_{323-339}$ peptide (1 μg/ml) and/or with the following Abs: 20 μg/ml of neutralizing Abs to IL-13 (recombinant goat anti-mouse, R&D Systems) and TGF-β (clone 1D11.16.8; ATCC), and/or 10 μg/ml of blocking mAbs to CD25 (clone PC 61.5.3; ATCC TIB-222). For the last 18 h of culture, cells were pulsed with [^{3}H] TdR.

In Vivo Neutralization of IL-23

To block IL-23 *in vivo*, mice dosed with 100 μg of PLP:OVA-pσ1 on day -14 and -7 before EAE challenge were given i.p. 0.5 ml of rabbit anti-mouse IL-23p19 serum (RS, made in-house) on day -1 before challenge, and 0.25 ml of anti-IL-23 RS on day 1 and 5 after EAE challenge with $PLP_{139-151}$. Control mice received i.p. injection of equal amount of normal rabbit serum (NRS) (Jackson ImmunoResearch Laboratories, West Grove, PA).

In Vitro T Cell Assays

Cell-sorted $CD4^+$ T cells isolated from CLNs, HNLNs, MLNs, and spleens were cultured, as described elsewhere (33). Cells were pulsed with ^{3}H-TdR (0.5 μCi/well),

and ^{3}H-TdR incorporation by proliferating $CD4^+$ T cells (triplicate cultures) was measured and expressed as a stimulation index (SI) (33). To assess cytokine production by T_{reg} cells and effector T cells, $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells (2×10^5) were stimulated *in vitro* with anti-CD3 mAb-coated wells (10 μg/ml; BD Pharmingen) and a soluble anti-CD28 mAb (5 μg/ml; BD Pharmingen) for 5 days (final volume of 300 μl in a 48-well plate). Capture ELISA was used to quantify triplicate sets of samples to measure cytokine production (33).

Statistical Analysis

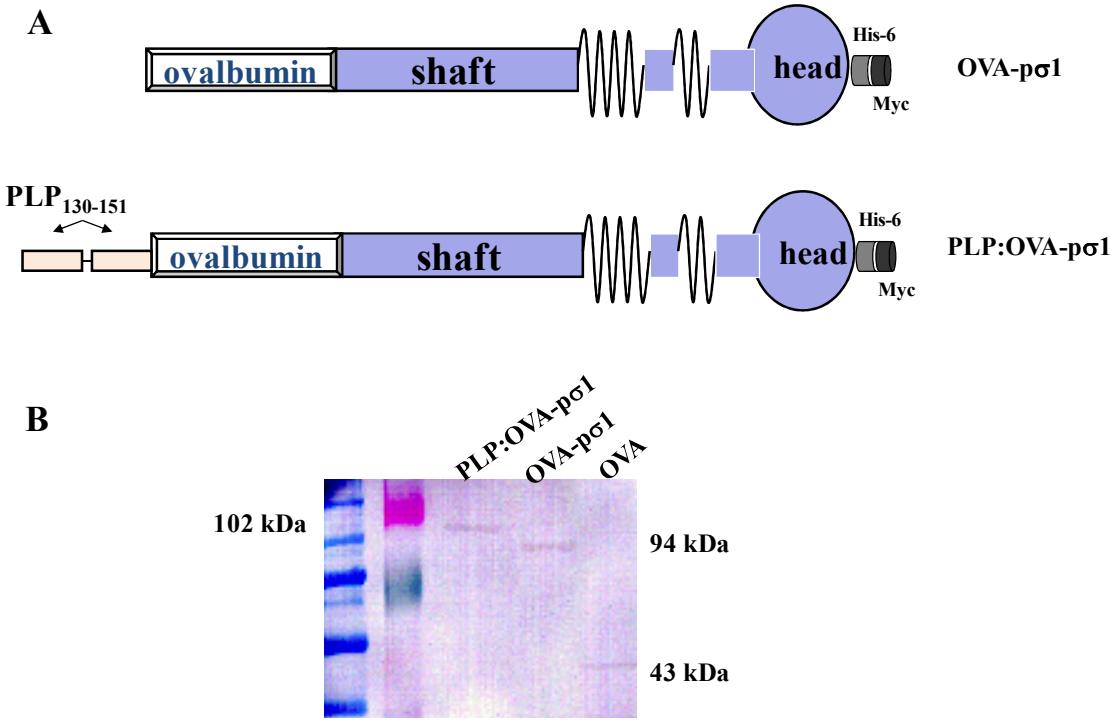
The ANOVA followed by posthoc Tukey test was applied to show differences in clinical scores in treated vs. PBS mice. The student *t* test was used to evaluate the differences between variations in cytokine level production, and *p*-values < 0.05 are indicated, unless specified otherwise.

Results

PLP:OVA-pσ1-Dosed Mice Remain Unresponsive to OVA upon Systemic Challenge with OVA in IFA

We have shown previously (89) that mucosal administration of OVA-pσ1 induces tolerance to OVA, as evidenced by lack of OVA-specific Ab titers in serum and OVA-specific delayed type hypersensitivity (DTH) response. To discern whether fusion of two copies of encephalitogenic proteolipid protein (PLP) peptide ($PLP_{130-151}$) to the N-terminus of OVA-pσ1 (PLP:OVA-pσ1; Figure 3.1*A* and *B*) will alter pσ1's ability of to induce tolerance to OVA, BALB/c mice were adoptively transferred with OVA-Tg $CD4^+$

T cells, and then nasally dosed with 50 μg of PLP:OVA-pσ1, OVA, or PBS twenty four hours later, and subcutaneously (s.c) challenged with OVA in IFA on day 3 post challenge (p.ch). Mice dosed with PLP:OVA-pσ1 showed strikingly depressed OVA-specific DTH responses measured five days after challenge (Figure 3.1*D*). Also, analysis of serum samples collected from these mice seven days after challenge revealed significant decreases of OVA-specific IgG in PLP:OVA-pσ1-dosed and challenged mice compared to the controls, suggesting that the N-terminus fusion of two copies of PLP:OVA-pσ1 to OVA-pσ1 did not amend pσ1- mediated tolerance to OVA (Figure 3.1*C* and *D*).



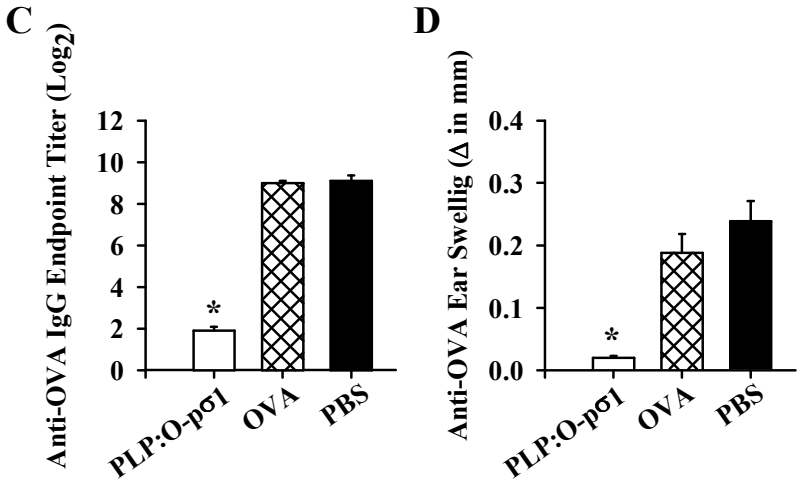
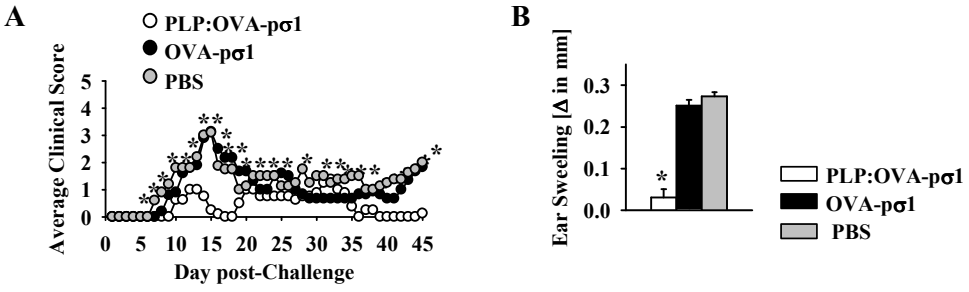


Figure 3.1. N terminal modification of OVA-pσ1 resulted in a functional protein (PLP:OVA-pσ1). (*A*) Schematic representation of OVA-pσ1 and PLP:OVA-pσ1. Two copies of $PLP_{130-151}$ were N terminally fused to OVA-pσ1, resulting in PLP:OVA-pσ1. (*B*) PLP:OVA-pσ1 (102 kDa), OVA-pσ1 (94 kDa) and OVA (43 kDa) were analyzed for the immunoreactivity with rabbit anti-mouse OVA pAbs (in home made). (*C, D*) N terminal fusion of myelin proteolipid protein (PLP) peptide ($PLP_{130-151}$) to OVA-pσ1 did not alter OVA-specific tolerance induced by OVA-pσ1. BALB/c mice were adoptively transferred with 5 x 10^6 cell per mouse of OVA-Tg (DO11.10) $CD4^+$ T cells. 24 h later mice were nasally dosed with 50 μg of PLP:OVA-pσ1, OVA or with PBS. Four days post adoptive transfer mice were subcutaneously (s.c.) challenged with 100 μg of OVA in IFA. Levels of OVA-specific IgG Abs in serum (*C*) and the presence of OVA-specific DTH response (*D*) was measured in these mice 7 d after challenge. Unlike, OVA- or PBS dosed and challenged mice, PLP:OVA-pσ1-dosed mice remained unresponsive to OVA even after peripheral challenged as demonstrated by significant reduction in OVA-specific IgG titers and lack of OVA-specific DTH response in these mice. Results depict mean ± SEM of 5 mice/group. *, $p < 0.001$ vs. PLP:OVA-pσ1-dosed mice.

Nasal Administration of PLP:OVA-pσ1 Ameliorates Clinical Manifestation of EAE

Nasal delivery of OVA fused to pσ1 induced IL-10-producing $CD25^+CD4^+FoxP3^+$ T regulatory (T_{reg}) cells and IL-4-secreting Th2 cells that significantly inhibited proinflammatory response in mice challenged with OVA in IFA (89). To test whether pσ1-delivered protein vaccine can prevent $PLP_{139-151}$-specific inflammatory response and clinical manifestation of EAE, susceptible SJL mice were nasally dosed three times with PLP:OVA-pσ1, OVA-pσ1, or PBS and subjected to a conventional $PLP_{139-151}$ challenge one week later. Mice were evaluated daily for the

development of clinical disease. PBS- and OVA-pσ1-dosed mice developed fully pronounced EAE with the average clinical score of >3 at the peak of the disease, relapsing-remitting disease onset, and they did not recover completely from the disease (Figure 3.2*A*, Table 3.1). In contrast, mice dosed with PLP:OVA-pσ1 showed delayed development and reduced duration of clinical disease, and the average clinical score at the peak of the disease was ~1 for these mice. Additionally, unlike PBS- or OVA-pσ1-dosed mice, PLP:OVA-pσ1-protected mice recovered completely from the acute disease, and complete recovery was observed after the first relapse. The $PLP_{139\text{-}151}$-specific DTH response measured 2 weeks after EAE induction confirmed significant reduction of $PLP_{139\text{-}151}$-specific Th1 response in mice dosed with PLP:OVA-pσ1 but not OVA-pσ1, suggesting the Ag-specificity of the anti-inflammatory response induced by pσ1-delivered protein vaccine (Figure 3.2*B*). These results indicate that nasally administered PLP:OVA-pσ1 can protect mice from development of $PLP_{139\text{-}151}$-induced EAE and that pσ1-delivered tolerance is restricted to the pσ1-fused Ag.



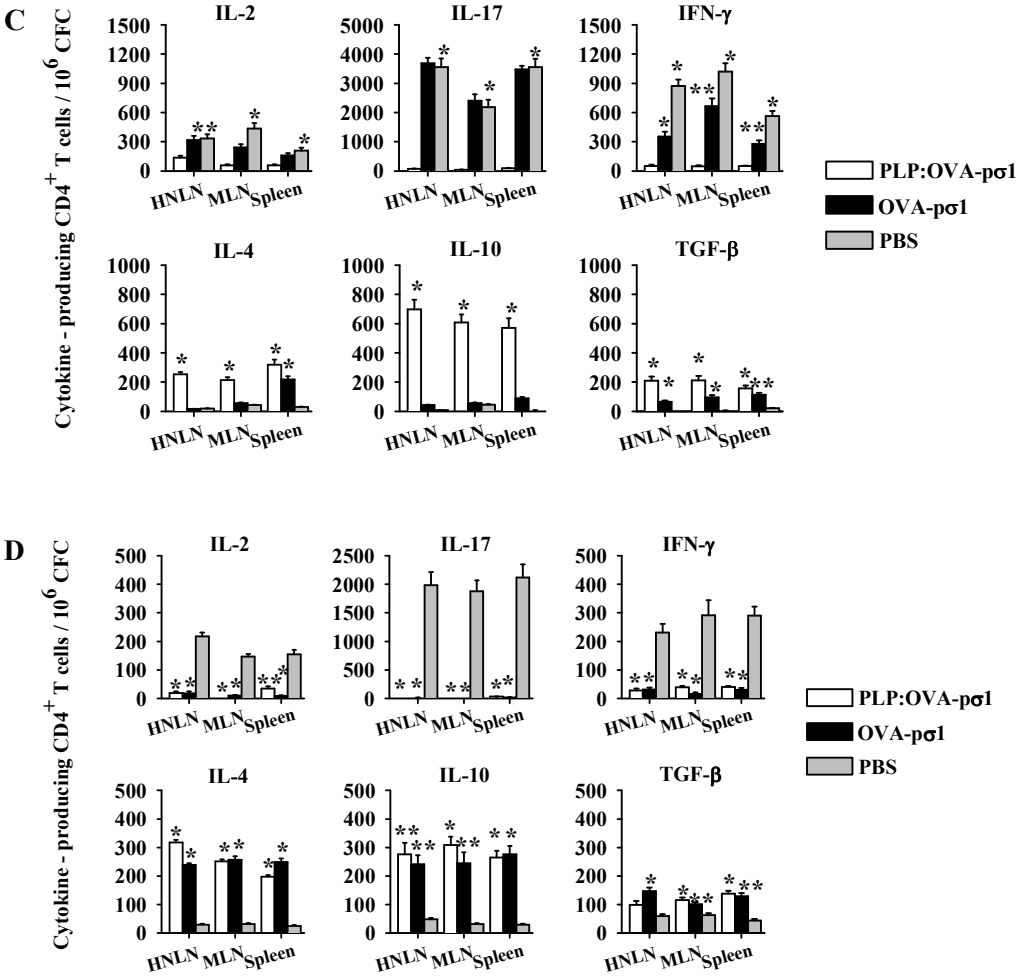


Figure 3.2. Nasal administration of PLP:OVA-pσ1 protects mice from clinical manifestation of EAE via inhibition of proinflammatory $CD4^+$ T cells and expansion of anti-inflammatory and regulatory $CD4^+$ T cell response. SJL mice were dosed with 100 μg of PLP:OVA-pσ1, OVA-pσ1 or PBS on day -21, -14 , and -7 relatively to the day of EAE induction. Mice were challenged with $PLP_{139-151}$ peptide on day 0 and monitored daily for the development of clinical disease. (*A*) PBS and OVA-pσ1-dosed mice developed fully pronounced EAE and never recovered completely. In contrast to these mice, PLP:OVA-pσ1-immunization prior to EAE induction significantly delayed and ameliorated clinical onset of the disease and all PLP:OVA-pσ1-dosed mice recovered completely from EAE. Average of 10-15 mice per group is shown. * $p < 0.05$ for PLP:OVA-pσ1 vs. PBS. (*B*) Anti-$PLP_{139-151}$ DTH response measured one week after EAE induction confirmed that unlike in PBS- or OVA-pσ1-dosed mice, $PLP_{139-151}$-specific Th1 response was diminished in PLP:OVA-pσ1-dosed mice. Mean ± SD of 10-15 mice per group is depicted. * $p <$ 0.001 for PLP:OVA-pσ1 vs PBS. *(C, D)* Mice were sacrificed at the peak of clinical disease and $CD4^+$ T cells from their HNLNs, MLNs and spleens were isolated, *in vitro* re-stimulated with 30 μg/ml of $PLP_{139-151}$ peptide (*C*) or with 1 mg/ml of OVA (*D*) and analyzed for the cytokine production. (*C*) $CD4^+$ T cells isolated from mice dosed with PBS or OVA-pσ1 at the peak of the clinical disease produced mostly proinflammatory cytokines (IL-2, IL-17, and IFN-γ).

Figure 3.2. – continued. In contrast to these mice, PLP:OVA-pσ1-immunization stimulated expansion of $CD4^+$ T cells that produced IL-10, IL-4, and TGF-β, but not inflammatory $CD4^+$ T cells. Results show mean ± SEM of 10-15 mice per group. *, $p < 0.001$; **, $p < 0.05$ for PLP:OVA-pσ1 vs. OVA-pσ1, and OVA-pσ1 vs. PBS (*D*) In contrast to $CD4^+$ T cells *in vitro* re-stimulated with $PLP_{139-151}$ peptide (*C*), $CD4^+$ T cells isolated from diseased OVA-pσ1-dosed mice produced anti-inflammatory cytokines in response to *in vitro* re-stimulation with OVA, and very little OVA-specific $CD4^+$ T cells that produced proinflammatory cytokines were detected in OVA-pσ1-dosed mice. Likewise, PLP:OVA-pσ1-protected mice displayed enhancement in $CD4^+$ T cells secreting anti-inflammatory cytokines, and no proinflammatory $CD4^+$ T cells were induced in these mice in response to OVA re-stimulation. In contrast to PLP:OVA-pσ1- and OVA-pσ1-dosed mice $CD4^+$ T cells isolated from PBS-dosed mice produced predominantly proinflammatory cytokines in response to *in vitro* stimulation with OVA. Results show mean ± SEM of 10-15 mice per group. *, $p < 0.001$; **, $p < 0.05$ for PLP:OVA-pσ1 or OVA-pσ1 vs. PBS.

Table 3.1. Nasal administration of PLP:OVA-pσ1 prior to $PLP_{139-151}$ challenge protects SJL mice from EAE[a]

Treatment[b]	EAE/Total[c]	Onset[d]	Max. score[e]	Cs[f]	Inflam[g]	Demyelin[h]
PBS	12/12	8.83 ± 1.7	5	55.29	2.4 ± 0.5	3.1 ± 0.4
PLP:OVA-pσ1	11/12	11.5 ± 1.1*	2	9.17*	0.5 ± 0.7*	0.7 ± 0.4*

[a] SJL mice were challenged s.c. with 200 μg $PLP_{139-151}$ in complete Freund's adjuvant plus 200 ng PT i.p. on days 0 and 2.
[b] Mice were nasally immunized 14 days prior to challenge with 100 μg of PLP:OVA-pσ1 or with PBS
[c] Number of mice with EAE/total in group.
[d] Mean day ± SD of clinical disease onset.
[e] Maximum (Max.) daily clinical score.
[f] Cumulative scores (CS) were calculated as the sum of all scores from disease onset to day 26 post-challenge, divided by the number of mice in each group. * $p < 0.001$ for PBS vs. PLP:OVA-pσ1-dosed mice.
[g] Mean score ± SEM of inflammation: the infiltration of nucleated cells into spinal cords was scored from 0 to 4 in each mouse separately, and the mean score and SEM were calculated. *, $p < 0.001$ for PBS vs. PLP:OVA-pσ1-dosed mice.
[h] Mean score ± SEM of demyelination: the demyelination in spinal cords was scored from 0 to 4 in each mouse separately, and the mean score and SEM were calculated. *, $p < 0.001$ for PBS vs. PLP:OVA-pσ1-dosed mice.

PLP:OVA-pσ1 Protects Mice from EAE via Induction of $PLP_{139-151}$-Specific Regulatory and Th2 Type Cytokines

Cytokine profiles of $CD4^+$ T cell isolated at the peak of the acute disease (day fourteen, p.ch.) from head and neck lymph nodes (HNLNs), mesenteric lymph nodes

(MLNs), and spleens of PLP:OVA-pσ1-, OVA-pσ1- , or PBS-dosed and challenged mice were analyzed after *in vitro* stimulation with $PLP_{139-151}$ peptide. In contrast to OVA-pσ1-dosed controls, mice dosed with PLP:OVA-pσ1 showed significant decreases of IL-2-producing $CD4^+$ T cells, over 8-fold reduction in IFN-γ-producing $CD4^+$ T cells, and virtual lack of IL-17-producing $CD4^+$ T cells (> 40-fold reduction) in all tested tissues (Figure 3.2*C*). When compared to PBS-dosed mice, ~ 2-fold reduction in IFN-γ-secreting $CD4^+$ T cells in HNLNs and spleens was observed in OVA-pσ1-dosed mice, but no differences in IL-2- and IL-17-producing $CD4^+$ T cells were detected between these PBS- and OVA-pσ1- dosed mice. In contrast to proinflammatory $CD4^+$ T cells, mice dosed with PLP:OVA-pσ1 showed strikingly raised IL-10-secreting $CD4^+$ T cells and significant enhancement in anti-inflammatory IL-4-, and TGF-β-producing $CD4^+$ T cells (Figure 3.2*C*). PLP:OVA-pσ1-dosed mice showed at least 8-fold increase in IL-10-producing $CD4^+$ T cells compared to OVA-pσ1- and PBS-dosed mice, and virtually no IL-10-producing $CD4^+$ T cells were evident by $PLP_{139-151}$ - stimulated $CD4^+$ T cells from OVA-pσ1- and PBS-dosed mice (Figure 3.2*C*). Compared to OVA-pσ1-dosed mice, a 4-fold increase in IL-4- and > 2-fold enhancement in TGF-β-secreting $CD4^+$ T cells were observed in the LNs of PLP:OVA-pσ1-dosed mice. In contrast to PBS-dosed mice, a significant increase was also detected in IL-4-producing $CD4^+$ T cells in spleens and TGF-β-producing $CD4^+$ T cells in all tested tissues of OVA-pσ1-dosed mice (Figure 3.2*C*). These results further suggest that PLP:OVA-pσ1-induced protection against

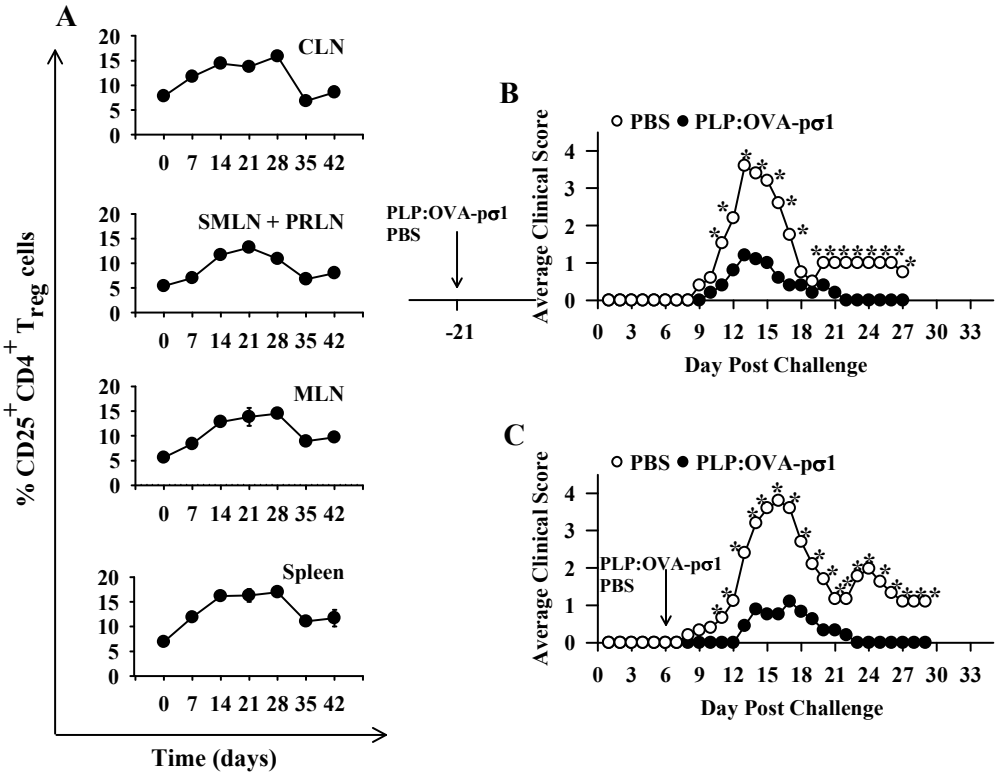
$PLP_{139-151}$-challenge is Ag-specific and is associated with increased production of regulatory cytokines and efficient suppression of inflammatory cytokines.

We have also assessed cytokine production by $CD4^+$ T cells isolated from PLP:OVA-pσ1-, OVA-pσ1-, or PBS-dosed and challenged mice after *in vitro* re-stimulation with OVA. $CD4^+$ T cells from mice protected by PLP:OVA-pσ1 produced anti-inflammatory cytokines also in response to OVA-re-stimulation (Figure 3.2*D*). Importantly, OVA-pσ1-dosed mice showed significant enhancement in $CD4^+$ T cells producing anti-inflammatory cytokines in response to OVA re-stimulation when compared to PBS-dosed mice (Figure 3.2*D*). No difference in numbers of $CD4^+$ T cells producing pro- or anti-inflammatory cytokines in response to *in vitro* re-stimulation with OVA were detected between PLP:OVA-pσ1- and OVA-pσ1-dosed mice, and these mice produced very little or no proinflammatory cytokines in response to OVA stimulation (Figure 3.2*D*). When compared to $CD4^+$ T cells from PBS-dosed mice, over 8-fold enhancement in IL-4-producing $CD4^+$ T cells, 5-fold rise in IL-10-producing $CD4^+$ T cells, and significant increase in $CD4^+$ T cells producing TGF-β was observed in PLP:OVA-pσ1- and OVA-pσ1-dosed mice. In contrast to PLP:OVA-pσ1- and OVA-pσ1-dosed mice, drastic increase in numbers of IL-17-producing $CD4^+$ T cells, over 4-fold enhancement in IL-2-producing $CD4^+$ T cells, and 7-fold raise in IFN-γ-secreting $CD4^+$ T cells was observed in PBS-dosed mice (Figure 3.2*D*). These results suggest that even though OVA-pσ1-dosed mice were not protected against the EAE challenge with $PLP_{139-151}$ peptide, OVA-specific tolerance was not compromised in these mice (Figure 3.2*D*).

Single Nasal Administration of
PLP:OVA-pσ1 Prevents and Treats against EAE

Our previous studies demonstrated the induction of nasal tolerance by OVA-pσ1 with as little as a single low dose of OVA-pσ1, and this was sufficient to generate a sustainable, long lasting tolerance that withstood a parenteral OVA challenge (89). To determine if a single dose of PLP:OVA-pσ1 could also protect mice against EAE, kinetics experiment was performed. SJL mice dosed with 100 μg of PLP:OVA-pσ1 on days 0, 7, and 14 were sacrificed after each PLP:OVA-pσ1-dose at seven day intervals, and FACS analysis was performed on the lymphocytes isolated from their cervical LNs (CLNs), submaxillary gland LNs (SMLNs), parotid gland LNs (PRLNs), and spleens. Even though the most pronounced enrichment in $CD25^+CD4^+$ T cells was observed after two nasal doses of PLP:OVA-pσ1 in all tested tissues (day 14), $CD25^+CD4^+$ T cells doubled after just after a single dose of PLP:OVA-pσ1 (day 7), except in SMLNs and PRLNs (Figure 3.3*A*). To test whether the ~ 2-fold enrichment of $CD25^+CD4^+$ T cells after a single nasal administration of PLP:OVA-pσ1 was sufficient to prevent and/or inhibit the inflammatory disease, SJL mice were dosed with a 100 μg of PLP:OVA-pσ1 or with PBS prior to EAE induction, or six days after challenge with $PLP_{139-151}$ (Figure 3.3*A* and *B*, Table 3.1). PBS-dosed control mice, displayed topical disease with the peak average clinical score of 4 independently of the day of PBS administration (Figure 3.3*A* and *B*). Additionally, all PBS-dosed mice developed clinical symptoms, and the maximal clinical score reached by these mice was 5 (Table 3.1). PLP:OVA-pσ1-protected and -treated mice showed ameliorated clinical manifestation of EAE, and at the peak of the

disease, the average clinical score ~ 1 in these mice. However, a significant difference was detected between mice dosed with 100 μg of PLP:OVA-pσ1 21 or 14 days prior to EAE induction (Figure 3.3*B* and Table 3.1). Administration of PLP:OVA-pσ1 on day -14 caused significant delay in the inauguration of clinical symptoms compared to PBS-dosed controls, whereas mice that received nasal administration of a vaccine or PBS on day - 21 developed EAE around nine days after challenge (Figure 3.3*B* and Table 3.1). Treatment of mice with PLP:OVA-pσ1 vaccine six days after EAE induction delayed the clinical onset of the disease and significantly ameliorated the symptoms, when compared to PBS-treated mice (Figure 3.3*C*). These results indicate that a single nasal dose of PLP:OVA-pσ1 given before EAE induction or during the pre-clinical stage of EAE is sufficient to protect mice from development of fully pronounced disease.



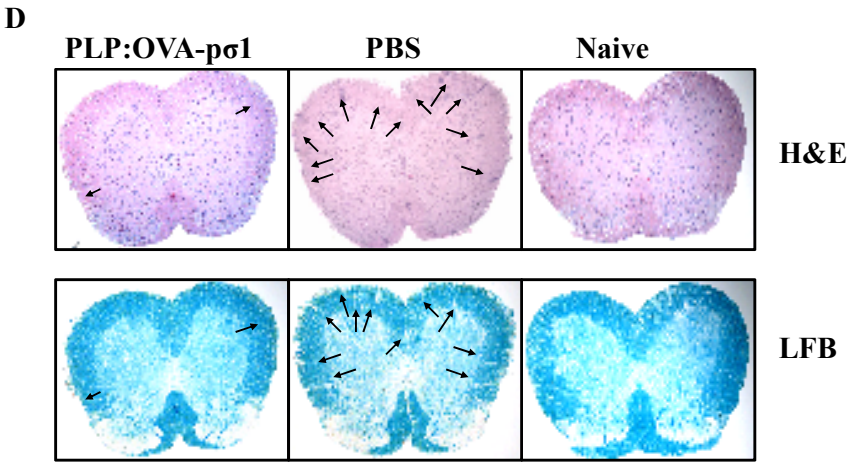


Figure 3.3. Single nasal dose of PLP:OVA-pσ1 protects mice from clinical EAE. (*A*) SJL mice were dosed with 100 μg of PLP:OVA-pσ1 up to 3 times in week intervals to determine optimal protective dose of PLP:OVA-pσ1. Three mice were sacrificed in seven day intervals starting on day 0 and lymphocytes isolated from their CLNs, SMLNs + PRLNs, MLNs, and spleens were analyzed by FACS for the percentages of $CD25^{+}CD4^{+}$ T_{reg} cells. (*B, C*) Mice were dosed with 100 μg of PLP:OVA-pσ1 or PBS on day -21 (*B*) or +6 (*C*) relative to the day of EAE induction. Unlike PBS, single dose administration of PLP:OVA-pσ1 before of after challenge with $PLP_{139-151}$ peptide significantly inhibited the occurrence and duration of the clinical EAE. *, $p < 0.05$ for PLP:OVA-pσ1vs.PBS. (*D*) Mice dosed with PLP:OVA-pσ1 or with PBS 21 d before EAE induction were sacrificed at the peak of the disease (day 14, p.ch) and histopathology of their spinal cords was determined by staining with LFB (demyelination) and H&E (mononuclear cell infiltration). Mice dosed with PLP:OVA-pσ1 showed significant reduction in the CNS tissue pathology (designated by arrows) comparing to PBS-dosed not protected mice. * $p < 0.001$ for PLP:OVA-pσ1 vs. PBS.

Nasally Delivered PLP:OVA-pσ1 Prevents from Neuropathology of EAE

The clinical manifestation of EAE is associated with autoreactive proinflammatory T lymphocytes, which during active phase of the disease infiltrate into the CNS and initiate inflammatory response accountable for the destruction of CNS myelin. SJL mice nasally dosed with 100 μg of PLP:OVA-pσ1 or with PBS were induced with EAE two to three weeks later. Mice were sacrificed at the peak of the clinical disease and evaluated for the CNS histopathology. Mice dosed with PLP:OVA-pσ1 prior to EAE induction showed minimal infiltration of mononuclear cell and

reduced demyelination compared to the PBS-dosed group ($p < 0.001$; Figure 3.3*D*, Table 3.1).

FACS analysis was performed on spinal cords' cells obtained from naive mice and from mice dosed with PLP:OVA-pσ1 or PBS and challenged with $PLP_{139-151}$ peptide. Consistent with the histological evaluation, mice dosed with PLP:OVA-pσ1 prior to EAE induction showed minimal infiltration of inflammatory cells (MHC $II^{+}CD45^{high}$) into the CNS, and in fact when compared with the naive controls, only a negligible percentage of macrophages (Mac-3^{+}) was able to penetrate the CNS in PLP:OVA-pσ1-dosed mice (Table 3.2). In contrast, PBS-dosed mice showed significant CNS infiltration with neutrophiles/granulocytes ($CD11b^{+}Gr\text{-}1^{+}$), lymphocytes ($CD4^{+}$ TCR-β^{+}), and macrophages (Mac-3^{+}) (Table 3.2). These results suggest that PLP:OVA-pσ1-mediated protection against EAE is due to suppression of encephalitogenic cell infiltration into the CNS.

Table 3.2. Nasal treatment with PLP:OVA-pσ1after EAE induction[a] reduce inflammatory cell infiltration[c] into the spinal cords

	MHC class II^+ $CD45^{high}$			
Treatment[b]	**% Infiltration**	**% $CD4^+TCR\text{-}\beta^+$**	**% $CD11b^+Gr\text{-}1^+$**	**% Mac-3^+**
Before EAE	0.94 ± 0.27	0.63 ± 0.06	0.56 ± 0.15	0.03 ± 0.02
PLP:OVA-pσ1	0.83 ± 0.2*	0.52 ± 0.09**	0.24 ± 0.06*	0.32 ± 0.14*
PBS	3.44 ± 0.4	2.78 ± 0.87	2.26 ± 0.18	1.54 ± 0.25

[a] SJL/J mice were challenged s.c. with 200 μg $PLP_{139\text{-}151}$ in complete Freund's adjuvant plus 200 ng PT i.p. on days 0 and 2.
[b] Mice were nasally treatment 6 days post-challenge with 100 μg of PLP:OVA-pσ1 or with PBS.
[c] Results are shown in percentage of MHC class II^+ $CD45^{high}$ cells from the total cells in spinal cords analyzed by FACS *, $p < 0.001$ **, $p < 0.05$ for PBS vs. PLP:OVA-pσ1-treated mice.

PLP:OVA-pσ1 Administration Significantly Enhances IL-10-Producing $CD25^+CD4^+FoxP3^+$ T_{reg} Cells and IL-4-Producing $CD25^-CD4^+FoxP3^+$ Th2 Cells that Inhibit Inflammatory $CD4^+$ T Cells

OVA-pσ1-induced tolerance was mediated by $CD25^+CD4^+FoxP3^+$ T_{reg} cells and supported by $CD25^-CD4^+FoxP3^+$ Th2 cells (89). To discern whether PLP:OVA-pσ1 induces enrichment of $CD4^+$ T regulatory cells in $PLP_{139-151}$-challenged mice, we analyzed $CD4^+$ T cells isolated from PLP:OVA-pσ1-protected and PBS-dosed diseased mice by FACS. As expected, nasal administration of PLP:OVA-pσ1 fourteen days before EAE induction induced major augmentation in $CD25^+CD4^+FoxP3^+$ T_{reg} cells and $CD25^-CD4^+FoxP3^+$ Th2 cells, when compared to PBS-dosed and challenged mice (Figure 3.4*A*). PLP:OVA-pσ1 induced > 25 % of $FoxP3^+$ T_{reg} cells and > 18 % of $FoxP3^+$ Th2 cells (Figure 3.4*A* and Table 3.3). In contrast, PBS-dosed mice showed only slight enrichment in $FoxP3^+CD25^+CD4^+$ T_{reg} cells (~10 %), but percentages of $FoxP3^+CD25^-CD4^+$ Th2 cells were not improved in these mice when compared to naive SJL mice (Figure 3.4*A* and Table 3.3). Unlike $CD4^+$ T cells from PBS-dosed mice that did not produce anti-inflammatory cytokines, over 85 % of PLP:OVA-pσ1-derived $CD25^+CD4^+FoxP3^+$ T_{reg} cells showed expression of IL-10, and almost as many $CD25^-CD4^+FoxP3^+$ Th2 cells obtained from these mice produced IL-4 (Figure 3.4*A* and *B* and Table 3.3). Production of IL-4 and IL-10 was not different between PLP:OVA-pσ1- and PBS-derived $CD25^+$ T_{reg} cells and PLP:OVA-pσ1- and PBS-derived $CD25^-$ Th2 cells, respectively, although PLP:OVA-pσ1-primed $CD25^-FoxP3^+$ Th2 cells produced

significantly more TGF-β than the $CD25^{-}FoxP3^{+}$ Th2 cells isolated from PBS-dosed mice (Figure 3.4*A*, and Table 3.3).

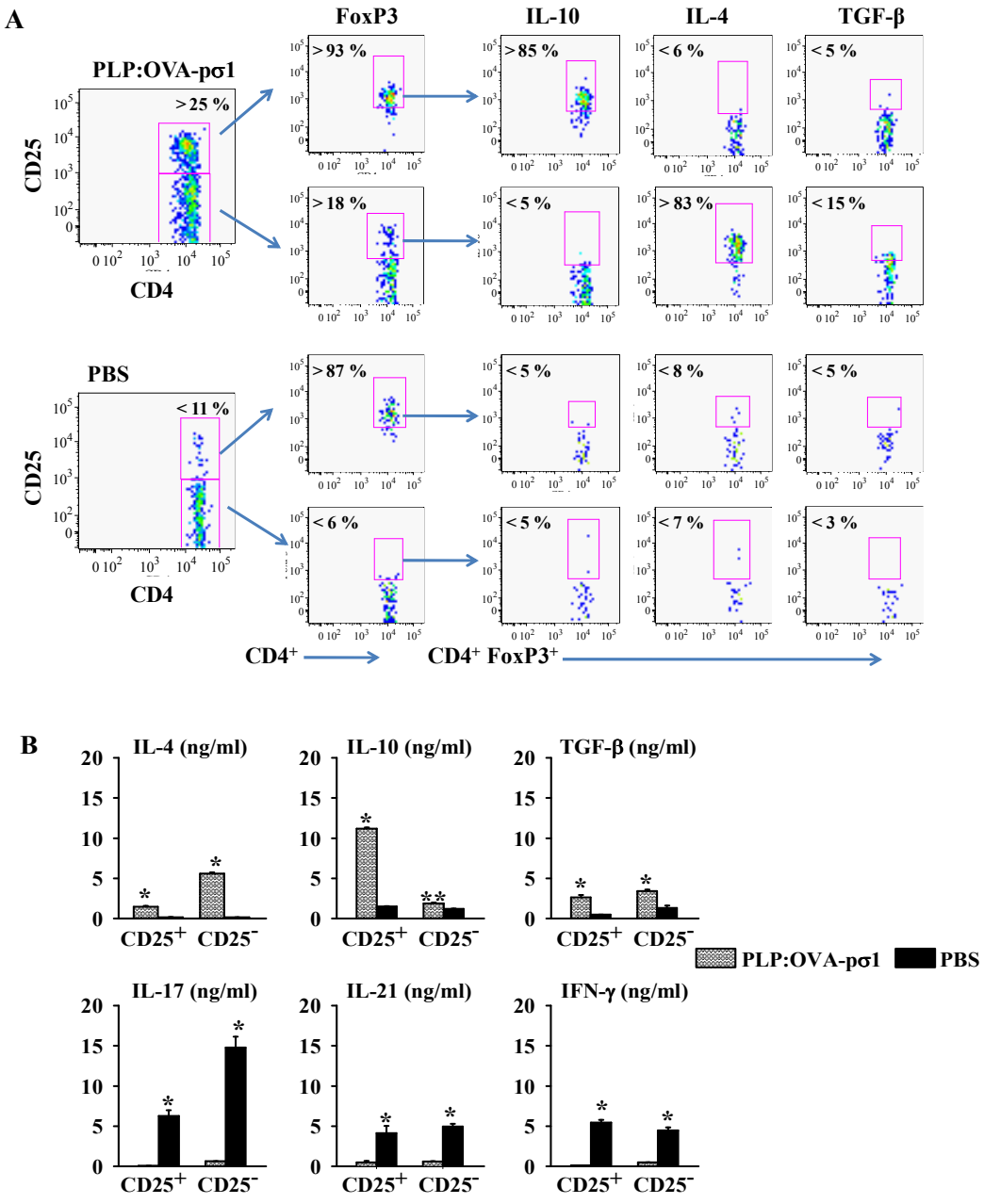


Figure 3.4. PLP:OVA-pσ1 induces expression of IL-10-producing $CD25^{+}CD4^{+}FoxP3^{+}$ T_{reg} cells and IL-4-producing $CD25^{-}CD4^{+}FoxP3^{+}$ Th2 cells. SJL mice were dosed with 100 µg of PLP:OVA-pσ1 or with PBS fourteen days before challenge with $PLP_{139-151}$ peptide.

Figure 3.4. – continued. (*A*) Lymphocytes from HNLNs and spleens were isolated, cultured with 30 μg /ml of $PLP_{139-151}$ for 3 d, and stained for expression of extracellular (CD25, CD4 and TGF-β), and intracellular markers (FoxP3, IL-10 and IL-4). Presented FACS plots show cells isolated from spleens. (*B*) $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were beads-sorted from combined HNLN-, MLN- and spleen-lymphocytes and *in vitro* stimulated with plate bound anti-CD3 and soluble anti-CD28 for 72 h. Collected supernatants were analyzed by cytokine ELISA. Unlike PBS-dosed mice, PLP:OVA-pσ1- administration induced significant enrichment in $FoxP3^+CD4^+$ $CD25^+$ T_{reg} cells and $CD25^-$ Th2 cells in mice. In contrast to PBS-derived $CD4^+$ T cells that produced mostly proinflammatory cytokines, $CD25^+CD4^+$ $FoxP3^+$ T_{reg} cells from PLP:OVA-pσ1-dosed mice produced predominantly IL-10, whereas PLP:OVA-pσ1-derived $CD25^-CD4^+FoxP3^+$ Th2 cells produced IL-4. Negligible amounts of proinflammatory cytokines were secreted by $CD4^+$ T cells isolated from PLP:OVA-pσ1-dosed and challenged mice. Mean ± SEM of 10 mice/group is depicted. *, $p < 0.05$ for PLP:OVA-pσ1 vs. PBS.

To further determine cytokine profiles of $CD4^+$ T cells obtained from PLP:OVA-pσ1 or PBS-dosed and challenged mice, supernatants isolated from the bead-selected $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were analyzed by ELISA. Consistent with the FACS analysis, $CD25^+CD4^+$ T_{reg} cells acquired from PLP:OVA-pσ1-protected mice produced mostly IL-10, and $CD25^-CD4^+$ T cells obtained from these mice secreted a majority of IL-4. Additionally, more IL-4 and TGF-β were produced by PLP:OVA-pσ1-derived $CD4^+$ T cells (either $CD25^-$ and $CD25^+$) than by the same cells from PBS-dosed diseased mice (Figure 3.4*B*, Table 3.3). Proinflammatory cytokines, IL-17, IL-21, and IFN-γ were primarily secreted by PBS-derived $CD25^-CD4^+$ and $CD25^+CD4^+$ T cells and in nominal amounts by $CD25^-CD4^+$ T cells acquired from PLP:OVA-pσ1-dosed mice (Figure 3.4*B*). Consistent with the mechanism of pσ1-mediated tolerance to OVA (89), nasal administration of PLP:OVA-pσ1 induced Ag-specific IL-10-producing $CD25^+CD4^+FoxP3^+$ T_{reg} cells and IL-4-secreting $CD25^-CD4^+FoxP3^+$ Th2 cells.

Table 3.3. Characterization of $CD4^+$ T cells from combined LNs and spleens of naïve, PBS- and PLP:OVA-pσ1-dosed SJL mice[a]

T cells	PLP:OVA-pσ1	PBS	Naïve
$CD25^+CD4^+$	25.10 ± 1.62*	10.69 ± 1.04	5.50 ± 0.68
$FoxP3^+$	93.10 ± 1.44*	71.55 ± 2.45	43.54 ± 3.72
TGF-β^+	7.09 ± 0.74	4.94 ± 1	4.42 ± 0.72
$GITR^+$	19.52 ± 1.3*	29.91 ± 0.97	34.54 ± 2.79
$CCR6^+$	67.88 ± 1.57*	25.52 ± 2.21	22.26 ± 1.92
CTLA-4^+	48.41 ± 5.34**	33.03 ± 3.52	27.72 ± 2.32
$ICOS^+$	20.54 ± 1.33	22.17 ± 1.81	22.44 ± 1.54
IL-10^+	86.35 ± 2.25*	17.02 ± 1.34	9.51 ± 1.19
IL-4^+	7.31 ± 0.88	6.29 ± 1.08	4.86 ± 1.01
OX-40^+	4.68 ± 0.75	4.42 ± 0.96	2.58 ± 0.38
$CD25^-CD4^+$			
$FoxP3^+$	19.89 ± 1.08*	7.84 ± 0.81	5.65 ± 0.34
TGF-β^+	13.44 ± 1.04*	4.02 ± 0.81	1.62 ± 0.42
$GITR^+$	17.49 ± 1.71**	11.77 ± 1.45	7.36 ± 0.43
$CCR6^+$	21.42 ± 2.39**	14.76 ± 1.94	9.35 ± 1.18
CTLA-4^+	5.54 ± 0.35	6.09 ± 0.8	4.41 ± 0.31
$ICOS^+$	6.51 ± 0.21**	8.37 ± 0.63	8.7 ± 0.86
IL-10^+	6.28 ± 1.11	6.98 ± 1.09	6.01 ± 0.98
IL-4^+	83.83 ± 2.64*	4.99 ± 0.54	5.98 ± 0.54
OX-40^+	6.85 ± 0.95*	34.73 ± 1.18	2.91 ± 0.5

[a] Mean ± SEM of 10 mice per group is presented. Percentages of $CD25^+CD4^+$ T_{reg} cell and $CD25^-CD4^+$ Th2 cell analyzed by FACS (Figure 3.4*A*).

*, $p < 0.001$, ** $p < 0.05$ for PBS vs. PLP:OVA-pσ1-dosed mice.

In Vitro Neutralization of IL-10 and IL-4 Reverses PLP:OVA-pσ1-Mediated Suppression of Proliferation of $PLP_{139-151}$-Stimulated $CD4^+$ T Cells

$CD4^+$ T cells obtained from mice given nasally PLP:OVA-pσ1 produced predominantly IL-10 and IL-4. We wanted to determine if these cytokines could alter proliferation of $PLP_{139-151}$-specific $CD4^+$ T cells *in vitro*. SJL mice induced with EAE were treated on day 6, p.ch with 100 μg of PLP:OVA-pσ1 or with PBS. Mice were sacrificed at the peak of the disease (14 d, p. ch), and isolated splenic $CD4^+$ T cells were cultured for 48 h with 30 μg/ml of $PLP_{139-151}$ peptide and one of the following Abs: anti-IL-10 mAb, anti-IL-4 mAb, or with rat IgG. *In vitro* proliferation of cultured $CD4^+$ T cells was measured 16 h after addition of [^{3}H] TdR to the culture plates. $PLP_{139-151}$-specific proliferation of the $CD4^+$ T cells isolated from PBS-dosed mice was not inhibited by co-culturing with anti-IL-10 or anti-IL-4 Abs (Figure 3.5*A*). In contrast to these mice, $CD4^+$ T cells obtained from PLP:OVA-pσ1-treated mice did not proliferate when incubated with $PLP_{139-151}$ peptide alone, or in combination with rat IgG. Co-culture of PLP:OVA-pσ1-derived $CD4^+$ T cells with $PLP_{139-151}$ peptide and anti-IL-10 mAbs completely restored proliferation of $CD4^+$ T cells inhibited by PLP:OVA-pσ1 (Figure 3.5*A*). Additionally, when PLP:OVA-pσ1-derived $CD4^+$ T cells were co-cultured with anti-IL-4 mAbs, the PLP:OVA-pσ1-induced inhibition of proliferation was also partially annulled. Therefore, IL-10 and IL-4 are important for PLP:OVA-pσ1-mediated suppression of $PLP_{139-151}$-specific $CD4^+$ T cells.

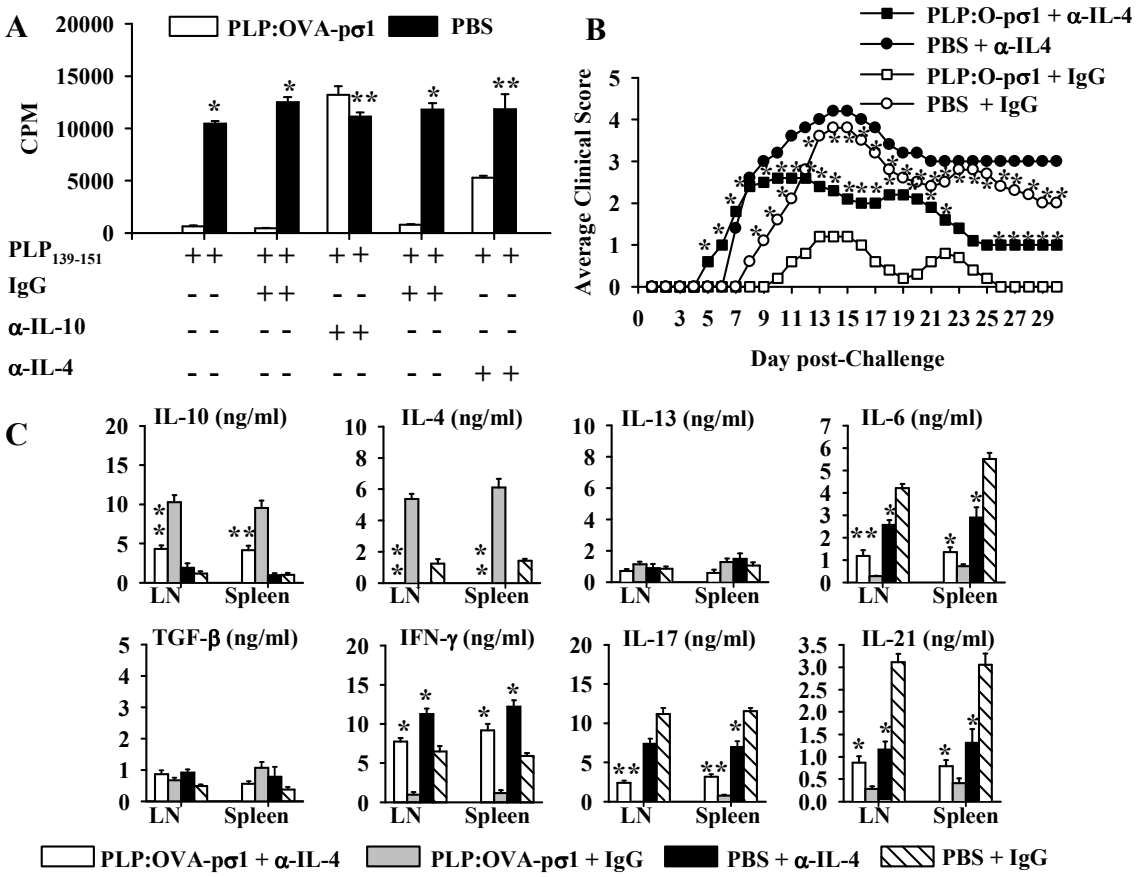


Figure 3.5. IL-10 and IL-4 are important for PLP:OVA-pσ1-mediated protection against EAE. (*A*) SJL mice were induced with EAE and 6 d later were treated with 100 μg of nasally given PLP:OVA-pσ1 or with PBS. Splenic $CD4^+$ T cells were isolated from mice at the peak of the disease and cultured for ~ 66 h in 1:1 ratio with the splenic feeder cells, $PLP_{139-151}$ peptide, and either anti-IL-4 mAb, anti-IL-10 mAb or with rat IgG. During the last 18 h of cultures cells were pulsed with [^{3}H] TdR. Results depict proliferation of cultured cells corrected over the proliferation of unstimulated $CD4^+$ T cell. Proliferation of $CD4^+$ T cells isolated from PBS-dosed mice was not inhibited by co-incubation with neutralizing mAbs. In contrast to these cells, $CD4^+$ T cells obtained from PLP:OVA-pσ1-treated mice did not proliferate in the response to $PLP_{139-151}$ peptide unless when treated with anti-IL10 or anti-IL-4 mAbs. *, $p < 0.001$; **, $p < 0.05$ for PLP:OVA-pσ1 vs. PBS. (*B, C*) Mice dosed with PLP:OVA-pσ1 or PBS on day -21 and -14 were i.p. injected with 1 mg of anti-IL-4 mAb or rat IgG on day -1 and +5 relatively to the day of challenge with $PLP_{139-151}$ peptide. (*B*) Mice dosed with PLP:OVA-pσ1 + IgG developed ameliorated and delayed EAE and all recovered from the disease. PBS + anti-IL-4-dosed mice developed EAE that started earlier and was more severe than in PBS + IgG-dosed mice. The disease course of mice dosed with PLP:OVA-pσ1 + anti-IL-4 mAbs was less rigorous than PBS + IgG-dosed mice, but significantly more severe when compared to PLP:OVA-pσ1 + IgG-dosed mice. *, $p < 0.05$ for PLP:OVA-pσ1 + IgG vs. PBS + IgG or PLP:OVA-pσ1+ anti-IL-4. (*C*) $CD4^+$ T cells isolated from HNLNs, MLNs, and spleens of these mice were incubated with feeder cells and $PLP_{139-151}$ peptide for 72 h. Cultured supernatants were analyzed for cytokine production by ELISA. Results show cytokine production by cultured $CD4^+$ T cells corrected over the cytokine production by unstimulated cells. PBS and PLP:OVA-pσ1-dosed mice treated with anti-IL-4 produced more IFN-γ and less IL-10 than their respective IgG-treated controls.

Figure 3.5. – contunied. PLP:OVA-pσ1 + anti-IL-4-dosed mice produced less IL-10 and more IFN-γ, IL-6 and IL-17 than PLP:OVA-pσ1 + IgG-dosed mice. *, $p < 0.05$ for PLP:OVA-pσ1 + anti-IL-4 vs. PBS + IgG or PLP:OVA-pσ1+ IgG, and PBS + IgG vs. PBS + anti-IL-4.

In Vivo Neutralization of IL-4 Partially Reverses PLP:OVA-pσ1-Mediated Protection against EAE Due to Induction of Proinflammatory Cytokines

To test whether the presence of IL-4 is required for PLP:OVA-pσ1-mediated protection against EAE, 11B11 mAb was used to neutralize *in vivo* IL-4. SJL mice dosed with 100 μg of PLP:OVA-pσ1 or with PBS on day -14 and -7 were injected intraperitoneally (i.p.) with 1 mg of anti-IL-4 mAb or rat IgG on day -1 and +5 relatively to the day of EAE induction, as described (145). Consistent with our previous results, mice dosed with PBS and injected i.p. with rat IgG developed typical EAE onset that started around day 8, p.ch, with the peak of the disease at day 14, p.ch, and the average peak clinical score of 4 (Figure 3.5*B*). Interestingly, *in vivo* neutralization of IL-4 accelerated the initiation of clinical disease and amplified the severity of symptoms in PBS-dosed mice (Figure 3.5*B* and Table 3.4). Vaccine-protected mice injected i.p. with IgG developed significantly delayed and less severe EAE, typically seen in PLP:OVA-pσ1-dosed mice. However, when mice given nasally PLP:OVA-pσ1 were also injected with anti-IL4 mAbs, the clinical disease began significantly earlier and was more severe than in PLP:OVA-pσ1 + IgG-dosed mice, but less pronounced than in PBS + IgG-dosed control mice (Figure 3.5*B* and Table 3.4).

Table 3.4. *In vivo* neutralization of IL-4 partially reverse PLP:OVA-pσ1-mediated protection against EAE[a]

Treatment[b]	EAE/Total[c]	Onset[d]	Max. score[e]	CS[f]
PBS + IgG	10/10	8.1 ± 0.87*	5	47.2
PLP:OVA-pσ1 + IgG	9/10	10.9 ± 1.19	2	10.4
PBS + anti-IL4	10/10	7.7 ± 1.63*	5	62.6
PLP:OVA-pσ1 + anti-IL4	10/10	6.6 ± 1.07*	5	40.4

[a] SJL/J mice were challenged s.c. with 200 μg $PLP_{139-151}$ in complete Freund's adjuvant plus 200 ng PT i.p. on days 0 and 2.
[b] Mice were nasally immunized 21 and 14 days prior to EAE challenge with 100 μg of PLP:OVA-pσ1 or with PBS (Figure 3.5*B* and *C*)
[c] Number of mice with EAE/total in group.
[d] Mean day ± SD of clinical disease onset.
[e] Maximum (Max.) daily clinical score.
[f] Cumulative scores (CS) were calculated as the sum of all scores from disease onset to day 26 post-challenge, divided by the number of mice in each group. *, $p < 0.001$ **, $p < 0.05$ for PBS vs. PLP:OVA-pσ1-dosed mice.

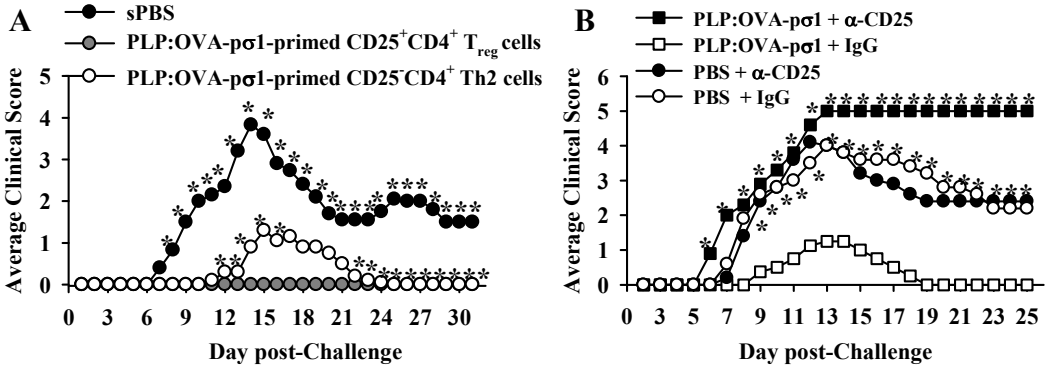
Cytokine profiles of $CD4^+$ T cells isolated from these mice at the peak of the disease revealed enhanced production of proinflammatory cytokines, IFN-γ, IL-6, and IL-17, and reduced production of IL-10 by anti-IL-4 mAb-treated PLP:OVA-pσ1-dosed mice when compared to PLP:OVA-pσ1 + IgG-dosed mice (Figure 3.5*C*). Production of IL-21 was induced in PLP:OVA-pσ1 + anti-IL-4 mAb-treated mice when compared to PLP:OVA-pσ1 + IgG-dosed mice, though this increase was not statistically significant. However, in contrast to PBS + IgG-dosed mice, anti-IL-4 mAb-treated PLP:OVA-pσ1-dosed mice produced significantly more IL-10, and considerably less IL-6, IL-17, IL-21, but not IFN-γ (Figure 3.5*C*). Finally, PBS-dosed, anti-IL-4 mAb-treated mice produced less IL-6 and IL-21 in all tested tissues, and less IL-17 in spleens than PBS + IgG-dosed

mice (Figure 3.5*C*). Additionally, more IFN-γ was produced by PBS + anti-IL-4 mAb-dosed mice when compared to mice dosed with PBS + IgG (Figure 3.5*C*). There was no difference in production of IL-13 and TGF-β between all tested groups. Overall, *in vivo* neutralization of IL-4 in either PBS- or PLP:OVA-pσ1-dosed mice induced more IFN-γ when compared to their respective IgG-injected control mice, and significantly less of IL-10 was produced in anti-IL-4 mAb-treated PLP:OVA-pσ1-dosed mice than in PLP:OVA-pσ1-protected mice.

Adoptive Transfer of PLP:OVA-pσ1-Derived T_{reg} Cells Entirely Protects Mice against EAE, but Adoptive Transfer of PLP:OVA-pσ1-Primed $CD25^-CD4^+$ T Cells Only Ameliorates Clinical Manifestation of EAE

PLP:OVA-pσ1-mediated protection against EAE is associated with induction of $FoxP3^+$ T_{reg} cells and $FoxP3^+$ $CD25^-CD4^+$ Th2 cells that produce IL-10 and IL-4, respectively. Neutralization of IL-10 *in vitro* and neutralization of IL-4 *in vitro* and *in vivo* reversed PLP:OVA-pσ1-mediated inhibition of a $PLP_{139-151}$-specific immune response, and PLP:OVA-pσ1-induced protection against EAE. To determine how important these $CD4^+$ T cell subsets are for PLP:OVA-pσ1-induced protection against EAE, an adoptive transfer study was performed. SJL mice were dosed with 100 μg or PLP:OVA-pσ1 on days 0 and 7 for optimal induction of T_{reg} cells, and $CD4^+$ T cell subsets were isolated 2 wks later. PLP:OVA-pσ1-primed bead sorted $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells (6×10^5 each) were adoptively transferred via intravenous (i.v.) injection into naive SJL recipient mice, and 24 h later, mice were induced with EAE.

Adoptive transfer of PLP:OVA-pσ1-derived T_{reg} cells fully protected recipient mice against EAE, since these mice never developed any clinical symptoms (Figure 3.6*A*). Interestingly, transfer of PLP:OVA-pσ1-primed $CD25^{-}CD4^{+}$ T cells significantly ameliorated and delayed the disease onset in recipient mice. EAE in $CD25^{-}CD4^{+}$ -transferred mice developed on day 11, p.ch, and the average clinical score at the peak of the disease was 1.5 in this group. In contrast to PBS-dosed mice, mice transferred with PLP:OVA-pσ1-derived $CD25^{-}CD4^{+}$ T cells recovered entirely from the acute disease and did not relapse. Consistent with previous results, PBS-dosed mice developed EAE about day 7, p.ch with severity of the disease increasing until day 14, p.ch when the average clinical score reached 4. Also, PBS-dosed mice never recovered completely from the relapsing-remitting disease (Figure 3.6*A*). These results clearly indicate the pivotal role for PLP:OVA-pσ1-derived T_{reg} cells in establishing protection against EAE, and highlight the supportive role of PLP:OVA-pσ1-induced Th2 cell bias in this process.



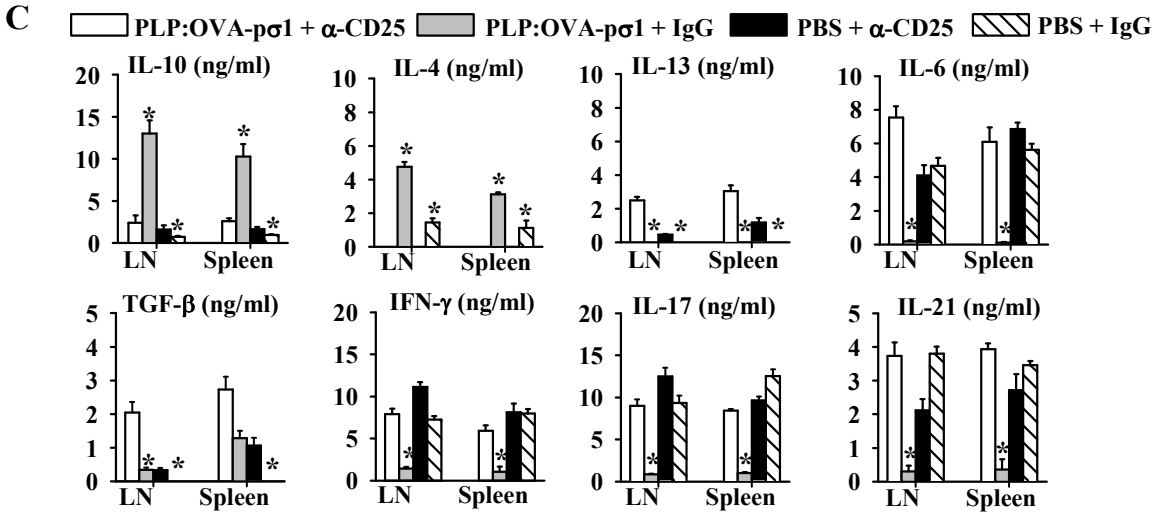


Figure 3.6. Functional $CD25^+CD4^+$ T_{reg} cells are required for PLP:OVA-pσ1-induced protection against EAE. (*A*) SJL mice dosed nasally with 100 μg of PLP:OVA-pσ1 on day 0 and 7, were sacrificed on day 14. 6 x 10^5 of $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells were isolated from these mice and adoptively transferred into naive SJL recipient mice that were induced with EAE 24 h later. Adoptive transfer of PLP:OVA-pσ1-primed $CD25^+CD4^+$ T_{reg} cells entirely protected mice from development of clinical EAE, but transfer of PLP:OVA-pσ1-derived $CD25^-CD4^+$ Th2 cells also significantly delayed and improved severity of EAE. Averaged clinical scores from 2 independent experiments (10 mice per group) are shown. *, $p < 0.05$ for the statistical differences vs. PBS-dosed mice. (*B, C*) SJL mice nasally dosed with 100 μg of PLP:OVA-pσ1 or with PBS on day -14 and -7 were i.p. injected with 0.5 mg of anti-CD25 mAbs or rat IgG on day -5 and -2 relatively to the day of EAE induction. (*B*) Clinical disease in PBS-dosed mice was not affected by administration of anti-CD25 mAbs. However, PLP:OVA-pσ1-dosed mice functionally depleted of $CD25^+CD4^+$ T_{reg} cells developed extremely rigorous EAE and all of these mice died at the peak of the disease. Averaged clinical scores from 2 independent experiments (10 mice per group) are shown. *, $p < 0.05$ for the difference between PLP:OVA-pσ1 + anti-CD25- and PBS + IgG- vs. PLP:OVA-pσ1 + IgG-treated mice. (*C*) $CD4^+$ T cells isolated from HNLNs, MLNs and spleens were cultured with feeder cells and $PLP_{139-151}$ peptide for 72 h. Production of cytokines by unstimulated $CD4^+$ T cells set as a background was subtracted from the measurements. Functional inactivation of T_{reg} cells in PLP:OVA-pσ1-dosed mice resulted in induction of proinflammatory $CD4^+$ T cells. Importantly, $CD4^+$ T cells obtained from PLP:OVA-pσ1-dosed anti-CD25-treated mice produced more IL-13 in LNs and spleens and TGF-β in LNs than any other experimental group. Mean ± SEM from 5 mice per group is shown. *, $p < 0.05$ for PLP:OVA-pσ1 + anti-CD25 vs. PLP:OVA-pσ1 + IgG and PBS + IgG.

Functional Inactivation of $CD25^+$ Cells *In Vivo* Entirely Abrogates PLP:OVA-pσ1-Mediated Protection against EAE

To further investigate the requirement for $CD25^+CD4^+$ T_{reg} cells in PLP:OVA-pσ1-induced protection against EAE, SJL mice dosed with PLP:OVA-pσ1 or PBS were *in vivo* treated with blocking mAbs to CD25 prior to EAE induction. *In vivo* depletion of

functional $CD25^+CD4^+$ cells did not affect the disease course in PBS-dosed mice, when compared to PBS + IgG-dosed mice (Figure 3.6*B*). EAE in mice dosed with PLP:OVA-pσ1 + IgG developed significantly later, around day 9, p.ch, and was considerably milder than in PBS + IgG-dosed mice. Anti-CD25 mAb-treatment of PLP:OVA-pσ1-dosed mice significantly accelerated the development of EAE (Figure 3.6*B*). Importantly, EAE was drastically more severe in PLP:OVA-pσ1-dosed, anti-CD25 mAb-treated mice than in any other tested group, and all PLP:OVA-pσ1 + anti-CD25 mAb-dosed mice died at the peak of the disease (Figure 3.6*B*).

Next we analyzed the cytokine production by $CD4^+$ T cell isolated from HNLNs, MLNs and spleens of PLP:OVA-pσ1- or PBS-dosed mice treated with anti-CD25 mAbs. PBS-dosed mice treated with anti-CD25 mAbs or with IgG developed classic proinflammatory response, and $CD4^+$ T cells isolated from these mice produced drastically more IFN-γ, IL-6, IL-17, and IL-21, and considerably less IL-10 and IL-4 compared to PLP:OVA-pσ1 + IgG-dosed mice (Figure 3.6*C*). Unlike PLP:OVA-pσ1 + IgG-treated mice that produced significant amounts of IL-10, IL-4, and almost no proinflammatory cytokines, $CD4^+$ T cells obtained from mice dosed with PLP:OVA-pσ1 and treated with anti-CD25 mAbs produced 3-fold less IL-10 and no IL-4. Additionally, anti-CD25 mAb-treated, PLP:OVA-pσ1-dosed mice produced 5-fold more of IFN-γ, 8-fold more of IL-17, and over 10-fold more of IL-21 and IL-6 than PLP:OVA-pσ1 + IgG-dosed mice (Figure 3.6*C*). Interestingly, $CD4^+$ T cells acquired from mice treated with PLP:OVA-pσ1 + anti-CD25 mAbs produced notably more of TGF-β in LNs and IL-13 in all tested tissues compared to any other tested group (Figure 3.6*C*). These results further

indicate that PLP:OVA-pσ1-mediated protection against EAE is critically dependent on the presence of PLP:OVA-pσ1-induced $CD25^+$ T_{reg} cells. Moreover, lack of protection in PLP:OVA-pσ1-dosed mice functionally depleted of T_{reg} cells is associated with enhanced proinflammatory cytokines but also correlates with increased production of IL-13 and TGF-β.

Combined Inactivation of TGF-β
and CD25 Restores PLP:OVA-pσ1-Mediated
Protection against EAE

PLP:OVA-pσ1-dosed mice injected i.p. with anti-CD25 mAb developed very severe clinical manifestation of EAE. On the other hand, $CD4^+$ T cells obtained from these mice 10 d p.ch revealed significantly increased production of IL-13 and TGF-β in addition to proinflammatory cytokines, IFN-γ, IL-6, IL-17, and IL-21. Taking advantage of the fact that PLP:OVA-pσ1 induces tolerance to $PLP_{139-151}$ peptide but also to OVA, we wanted to determine if TGF-β and/or IL-13 can support OVA-specific proliferation of DO11.10 $CD4^+$ T cells incubated with PLP:OVA-pσ1 and anti-CD25 mAb. OVA-Tg $CD4^+$ T cells isolated from spleens of naive DO11.10 mice were cultured *in vitro* with PLP:OVA-pσ1 and various combinations: anti-TGF-β mAb, anti-IL-13 neutralizing pAb, and/or anti-CD25 mAb, or with the appropriate isotype control Abs. During the last 18 h of cultures, [^{3}H] TdR was added, and proliferation of OVA- Tg $CD4^+$ T cells was determined. PLP:OVA-pσ1-suppressed proliferation of OVA-Tg $CD4^+$ T cells was reversed by incubation of these cells with anti-CD25 mAbs (Figure 3.7*A*). Co-culture of PLP:OVA-pσ1-stimulated cells with neutralizing Abs to IL-13 and/or TGF-β did not

alter PLP:OVA-pσ1-mediated suppression of $CD4^+$ T cells proliferation. However, simultaneous inactivation of TGF-β and CD25, or TGF-β, IL-13, and CD25 in PLP:OVA-pσ1-stimulated cultures re-established PLP:OVA-pσ1-mediated inhibition of proliferation of the Tg $CD4^+$ T cells (Figure 3.7*A*). In contrast to concurrent inactivation of CD25 and TGF-β, synchronized blockade of IL-13 and CD25 did not reverse anti-CD25 mAb-mediated proliferation of PLP:OVA-pσ1-stimulated Tg $CD4^+$ T cells (Figure 3.7*A*).

Next we wanted to determine if *in vivo* co-administration of anti-TGF-β mAb and anti-CD25 mAbs would re-establish PLP:OVA-pσ1-mediated protection against EAE. SJL mice nasally given PLP:OVA-pσ1 or PBS were injected i.p. with anti-TGF-β mAb and anti-CD25 mAbs or with the isotype control Abs (Figure 3.7*B*). No difference in onset and severity of clinical EAE was observed between mice dosed with PBS and injected i.p. with different combinations of mAbs (Figure 3.7*C* - bottom panel). However, mice dosed with PBS + anti-TGF-β mAb remitted further from the acute disease (Figure 3.7*C* - bottom panel). PLP:OVA-pσ1-dosed mice injected i.p. with anti-CD25 mAbs developed very aggressive disease, and all mice died at the peak of clinical onset (Figure 3.7*C* - upper panel). In contrast to PLP:OVA-pσ1 + anti-CD25 mAb-dosed mice, mice given PLP:OVA-pσ1 + IgG or PLP:OVA-pσ1 + anti-TGF-β mAbs developed very mild disease with the average peak clinical score of ~ 1.5; all of these mice recovered from acute EAE. Interestingly, clinical appearance of EAE in PLP:OVA-pσ1-dosed mice treated with combination of anti-CD25 mAb and anti-TGF-β mAb resembled

ameliorated disease seen in PLP:OVA-pσ1 + IgG-dosed mice (Figure 3.7*C* - upper panel).

Therefore, we propose that a very aggressive clinical onset of EAE observed in mice nasally dosed with PLP:OVA-pσ1 and functionally depleted of $CD25^+$ T_{reg} cells is due to uncontrolled induction of proinflammatory Th17 cells, suggesting that our model is critically dependent on the presence of TGF-β.

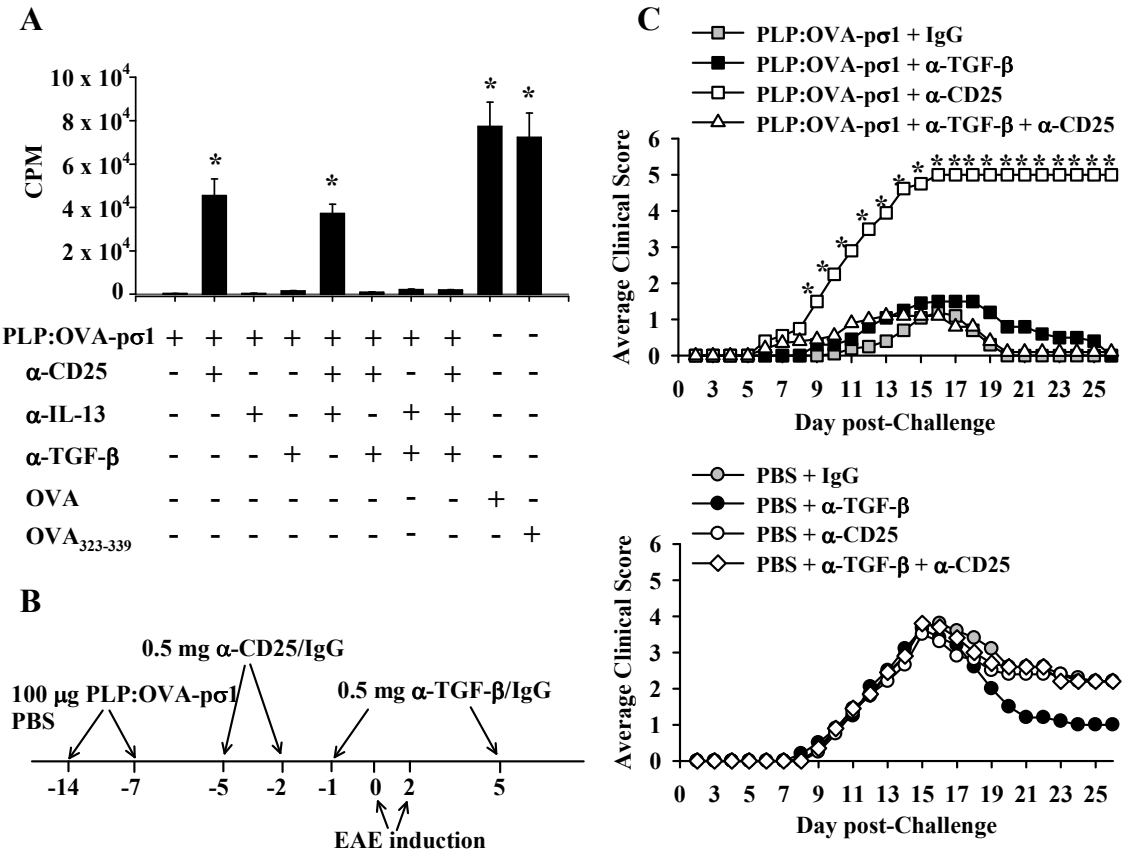


Figure 3.7. Eradicated protection against EAE in anti-CD25-treated PLP:OVA-pσ1-dosed mice is restored by neutralization of TGF-β *in vivo*. (*A*) Splenic $CD4^+$ T cells isolated from naive DO11.10 OVA Tg mice were cultured for a total of 66 h with 1:1 ratio of feeder cells, 50 µg/ml of PLP:OVA-pσ1 and with Abs to CD25 (10 µg/ml), IL-13 (20 µg/ml) and/or TGF-β (20 µg/ml) alone or in amalgamation. Wells containing Tg $CD4^+$ T cells and feeder cells stimulated with 1 mg/ml of OVA or 1 µg/ml of $OVA_{323-339}$ peptide were set for positive proliferation control. During the last 18 h of cultures cells were pulsed with [^{3}H] TdR.

Figure 3.7. – continued. Results depict proliferation of cultured cells corrected over the proliferation of unstimulated Tg $CD4^+$ T cell, and cell cultured with isotype control Abs. PLP:OVA-pσ1-stimulated Tg $CD4^+$ T cells cultured alone or in combination with anti-IL-13 pAbs, and/or anti-TGF-β mAbs did not proliferate. However, Tg $CD4^+$ T cells incubated with PLP:OVA-pσ1 + anti-CD25 mAbs or PLP:OVA-pσ1 + anti-CD25 mAb + anti-IL-13 pAbs proliferated profoundly. Simultaneous neutralization of TGF-β and CD25 in cultures stimulated with PLP:OVA-pσ1 re-established PLP:OVA-pσ1-mediated suppression of proliferation of OVA Tg $CD4^+$ T cell. Results depict mean ± SEM of 6 wells per treatment. *, $p < 0.001$ for proliferation of Tg $CD4^+$ T cells vs. PLP:OVA-pσ1-stimulated cells (*B*) Experimental design for combined neutralization of TGF-β and CD25 *in vivo*. (*C*) PBS-dosed mice treated with different combination of neutralizing Abs (see Figure 3.7*B*) developed classical manifestation of EAE. Anti-CD25 mAbs treatment of PLP:OVA-pσ1–dosed mice abrogated PLP:OVA-pσ1-mediated protection against EAE, but concomitant injection of anti-TGF-β and anti-CD25 mAbs restored PLP:OVA-pσ1-induced protection. Mean of 10 mice per group is shown. *, $p < 0.05$ for differences vs. PLP:OVA-pσ1 + IgG-dosed mice (upper panel), or vs. PBS + IgG-dosed mice (bottom panel).

Concomitant Neutralization of CD25 and TGF-β Restores PLP:OVA-pσ1-Mediated Protection against EAE via Reduction of Th17-Related Proinflammatory Cytokines

Mice dosed with PLP:OVA-pσ1 or PBS and treated with anti-TGF-β mAb and/or anti-CD25 mAb were sacrificed at day 10, p.ch. Cytokine production by whole lymphocytes or bead-isolated $CD4^+$ T cells obtained from their HNLNs was assessed by ELISA after *in vitro* stimulation with $PLP_{139-151}$ peptide. Lymphocytes acquired from PBS-dosed groups produced enhanced amounts of proinflammatory cytokines IL-6, IL-17, IL-21, and IL-23, and very little to no anti-inflammatory IL-10 and IL-4 (Figure 3.8*A*). In contrast to PLP:OVA-pσ1 + IgG -treated mice, PLP:OVA-pσ1-dosed mice that received combination of anti-CD25 mAb and anti-TGF-β mAb produced > 8-fold less of IL-10 and almost 2-fold less of IL-22, which was consistent with the lack of functional IL-10-producing T_{reg} cells in mice dosed with PLP:OVA-pσ1 and treated with anti-TGF-β mAb and anti-CD25 mAb (Figure 3.8*A*). Concomitant with depressed production of IL-10 and IL-22 by lymphocytes from PLP:OVA-pσ1 + anti-CD25 mAb + anti-TGF-β mAb-treated mice, significantly more IL-21 was secreted by lymphocytes obtained from

these mice when compared to PLP:OVA-pσ1-dosed mice treated with control IgG Ab (Figure 3.8*A*). Mice dosed with PLP:OVA-pσ1 and treated with combination of anti-CD25 mAb and anti-TGF-β mAb were protected against EAE despite lacking functional T_{reg} cells. Interestingly, lymphocytes isolated from PLP:OVA-pσ1-dosed mice treated with anti-CD25 mAb and anti-TGF-β mAb produced over 6-fold more of IL-28 and IL-4 when compared to diseased mice dosed with PLP:OVA-pσ1 and treated with anti-CD25 mAbs, implicating that in the absence of functional T_{reg} cells PLP:OVA-pσ1-induced protection against EAE is mediated by Th2-type cells. Equal amount of IL-28 and IL-4 were secreted by lymphocytes isolated from mice dosed with PLP:OVA-pσ1 and treated with IgG, anti-TGF-β mAb, or with the combination of anti-CD25 mAb and anti-TGF-β mAb, further suggesting that Th2 cell bias supports PLP:OVA-pσ1-mediated protection against EAE. Additionally, more IL-6 and IL-23 was produced by lymphocytes from protected PLP:OVA-pσ1 + anti-TGF-β mAb-dosed mice when compared to mice that received PLP:OVA-pσ1 + IgG, implying supportive, but not essential role of TGF-β in protection against EAE mediated by PLP:OVA-pσ1-primed T_{reg} cells. Additionally, the lack of differences in secretion of IL-4 between cells isolated from mice dosed with PLP:OVA-pσ1 + IgG and PLP:OVA-pσ1 + anti-TGF-β mAb + anti-CD25 mAb, suggests that the presence of T_{reg} cells is not required for IL-4 production (Figure 3.8*A* and *B*). Consistent with ameliorated disease exhibited by PLP:OVA-pσ1-protected mice (Figure 3.7*C* - upper panel), lymphocytes isolated from these mice produced negligible amounts of IL-6, IL-17, IL-21, and IL-23 (Figure 3.8*A*). IL-28 was not produced by the

lymphocytes isolated from diseased mice dosed either with PBS and various mAbs, or with PLP:OVA-pσ1 + anti-CD25 mAbs, further suggesting the consistent anti-inflammatory character of this cytokine in our experimental system (Figure 3.8*A*).

Consistent with the whole cell cultures, $CD4^+$ T cells isolated from PBS-dosed mice independent of treatment produced predominantly proinflammatory cytokines IFN-γ, IL-17, and IL-21, whereas production of anti-inflammatory IL-4, IL-10, IL-13, and TGF-β was depressed in these mice (Figure 3.8*B*). Interestingly, $CD4^+$ T cells obtained from mice dosed with PBS and treated with anti-TGF-β mAb produced nearly 2-fold less IFN-γ, reduced IL-17, but almost 2-fold more of IL-13 when compared to PBS + IgG-dosed mice, which was consistent with the higher recovery rate from clinical EAE exhibited by the PBS + anti-TGF-β mAb-dosed mice (Figure 3.7*C* -lower panel, and Figure 3.8*B*). Interestingly, PLP:OVA-pσ1 + anti-TGF-β mAb-treated mice, even though protected against EAE, produced 4-fold more of IFN-γ, ~ 2-fold more IL-17 and IL-21, but also 7-fold more of IL-13 than PLP:OVA-pσ1 + IgG-dosed mice (Figure 3.8*B*). $CD4^+$ T cells isolated from PLP:OVA-pσ1-protected mice showed an overall pattern contrary to PBS-dosed mice by the way these $CD4^+$ T cells produced anti-inflammatory, but little or no pro-inflammatory cytokines (Figure 3.8*B*). When $CD4^+$ T cells isolated from PLP:OVA-pσ1 + IgG- and PLP:OVA-pσ1 + anti-CD25 mAb + anti-TGF-β mAb-dosed mice produced similar levels IL-4, PLP:OVA-pσ1 + IgG-dosed mice produced 10 times more IL-10, but 5-fold less IL-13 when compared to PLP:OVA-pσ1 + anti-CD25 mAb + anti-TGF-β mAb-treated mice, further suggesting that protection against EAE in PLP:OVA-pσ1-dosed mice treated with anti-TGF-β mAb + anti-CD25

mAb is restored by induction of Th2 cells bias (Figure 3.7*C* and Figure 3.8*A* and *B*). Consistent with results presented in Figures 3.6 and 3.7, PLP:OVA-pσ1 + anti-CD25 mAb-treated mice showed over 9-fold enhancement in IFN-γ and IL-17, and 4-fold rise in IL-21, and TGF-β when compared to PLP:OVA-pσ1 + IgG- or PLP:OVA-pσ1 + anti-CD25 mAb + anti-TGF-β mAb-treated mice (Figure 3.8*B*). Interestingly, production of IL-13 by the unprotected PLP:OVA-pσ1 + anti-CD25 mAb-treated mice was elevated compared to PLP:OVA-pσ1 + IgG-treated mice, but reduced in comparison to PLP:OVA-pσ1 + anti-CD25 mAb + anti-TGF-β mAb-rescued mice, further confirming that Th2-type cells are induced in mice dosed with PLP:OVA-pσ1 and treated with anti-CD25 mAb + anti-TGF-β mAb (Figure 3.8*B*). These results suggest that concomitant neutralization of TGF-β and functional depletion of CD25 restores PLP:OVA-pσ1-mediated protection against EAE via induction of Th2 cell bias, as evidenced by increased production of IL-4, IL-13, and IL-28 in these mice compared to PLP:OVA-pσ1 + anti-CD25 mAb-treated diseased mice.

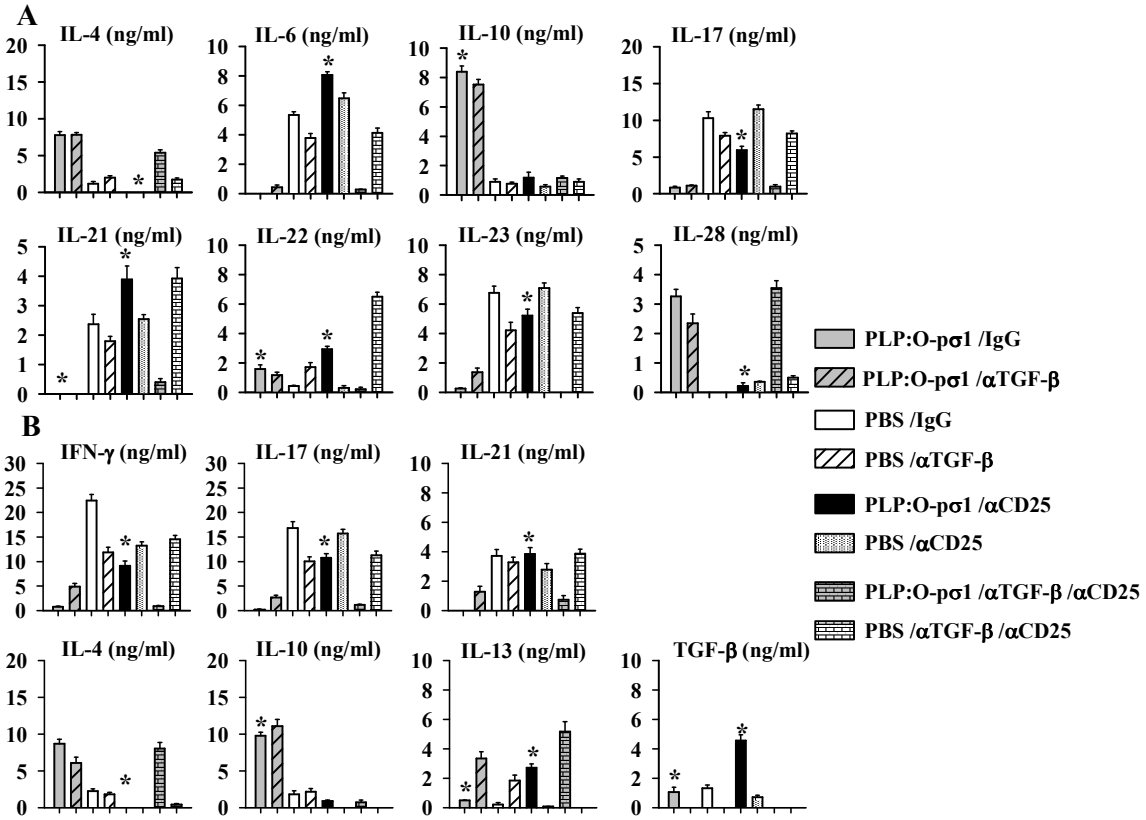


Figure 3.8. Concomitant neutralization of TGF-β and CD25 *in vivo* induces Th2-type cytokines. SJL mice were immunized and treated as described in Figure 3.7*B*. Whole lymphocytes (*A*) or $CD4^+$ T cells (*B*) isolated from their HNLNs on day 10 post EAE induction were cultured with $PLP_{139-151}$ peptide for 72 h, in the presence of feeder cells (*B*). Lymphocytes obtained from PBS-dosed mice independently on Ab treatment produced enhanced amounts of proinflammatory cytokines IFN-γ, IL-6, IL-17, IL-21 and IL-23, and little to no IL-4, IL-10, IL-13 and TGF-β. However, levels of IFN-γ and IL-17 produced by $CD4^+$ T cells were diminished in PBS + anti-TGF-β-treated mice when compared to PBS mice injected with isotype Ab control. PLP:OVA-pσ1-protected mice treated with IgG, anti-TGF-β mAbs or with combination of anti-TGF-β and anti-CD25 mAbs, produced enhanced amounts of IL-4 and IL-28 and very little to no of IFN-γ, IL-6, IL-17, IL-21 and IL-23. Additionally, mice dosed with PLP:OVA-pσ1 + anti-CD25 + anti-TGF-β produced significantly more IL-13 and IL-4 when compared to diseased PLP:OVA-pσ1 + anti-CD25-dosed mice. Mean ± SEM of 5 mice per group is shown * $p < 0.05$ for the PLP:OVA-pσ1 + anti-TGF-β + anti-CD25 vs. PLP:OVA-pσ1 + IgG and PLP:OVA-pσ1 + anti-CD25.

Mice nasally dosed with PLP:OVA-pσ1 and treated with anti-CD25 mAb displayed two-thirds fewer $FoxP3^+CD25^-CD4^+$ T cells when compared to PLP:OVA-pσ1-protected mice (Table 3.5). Interestingly, concomitant injection of PLP:OVA-pσ1-dosed mice with anti-TGF-β mAb and anti-CD25 mAb restored the expression of

FoxP3$^+$CD25$^-$CD4$^+$ T cells as seen with the PLP:OVA-pσ1 + IgG-dosed (protected) mice (Table 3.5). Though not as drastic as in PLP:OVA-pσ1 + anti-CD25 mAb-treated mice, a significant decrease in FoxP3$^+$CD25$^-$CD4$^+$ T cells isolated from protected PLP:OVA-pσ1 + anti-TGF-β mAb-treated mice was observed. These results suggest that in the presence of functional T_{reg} cells TGF-β may be important for induction of FoxP3-expressing CD25$^-$CD4$^+$ suppressor T cells. Anti-CD25 mAb- anti-TGF-β mAb-treated PLP:OVA-pσ1-dosed mice, even though protected against EAE, showed pronounced production of IL-4 (Figure 3.8*A* and *B*), and increased secretion of IL-13 when compared to PLP:OVA-pσ1 + IgG-dosed mice (Figure 3.8*B*). Additionally, since over 25 % of CD25$^-$CD4$^+$ T cells isolated from these mice expressed FoxP3, it is possible that in the absence of T_{reg} cells the induction of FoxP3 expression on CD25$^-$CD4$^+$ T cells may due to the other cytokines, perhaps IL-4.

Table 3.5. *In vivo* neutralization of TGF-β and CD25 induces FoxP3 expression of PLP:OVA-pσ1-primed Th2 cells

Treatment[a]	% FoxP3$^+$CD25$^-$CD4$^+$[b]
PLP:OVA-pσ1 + IgG	24.78 ± 0.96*,§
PLP:OVA-pσ1 + α-TGF-β	19.73 ± 1.31**
PLP:OVA-pσ1 + α-CD25	8.12 ± 1.16
PLP:OVA-pσ1 + α-TGF-β + α-CD25	26.68 ± 0.51*,§

[a] SJL/J mice were challenged s.c. with 200 μg $PLP_{139-151}$ in complete Freund's adjuvant plus 200 ng PT i.p. on days 0 and 2, and were nasally dosed 14 and 7 days prior to EAE challenge with 100 μg of PLP:OVA-pσ1 or with PBS and treated as described in Figure 3.7*B*

[b] Average percentages ± SD of FoxP3$^+$CD25$^-$CD4$^+$ T cells as a fraction of CD4$^+$ T cells from 3 mice per group are depicted, *, $p < 0.001$, **, $p < 0.05$ vs. PLP:OVA-pσ1 + anti-CD25, §, $p < 0.05$ vs. PLP:OVA-pσ1 + anti-TGF-β

In Vivo Neutralization of IL-23 Did Not Affect
PLP:OVA-pσ1-Mediated Protection against EAE

According to the literature, Th17 cells can be induced either in an IL-23-dependent or -independent manner, the latter activation occurring in the presence of TGF-β and/or additional proinflammatory cytokines (121, 124, 126, 205, 337, 362). Treatment of PLP:OVA-pσ1-dosed mice with anti-CD25 mAb reversed PLP:OVA-pσ1-mediated protection against EAE via induction of TGF-β-dependent proinflammatory Th17 cells. To determine if IL-23 is necessary for stimulation of proinflammatory response in PLP:OVA-pσ1 + anti-CD25 mAb-treated mice, we blocked IL-23 in PLP:OVA-pσ1- or PLP:OVA-pσ1 + anti-CD25 mAb-dosed mice *in vivo*. Neutralization of IL-23 had no effect on clinical onset and severity of EAE in PLP:OVA-pσ1-dosed mice (Figure 3.9). However, PLP:OVA-pσ1-dosed mice treated with rabbit anti-IL-23 serum in combination with anti-CD25 mAb developed milder clinical disease when compared to PLP:OVA-pσ1 + anti-CD25 mAb-treated mice, although these difference were not statistically significant (Figure 3.9).

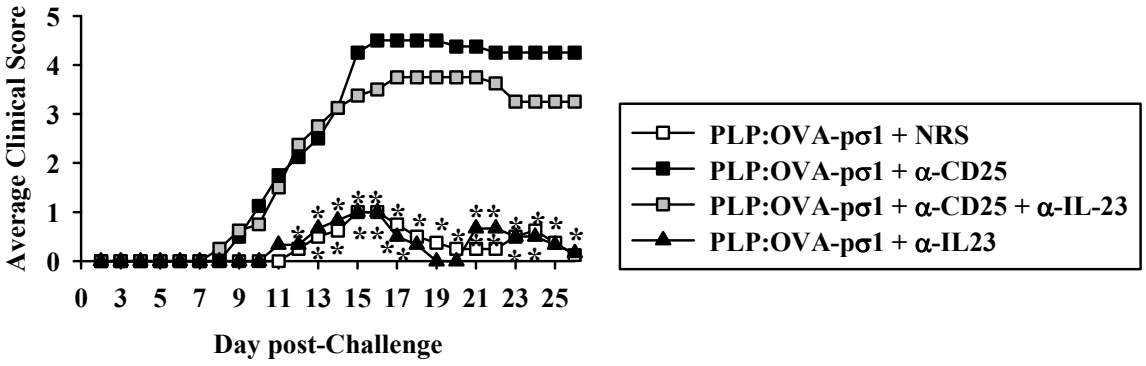


Figure 3.9. Induction of Th17 cells in PLP:OVA-pσ1-dosed mice functionally depleted of $CD25^+$ T_{reg} cells is independent of IL-23.

Figure 3.9. – continued. SJL mice were nasally given 100 μg of PLP:OVA-pσ1 or PBS on day -14 and -7 in relation to EAE induction. Mice were i.p. injected with anti-CD25 /rat IgG on day -5 and -2, and with anti-IL-23 RS/NRS on day -1, 1, and 5. Neutralization of IL-23 did not suppressed clinical disease in $CD25^+$ T_{reg} cells-inactivated PLP:OVA-pσ1-dosed mice. Mean of up to 5 mice per group is shown. * $p < 0.05$ vs. PLP:OVA-pσ1 + anti-CD25-dosed mice.

Discussion

Previously we showed that nasal administration of a single low dose of OVA-pσ1 induces tolerance to OVA in mice (89). Here we report that N-terminal modification of OVA-pσ1 by fusion with two copies of immunodominant T cell epitope, $PLP_{130-151}$, resulted in a functional protein (PLP:OVA-pσ1), that when applied nasally protected susceptible SJL mice against EAE. OVA-specific tolerance was not altered in PLP:OVA-pσ1-dosed mice, as evidenced by consistent inhibition of OVA-specific IgG Ab titers in serum and reduction of OVA-specific DTH response in these mice. We have shown here that a low dose of PLP:OVA-pσ1 given nasally significantly delayed clinical onset of the disease and greatly reduced clinical manifestation and the CNS histopathology in mice induced with EAE, when compared to PBS-dosed unprotected mice. Moreover, we have demonstrated that PLP:OVA-pσ1-mediated protection against EAE is Ag-specific evidenced by uninhibited EAE development in mice nasally given OVA-pσ1. In contrast to OVA-pσ1- and PBS-dosed and $PLP_{139-151}$-challenged mice, PLP:OVA-pσ1-dosed mice showed steady and major reduction in $CD4^+$ T cells secreting proinflammatory cytokines in response to *in vitro* re-stimulation with $PLP_{139-151}$-peptide, whereas $CD4^+$ T cells secreting regulatory and anti-inflammatory cytokines were enhanced. Importantly, even though OVA-pσ1-dosed mice were not protected against

EAE, these mice remained tolerant to OVA, as evidenced by significant increase in $CD4^+$ T cells producing anti-inflammatory cytokines, and concomitant depression in proinflammatory $CD4^+$ T cells after *in vitro* re-stimulation with OVA. Consistent with the reported generation of a single dose tolerance to OVA in OVA-pσ1-dosed mice (89), only a 100 μg of PLP:OVA-pσ1 given nasally were sufficient to significantly inhibit clinical manifestation of EAE in either a therapeutic or prophylactic paradigm. Like OVA-pσ1-induced tolerance to OVA, PLP:OVA-pσ1-mediated protection against EAE was facilitated by IL-10-secreting $FoxP3^+CD25^+CD4^+$ T cells and supported by $FoxP3^+CD25^-CD4^+$ Th2 cells that secreted predominantly IL-4. The role of IL-10 and IL-4 in protection against EAE was evaluated *in vitro* and *in vivo*. *In vitro* inhibition of IL-10 or IL-4 restored $PLP_{139-151}$-specific proliferation by $CD4^+$ T cells isolated from PLP:OVA-pσ1-dosed mice. Neutralization of IL-4 *in vivo* partially compromised PLP:OVA-pσ1-mediated protection against EAE via down regulation of IL-10-production by $CD4^+$ T cells and concomitant induction of proinflammatory Th1 and Th17 type cytokines. Adoptive transfer of PLP:OVA-pσ1-derived T_{reg} cells prior to the challenge with $PLP_{139-151}$ peptide entirely protected mice from development of EAE, but transfer of PLP:OVA-pσ1-primed $CD25^-CD4^+$ Th2 only cells significantly delayed the disease onset, but did improve disease severity in EAE-induced mice. Moreover, functional inactivation of T_{reg} cells prior to the challenge with $PLP_{139-151}$ peptide greatly impaired the PLP:OVA-pσ1-induce protection against EAE. Potent disease was observed in mice dosed with PLP:OVA-pσ1 and treated with anti-CD25 mAb associated with the enhanced production of IL-13, TGF-β , IL-6, IL-21, IL-17, and IFN-γ, and

concomitant drop in IL-4 and IL-10. *In vitro* studies confirmed the crucial role for TGF-β, but not IL-13, in anti-CD25 mAb-mediated neutralization of PLP:OVA-pσ1-dosed mice. In contrast to simultaneous neutralization of CD25 and IL-23 *in vivo*, simultaneous neutralization of TGF-β and CD25 re-established PLP:OVA-pσ1-induced protection suggesting that anti-CD25-induced Th17 inflammatory response in PLP:OVA-pσ1-dosed mice is activated via TGF-β- rather than IL-23-dependent pathway.

Previously we reported that recombinant pσ1 can be engineered to modulate immune response to mucosally delivered Ags (82, 89, 154). We demonstrated that chemically modified pσ1 significantly enhanced immunity to delivered DNA (82, 154), whereas genetic alteration of pσ1 renders it to induce tolerance to the fused protein Ag (89). Here we have demonstrated that protein vaccine generated by genetic fusion of encephalitogenic $PLP_{130-151}$ peptide to tolerogenic OVA-pσ1 when given nasally to EAE susceptible mice can efficiently suppress the development of $PLP_{139-151}$-induced disease. Pσ1-mediated protection against EAE was restricted to the delivered Ag, since nasal administration of PLP-carrying PLP:OVA-pσ1, but not OVA-pσ1, was able to protect mice against clinical manifestation of EAE. Likewise, diseased OVA-pσ1-, and PBS-dosed mice, but not PLP:OVA-pσ1-protected mice, developed significant PLP-specific DTH response, further suggesting the Ag-specific protection against EAE delivered by nasally given pσ1. Importantly, mice dosed with OVA-pσ1 and induced with EAE, despite developing uninhibited disease remained tolerant to OVA, as demonstrated by significant drop in numbers of $CD4^+$ T cells that secreted proinflammatory cytokines, but

important enhancement in $CD4^+$ T cells secreting anti-inflammatory and regulatory cytokines in response to *in vitro* re-stimulation with OVA.

We have shown previously that as little as a single dose of OVA-pσ1 given nasally induces sustainable tolerance to OVA (89). Similarly, only a single nasal dose of PLP:OVA-pσ1 administered to mice before EAE induction, or during its pre-clinical stage, prevented clinical manifestation of the disease and CNS histopathology. Induction of a low-dose tolerance to mucosally delivered encephalitogenic proteins or peptides has been described by others (24, 25, 37, 56). However, multiple and/or large doses of these Ags were required for protection against EAE (22, 25, 56). Surprisingly, feeding the patients with RR MS bovine myelin preparation did not result in a significant improvement in the number and severity of the relapses (22).

Enhanced protection against autoimmune diseases via delivery of encephalitogenic peptides by a carrier molecule has been described. Systemic injection of aggregated Ig chimeras carrying immunodominant $PLP_{139-151}$ or MOG_{35-55} peptides was shown to ameliorate the clinical manifestation of PLP- or MOG-induced EAE in susceptible mice (31, 146). Although successful, systemic administration of an Ag is often associated with the significant risk of contamination, pain, and unpleasant effects, and is not cost efficient. The alternative strategy of tolerance induction by mucosal administration of small amounts of an Ag chemically coupled to mucosa-binding molecule has been evaluated (67, 68, 151). Cholera toxin subunit B (CTB)-coupled human insulin when delivered orally or nasally is able to efficiently suppress destruction of pancreatic beta cells and reduce clinical manifestation of diabetes in mice (68, 151).

Oral administration of significantly smaller amounts of MBP coupled to CTB than MBP alone has been demonstrated to suppress ongoing EAE (67). Interestingly, in one study, MBP-CTB conjugates induced significant amounts of proinflammatory cytokine IFN-γ (67), whereas in another investigation nasal administration of insulin-CTB induced expression of TGF-β and IL-10 but did not affect the levels of IFN-γ or IL-4 (68). This inconsistent immunomodulatory effect of chemically coupled CTB was explored by Bergquist *et al*. This group has demonstrated that nasal administration of CTB-coupled dextran can induce either dextran-specific immune response (73) or tolerance (74), depending on the presence of pre-existing immunity to CTB. It is a general understanding that native cholera toxin (CT) is a strong mucosal adjuvant (152). Interestingly, also CTB can be used as a mucosal adjuvant (365, 366) because it lacks the toxicity associated with native CT (64). Moreover, it is possible that chemical modifications of CTB-conjugates can affect the immunogenicity of an Ag, which may result in a different immunomodulatory outcome following its administration to mice (35). Alternatively to chemical modification, it has been demonstrated that genetic fusion of $PLP_{139\text{-}151}$ to CTB can suppress ongoing EAE in mice and inhibit the CNS inflamation (35). Even though successful, this approach requires multiple nasal administrations of recombinant PLP-CTB for establishment of protection against EAE.

In contrast to these studies, we have shown that just a single nasal administration of $PLP_{139\text{-}151}$ fused to OVA-pσ1 can induce PLP-specific tolerance that effectively protects mice against EAE, via induction of anti-inflammatory cytokines, IL-10 and IL-4, and inhibition of Th1- and Th17-type proinflammatory cytokines. Induction of OVA-

pσ1-mediated tolerance to OVA has been associated with generation of IL-10-producing $FoxP3^+CD25^+CD4^+$ T_{reg} cells and IL-4-secreting $CD25^-CD4^+$ Th2 cells that could suppress OVA-induced proliferation of $CD4^+$ T effector cells *in vitro* and *in vivo* (89). Other studies have also found that low-dose mucosal tolerance is mediated by T_{reg} cells that express FoxP3 transcription factor (3, 37, 60, 109, 368), and FoxP3-expressing $CD25^+$ or $CD25^-$ T_{reg} cells can prevent and/or treat autoimmune diseases (33, 34, 114). Additionally, mucosally-induced $CD25^-CD4^+$ T cells have been shown to support recovery from EAE (33, 78). Consistent with these findings, nasal administration of PLP:OVA-pσ1 induces significant enhancement of $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells that expressed FoxP3.

The vast majority of these $FoxP3^+$ T_{reg} cells isolated from PLP:OVA-pσ1-protected mice produce IL-10, but not IL-4 or TGF-β, in response to the challenge with $PLP_{139-151}$. Induction of IL-10-producing T_{reg} cells after nasal administration of protein Ags has been reported previously (24, 89). Additionally, the importance of IL-10 for protection against EAE has been highlighted by a number of investigators (24, 105, 114, 128). Administration of IL-10 has been shown to ameliorate clinical and histopathological manifestation of EAE via induction of T_{reg} cells (129). Mice deficient in IL-10 or IL-10R were shown to develop more severe EAE, whereas IL-10 Tg mice exhibited resistance to EAE induction (131, 132). Moreover, we have previously reported, that pσ1-mediated tolerance could not be established in IL-10-deficient mice presumably due to the failure in generation of $FoxP3^+$ T_{reg} cells in IL-10-deficient mice given nasally OVA-pσ1 (89). Consistent with our previous findings and established

protective role of IL-10 against EAE, *in vitro* neutralization of IL-10 reversed PLP:OVA-pσ1-mediated suppression of $PLP_{139-151}$-specific proliferation of $CD4^+$ T cells.

In agreement with our previous study in which OVA-pσ1-mediated tolerance was supported by IL-4-producing $CD25^-CD4^+$ Th2 cells, PLP:OVA-pσ1 given nasally to the mice induced significant enhancement in $FoxP3^+CD25^-CD4^+$ Th2 cells in which over 80 % of them produced IL-4. The anti-inflammatory potential of IL-4 has been well characterized (168, 169, 177, 194), and stimulation of regulatory $FoxP3^+CD25^-CD4^+$ T cells has been reported by us and by others (33, 89, 114, 122). Alternatively, it is plausible that PLP:OVA-pσ1-induced $FoxP3^+CD25^-CD4^+$ T cells are undergoing conversion to the $FoxP3^+CD25^+CD4^+$ phenotype, since converting $FoxP3^+CD25^-CD4^+$ T cells were shown to be potent inhibitors of $CD4^+$ T cells expansion *in vivo* and proliferation *in vitro* (122, 220). It has been reported that IL-4 can supplement suppressive function of TGF-β-secreting regulatory Th3 cells (22, 23, 54). This cytokine has also been implicated in triggering expression of FoxP3 on naive peripheral $CD25^-CD4^+$ T cells (117), and when secreted by Th2 cells, IL-4 was shown to support the inhibition of $PLP_{139-151}$-specific proinflammatory response (33). Surprisingly, the deficiency of IL-4 has not been associated with increased susceptibility to development of autoimmune diseases, suggesting only a moderate supporting role for IL-4 in suppression of inflammatory responses against self-Ags (6, 130). Consistent with these findings, *in vitro* blocking of IL-4 partially reversed PLP:OVA-pσ1-mediated suppression of proliferation of encephalitogenic $CD4^+$ T cells in response to $PLP_{139-151}$ re-stimulation. Furthermore, *in vivo* neutralization of IL-4 significantly accelerated clinical

manifestation of EAE in PLP:OVA-pσ1- and PBS-dosed mice. PLP:OVA-pσ1-dosed mice treated with neutralizing anti-IL-4 mAbs developed disease, but was much less severe compared to PBS-dosed mice, suggesting a positive, but not a pivotal role for IL-4 in PLP:OVA-pσ1-mediated protection against EAE. Additionally, PLP:OVA-pσ1-dosed anti-IL-4-treated mice produced significantly less of IL-10, resulting in more IL-6, IL-21, IL-17, and IFN-γ when compared to PLP:OVA-pσ1-protected mice. Therefore, consistent with the supportive role of IL-4 in induction of $FoxP3^+$ T_{reg} cells (117), it is possible that neutralization of IL-4 down regulated IL-10-producing T_{reg} cells by PLP:OVA-pσ1-dosed mice. Alternatively, IL-4 and IFN-γ were shown to negatively regulate induction of Th17 cells (168, 169) and each other (194), and IL-4 was shown to promote IL-10 production by monocytes (388). Therefore, it is possible that the immune imbalance generated by neutralization of IL-4 via inhibition of Ag-specific T_{reg} cells at the time of EAE induction triggered the proinflammatory Th1 and Th17 responses.

Further evidence for the pivotal role of IL-10-producing T_{reg} cells and the supportive role of IL-4-secreting $CD25^-CD4^+$ T cells in PLP:OVA-pσ1-induced protection against EAE, was provided by the adoptive transfer study. We have shown that adoptive transfer of PLP:OVA-pσ1-derived $CD25^+CD4^+$ T cells prior to EAE induction entirely protected mice against the clinical disease. These results are in agreement with our previous data in which adoptive transfer of OVA-pσ1-primed $CD25^+CD4^+$ T cells efficiently inhibited OVA-specific proliferation of effector $CD4^+$ T cells *in vivo* (89). Induction of myelin-specific or nonspecific $CD25^+CD4^+$ T_{reg} cells has been demonstrated to protect and/or treat mice against autoimmune disorders (22, 33, 34,

54). Moreover, mice depleted of naturally occurring $CD25^+CD4^+$ T_{reg} cells were shown to spontaneously develop autoimmune diseases (3, 107), whereas functional inactivation of mucosally induced myelin-specific or -nonspecific $CD25^+CD4^+$ T cells render mice more susceptible to development of autoimmune diseases (33, 112). Consistent with the supportive role of $CD25^-CD4^+$ Th2 cells in OVA-pσ1-mediated tolerance (89), here we have demonstrated that adoptive transfer of PLP:OVA-pσ1-induced $CD25^-CD4^+$ Th2 cells prior to EAE induction significancy ameliorated and delayed the clinical onset of the disease. These results are not surprising since induction of Ag-specific (89, 141, 142, 266) or -nonspecific Th2 (33, 78, 270, 271, 275, 276) cell bias has been previously reported to support anti-inflammatory response and recovery from EAE. Also, $CD25^-CD4^+$ Th2 cells induced in response to oral infection with *Salmonella*-CFA/I vaccine were shown to partially suppress development of $PLP_{139-151}$-induced EAE in a myelin-nonspecific fashion (33).

The requirement for $CD25^+CD4^+$ T cells in PLP:OVA-pσ1-mediated protection against EAE was further confirmed by functional inactivation of these T_{reg} cells in PLP:OVA-pσ1-dosed mice. Unlike PBS-dosed mice that developed a similar course of EAE independently of Ab treatment, mice dosed with PLP:OVA-pσ1 and functionally depleted of T_{reg} cells developed very severe EAE with all of the mice succumbing at the peak of the acute disease. In agreement with these findings were other studies showing that functional inactivation of T_{reg} cells can increase susceptibility to development of autoimmune diseases (107, 112). Moreover, our group has reported previously that *in*

vivo depletion of T_{reg} cells exacerbates disease in otherwise protected *Salmonella*-CFA/I-treated mice (33).

Our further investigation revealed that $CD4^+$ T cells obtained from mice dosed nasally with PLP:OVA-pσ1 and i.p. treated with anti-CD25 mAb produced significantly more Th17 cell-associated cytokines, including IL-17, IL-21, and IL-6, whereas levels of IL-4 and IL-10 were significantly depressed in these mice, when compared to PLP:OVA-pσ1-dosed, IgG-treated mice. The development of proinflammatory Th17 cells can be established in either an IL-23-dependent or -independent fashion (126, 189, 190-192, 205, 210). The requirement for IL-23 in the induction of encephalitogenic Th17 cells has been demonstrated in one study in which mice lacking IL-23p19, but not IL-12p35, were shown to be resistant to EAE development (192, 205). Additionally, TGF-β alone (124), or in combination with IL-6 (126), IL-1 (208), or IL-21 (190) has been implicated in the induction of Th17 cells. Interestingly, the levels of TGF-β and IL-13 were also significantly enhanced in anti-CD25 mAb-treated PLP:OVA-pσ1- or PBS-dosed mice when compared to IgG-treated mice. The role of IL-13 has not been studied very extensively in EAE. This Th2 type cytokine is typically associated with induction of anti-inflammatory response (216, 273). Recently our group has reported induction of IL-13-producing T_{reg} cells in response to the oral infection with *Salmonella*-CFA/I_{IC} vaccine that expresses enterotoxigenic *E. coli* colonization factor Ag I (CFA/I) in the intracellular compartment (IC), as oppose to its extracellular expression in *Salmonella*-CFA/I vaccine (34). We have demonstrated that these IL-13-producing T_{reg} cells efficiently inhibited clinical manifestation and the CNS histopathology caused by $PLP_{139-151}$-induced EAE,

and neutralization of IL-13 reversed *Salmonella*-CFA/I_{IC}-mediated protection against EAE (34). In another study, it has been shown that enhanced secretion of IL-13 and other anti-inflammatory cytokines is associated with partial protection against EAE using recombinant TCR with covalently bound encephalitogenic peptide (217). Additionally, administration of human recombinant IL-13 prior to EAE induction in Lewis rats was shown to significantly ameliorate clinical EAE (218). In contrast to its anti-inflammatory role, IL-13 was also shown to promote MHC II expression on macrophages and thus trigger development of Th1 cells (219).

The immunomodulatory role of TGF-β is even more complex. On one hand, TGF-β is one of the two major regulatory cytokines secreted by T_{reg} cells, and its production has been linked to the forceful suppression of inflammatory response (3, 33, 56, 80). Regulatory potential of TGF-β has been implicated in both prevention and treatment of EAE (3, 33, 56, 80). Moreover, TGF-β was shown to trigger conversion of $CD25^{-}CD4^{+}$ T cells into $CD25^{+}CD4^{+}$ T_{reg} cells via up regulation of FoxP3 expression (122, 123). Interestingly, TGF-β alone or accompanied by IL-6 or IL-1 can induce production of Th17 cells (124, 126), whereas TGF-β and IL-2 have been recently implicated in induction of $FoxP3^{+}CD25^{+}CD4^{+}$ T_{reg} cells (121).

In our *in vitro* studies, neutralization of IL-13 alone, or in combination with TGF-β had no effect on proliferation of OVA Tg $CD4^{+}$ T cells incubated with PLP:OVA-pσ1 and/or anti-CD25 mAbs. Contrary to IL-13, simultaneous neutralization of TGF-β and blockade of CD25 restored the PLP:OVA-pσ1-mediated inhibition of proliferation of Tg $CD4^{+}$ T cells abrogated by neutralization of CD25. Consistent with the *in vitro* data,

when PLP:OVA-pσ1-dosed anti-CD25-treated mice were also treated with anti-TGF-β neutralizing mAb, but not with rabbit anti-IL-23 serum, the PLP:OVA-pσ1-mediated protection against EAE was restored. These results clearly indicate that induction of Th17 cells in mice dosed with PLP:OVA-pσ1 and depleted of T_{reg} cells is driven by TGF-β and not by IL-23.

Interestingly, when compared to PLP:OVA-pσ1 + IgG-dosed mice, PLP:OVA-pσ1-dosed anti-TGF-β mAb-treated mice were equally protected against clinical onset of EAE and displayed a very similar cytokine profiles, except that significantly more of IL-13 was produced by $CD4^+$ T cells isolated from PLP:OVA-pσ1 + anti-TGF-β mAb-dosed mice. Additionally, these anti-TGF-β mAb-treated PLP:OVA-pσ1-protected mice showed moderate, although a significant decrease in $FoxP3^+CD25^-CD4^+$ T cells when compared to PLP:OVA-pσ1 + IgG-dosed mice, but a significant increase in these cells when compared to anti-CD25 mAb-treated PLP:OVA-pσ1-dosed mice. This observation is consistent with the previous findings that TGF-β (118, 121-123), but also IFN-γ (127) and IL-4 (117) can support conversion of $CD25^-CD4^+$ T cells to $CD25^+CD4^+$ T_{reg} cells via the enhancement of FoxP3 expression. Therefore, the reduction in $FoxP3^+CD25^-CD4^+$ T cells in PLP:OVA-pσ1 + anti-TGF-β mAb-treated mice when compared to PLP:OVA-pσ1 + IgG-dosed mice can be explained by the lack of TGF-β-mediated conversion of $CD25^-CD4^+$ T cells to $FoxP3^+$ suppressive phenotype. However, since these PLP:OVA-pσ1 + anti-TGF-β-treated mice were producing IL-4 and IFN-γ, it is plausible that both or either of these cytokines stimulated the conversion in the absence of TGF-β. These results implicate that in the presence of functional PLP:OVA-pσ1-induced

T_{reg} cells the proinflammatory potential of TGF-β is inhibited, or alternatively, that TGF-β can play either pro- or anti-inflammatory role in a given system depending on the conditions. In contrast to our observations, simultaneous depletion of TGF-β and CD25 in mice fed with high or low doses of OVA was shown to prevent the induction of tolerance, suggesting the complementary role of $CD25^+$ T_{reg} cells and TGF-β in establishment of oral tolerance to OVA (389).

The analysis of cytokines produced by mice dosed with PLP:OVA-pσ1 + anti-CD25 mAb and rescued with anti-TGF-β mAb revealed inhibition of Th17-restricted proinflammatory cytokines and IFN-γ, but concomitant induction of IL-4, IL-13, and IL-28. Additionally, enhanced expression of FoxP3 on $CD25^-CD4^+$ T cells isolated from these mice when compared to anti-CD25 mAb-treated PLP:OVA-pσ1-dosed mice implicates activation of alternative regulatory pathway for PLP:OVA-pσ1-mediated tolerance that is independent on the presence of conventional T_{reg} cells.

The role for IL-28 has not yet been evaluated in EAE, although IL-28 was shown to prime tolerogenic DCs *in vitro* (224). Based on the established anti-inflammatory and anti-encephalitogenic roles of IL-4 and IL-13, we concluded that, neutralization of TGF-β in PLP:OVA-pσ1-dosed anti-CD25 mAb-treated mice reestablished protection against EAE via induction of Ag-specific Th2 cell bias.

In summary, we have shown that a single 100 μg dose of PLP:OVA-pσ1 given nasally prior or after EAE induction protects mice against the clinical manifestation of the disease and associated CNS histopathology. In contrast, mice dosed nasally with OVA-pσ1 developed fully pronounced disease, suggesting that pσ1-mediated tolerance is

Ag-specific. We have demonstrated here that IL-10-producing $FoxP3^{+}CD25^{+}CD4^{+}$ T_{reg} cells and IL-4-secreting $FoxP3^{+}CD25^{-}CD4^{+}$ Th2 cells were induced by nasal administration of PLP:OVA-pσ1 and that these cells to a different degree were involved in protection against EAE. Adoptive transfer of PLP:OVA-pσ1-derived $CD25^{+}CD4^{+}$ T cells entirely prevented EAE development in recipient mice upon challenge. PLP:OVA-pσ1-derived $CD25^{-}CD4^{+}$ T cells, when adoptively transferred, significantly delayed and ameliorated $PLP_{139-151}$-induced EAE. *In vitro* neutralization of IL-10 completely restored PLP-specific proliferation of PLP:OVA-pσ1-derived cells, whereas neutralization of IL-4 only partially reversed the PLP:OVA-pσ1-mediated suppression of $CD4^{+}$ T cell proliferation, suggesting the pivotal role for IL-10 and supportive function of IL-4 in PLP:OVA-pσ1-induced tolerance. Likewise, *in vivo* neutralization of IL-4 partially abrogated PLP:OVA-pσ1-mediated protection against EAE. PLP:OVA-pσ1-induced T_{reg} cells were crucial for the protection against EAE, and functional inactivation of these cells *in vivo* rendered mice highly susceptible to EAE development. This abrogation of PLP:OVA-pσ1-mediated protection in the absence of T_{reg} cells was due to the TGF-β-mediated induction of Th17 cells, since neutralization of TGF-β reestablished PLP:OVA-pσ1-mediated protection against EAE via suppression of Th17 type cytokines. Interestingly, in the absence of functional $CD25^{+}$ T_{reg} cells and TGF-β, PLP:OVA-pσ1-induced protection against EAE which was mediated by $CD25^{-}CD4^{+}$ Th2 cells that produced IL-13, IL-4, and IL-28 in response to $PLP_{139-151}$ re-stimulation, and also expressed FoxP3. These results implicate that a single low dose nasal tolerance mediated by genetically modified pσ1 can be successfully applied to prevent and/or treat

autoimmune diseases and allergies. Additionally, the ability to protect mice against EAE by just a single nasal dose of a vaccine may be crucial to overcome potential health risks such as allergy development.

NOTE: Histology sections presented in Figure 3.3*D* and incorporated into Table 3.1were made by Gayle Callis.

PROTEIN σ1 – BASED MUCOSAL VACCINE TREATS ACUTE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE) VIA INDUCTION OF IL-10-SECRETING ANTIGEN-SPECIFIC T_{REG} CELLS AND SELECTIVE APOPTOSIS OF AUTOREACTIVE EFFECTOR $CD4^+$ T CELLS

Introduction

Experimental autoimmune encephalomyelitis (EAE), a rodent model of multiple sclerosis (MS) (6), is induced in susceptible mice strains and is mediated by autoreactive proinflammatory $CD4^+$ T cells (6,135, 174). Suppression and recovery from EAE has been associated with induction of regulatory $CD4^+$ T cells (22, 24, 33, 34, 56, 105) that actively inhibit Th17 cell-mediated inflammation, and also $CD8^+$ T cells (75, 100, 101, 174, 234, 390), B cells (98, 236, 241), and DCs (31, 207, 250).

Aside from the well-established pathogenic role of B cells and auto-Abs in EAE (6, 235, 314, 391), these cells have been proven beneficial in suppressing inflammation and mediating recovery from EAE (98, 236). B cells can inhibit autoimmunity via direct interaction with pathogenic T cells, as well as by production of cytokines (98). Induced under inflammatory conditions (98) IL-10- (98, 236) and/or TGF-β– (236, 242) producing B regulatory (B_{reg}) cells, and IL-4-producing B effector 2 (Be2) have been implicated in the induction of low dose oral tolerance (241, 242). Induction of EAE in mice deficient of functional B cells (μMT mice) has resulted in more severe and exacerbated disease when compared to the wild type mice (236, 238).

Recently, attention has focused on the role of certain subsets of DCs in mucosal tolerance induction (39, 47, 99, 392) and protection against EAE (31, 250). IL-10-producing $CD8\alpha^-$ DCs were shown to mediate recovery from EAE (31), and $CD11b^+$

$CD8\alpha^-$ DCs that also produce IL-10 were capable of inducing IL-4 and IL-10-secreting Th2 cells *in vitro* (47). Integrin CD11b (α_M, CD18, Mac-1, CR3) has been linked to the tolerogenic role of DCs (47, 393) and monocytes (394, 395). Additionally, $CD11b^+$ expression on APCs was shown to be essential for induction of peripheral tolerance (99, 393) and suppression of autoimmune inflammatory diseases (393-395). In contrast to these reports, one group has shown that Mac-1 expression on T cells and accessory cells is required for development of EAE, and Mac-1 homozygous-deficient, mice develop delayed and ameliorated EAE (396).

Induction of $CD4^+$ regulatory T cells by mucosally administered protein Ags (61, 89, 389, 397) and the role of these cells in treatment of autoimmune diseases (9, 24, 25, 56, 76, 109, 112, 398) has been well-established. IL-10-producing Tr1 (Th10) cells that develop in response to a chronic exposure to a nasally given protein Ag (22) have been implicated in suppression of EAE following soluble peptide therapy (105). It has been demonstrated that depletion of mucosally-induced $CD25^+CD4^+$ T_{reg} cells can lead to a variety of autoimmune disorders (107, 112).

Mucosal administration of myelin components has been widely applied for treatment and/or protection against EAE (24, 56, 76, 156). Even though experimentally successful, generation of myelin-specific tolerance requires multiple and/or large Ag doses, which introduced a risk of allergy development. Previous work performed in our laboratory demonstrated that reovirus recombinant protein sigma 1 ($p\sigma1$) can be successfully applied as a delivery vehicle for induction of a long-lasting and sustainable tolerance to the fused protein Ag (89).

In a previous chapter, we reported that pσ1-mediated tolerance to the fused protein Ag can be easily adapted to prevent autoimmunity. $PLP_{130-151}$-bearing OVA-pσ1, termed PLP:OVA-pσ1, but not OVA-pσ1 alone, successfully ameliorates $PLP_{139-151}$-induced EAE, demonstrating the Ag-specificity of pσ1-mediated tolerance. We have shown that $FoxP3^+$ $CD25^+CD4^+$ T_{reg} cells that secrete IL-10 are essential for PLP:OVA-pσ1-mediated protection against EAE, although IL-4-secreting $CD25^-CD4^+$ Th2 cells, induced by nasally applied PLP:OVA-pσ1 support the observed tolerance.

Here we show that a single dose of $rMOG_{29-146}$-pσ1 fusion protein (MOG-pσ1), but not rMOG alone, when administered to mice, entirely prevents CNS pathology and clinical manifestation of MOG_{35-55}-induced EAE. Nasally applied MOG-pσ1 induces production of IL-10 by T_{reg} cells and IL-4 by $FoxP3^+$ Th2 cells. Moreover, we have determined that B cells from MOG-pσ1-protected mice produce both of these anti-inflammatory cytokines in response to the challenge with MOG_{35-55} peptide. Consistent with this finding, MOG-pσ1-mediated protection against EAE is partially abrogated in B cell-deficient (μMT) mice. We have shown that IL-10 secreted in response to MOG-pσ1-tolerization is crucial for protection against EAE, as evidenced by the inability to prevent EAE in MOG-pσ1-dosed $IL\text{-}10^{-/-}$ mice. Consistent with reports by others of the failure to induce mucosal tolerance in $CD11b^{-/-}$ mice (99), nasal delivery of MOG-pσ1 did not protect $CD11b^{-/-}$ mice from MOG_{35-55}-induced EAE. Importantly, we have shown that as little as 50 μg of MOG-pσ1 administered to the mice at the peak of clinical disease can significantly reduce ongoing EAE and accelerate recovery from disease.

Materials and Methods

Preparation of MOG-pσ1 and Recombinant MOG (rMOG)

Protein σ1 was prepared, as previously described (89). A synthetic gene encoding extracellular domain (amino acids 29-146) of mouse MOG protein (399) was synthesized by GenScript, Inc (Piscataway, NJ). To obtain the fusion protein $rMOG_{29-146}$-pσ1, rMOG was amplified with appropriate pairs of primers. Both 5' and 3' primers contained *EcoR*I site for cloning. The 5' primer also contained ATG initiation codon embedded into an optimal Kozak's sequence. The 3' primer was designed to be in-frame to pσ1. The PCR product was gel-purified and cloned into an intermediate topocloning vector. The insert was excised by cutting with *EcoR*I and gel-purified again, resulting in *EcoR*I ends. This fragment was then inserted into pPICσ1-1, a yeast expression cassette featuring the whole pσ1 coding region with an upstream *EcoR*I site. In pPICσ1-1, pσ1 is produced as a His-tag fusion protein for affinity purification on Talon metal affinity resin (BD Bioscience, Palo Alto, CA). Correct orientation of rMOG was determined by DNA sequencing. The junction between the rMOG and pσ1 featured a flexible linker (Pro-Gly) to minimize steric hindrance between the components. To produce recombinant MOG (rMOG) without pσ1, MOG_{29-146} was amplified with 5' and 3' primers containing and *EcoR*I and *Sal*I site, respectively. The PCR product was gel-purified and cloned into an intermediate topocloning vector. After digestion with *EcoR*I and *Sal*I rMOG was cloned into pPICB vector cut with *EcoR*I and *Xho*I. The resulting constructs were sequenced and expressed in the yeast *P. pastoris*, according to the manufacturer's

directions (Invitrogen Corp.). Recombinant proteins were extracted from yeast cells by a bead-beater (Biospec Products, Bertlesville, OK) and purified on a Talon metal affinity resin (BD Biosciences), according to manufacturer's instructions. Proteins were assessed for purity and quality by Coomassie-stained polyacrylamide gels and by Western blot analysis, using a monoclonal mouse anti-His C-terminal (Invitrogen) or a polyclonal rabbit anti-pσ1 Ab (produced in-house). All recombinant proteins migrated as a single band of the expected MW and showed proper immuno-reactivities.

Mice

Female six-week old C57BL/6N mice (Frederick Cancer Research Facility, National Cancer Institute, Frederick, MD) were used thought the study. IL-$10^{-/-}$ mice breeder pairs were obtained from The Jackson Laboratory to establish our breeding colony. Mac-$1^{-/-}$ and μMT mice were a generous gift from Dr. Allen Harmsen at Montana State University, Bozeman, Montana. All mice were maintained at Montana State University Animal Resources Center under pathogen-free conditions in individual ventilated cages under HEPA-filtered barrier conditions and were fed sterile food and water *ad libitum*. The mice were free of bacterial and viral pathogens, as determined by antibody screening and histopathological analysis of major organs and tissues. All animal care and procedures were in accordance with institutional policies for animal health and well-being.

Tolerance Induction, and MOG-pσ1 Treatment

For tolerance induction, mice (5 mice/group) were nasally dosed before EAE challenge, as described in the text, with a single dose of 50 μg of MOG-pσ1. Control groups were nasally dosed with PBS, or with rMOG (50 μg or 12.5 μg).

For MOG-pσ1 treatment, mice were nasally dosed with 50 μg of MOG-pσ1 10, 11, 17, or 20 days after EAE induction, as designated in the text. Control groups were treated with PBS. MOG-pσ1 was administered nasally in a volume of no more than 20 μl / dose up to four times a day, with no less than 2.5 hour intervals between each administration (89).

EAE Induction

For EAE induction, mice were challenged s.c. in flank with 150 μg of the encephalitogenic MOG peptide (MOG_{35-55}; MEVGWYRSPFSRVVHLYRNGK; Global Peptide Services, LLC, Ft. Collins, CO; HPLC-purified to > 90 %) in 100 μl of CFA (Sigma-Aldrich) containing 4 mg/ml *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI, USA) on day 0. On day 7, mice received second 150 μg dose of MOG_{35-55} peptide in CFA containing 1.5 mg/ml of *M. tuberculosis*. On days 0 and 2 post-primary challenge, mice received i.p. 200 ng of *Bordetella pertussis* toxin (PT; List Biological Laboratories, Campbell, CA) as described (235). Mice were monitored and scored daily for disease progression (78): 0, normal; 1, a limp tail; 2, hind limb weakness; 3, hind limb paralysis; 4, quadriplegia; 5, death.

Ab ELISA

Serum and fecal samples collection was performed, as previously described (89). rMOG-specific endpoint Ab titers were measured by ELISA, as previously described (46), using purified rMOG as coating Ag. Specific reactivity to rMOG was determined using HRP conjugates of goat anti-mouse IgG-, and IgA-specific Abs (1.0 μg/ml; Southern Biotechnology Associates, Birmingham, AL), and ABTS (Moss Inc., Pasadena, CA) enzyme substrate. The absorbences were measured at 415 nm on a Kinetics Reader model ELx808 (Bio-Tek Instruments). Endpoint titers were expressed as the reciprocal dilution of the last sample dilution, giving an absorbence of 0.1 OD units above the OD_{415} of negative controls after 1 h incubation (78, 357).

Measurement of Delayed-Type Hypersensitivity (DTH) Responses

To measure MOG_{35-55}- specific DTH responses *in vivo* (46), 10 μg of MOG_{35-55} were injected into the left ear pinna, and PBS alone (20 μl) was administered to the right ear pinna as a control. Ear swelling was measured 24 h later with an electronic digital caliper (World Precision Instruments, Sarasota, FL). The DTH response was calculated as the increase in ear swelling after Ag injection following subtraction of swelling in the control site injected with PBS.

Histological Evaluation of Spinal Cords

For histological evaluation of tissue pathology, spinal cords were removed 18 days after challenge and fixed with neutral buffered formalin (VWR International, West Chester, PA), embedded into paraffin, and sectioned at 5 μm. Cross sections of spinal

cords were stained with H&E for pathological changes and inflammatory cell infiltration. Adjacent sections were stained with luxol fast blue (LFB) and examined for loss of myelin. Pathological manifestations were scored separately for cell infiltrates and demyelination. Each H&E section was scored from 0 to 4: 0, normal; 1, cell infiltrate into the meninges; 2, one to four small focal perivascular infiltrates; 3, five or more small focal perivascular infiltrates and/or one or more large infiltrates invading the parenchyma; 4, extensive cell infiltrates involving 20 % or more of the white matter (33, 78). In each LFB stained section, myelin was also scored from 0 to 4: 0, normal; 1, one small focal area of demyelination; 2, two or three small focal areas of demyelination; 3, one to two large areas of demyelination; 4, extensive demyelination involving 20 % or more of white matter (33, 78).

Cytokine ELISA

Spleens, mesenteric lymph nodes (MLNs), and head and neck LNs (HNLNs) were aseptically removed 18 - 21 days after EAE induction from PBS-, MOG-pσ1- and rMOG- dosed mice. Lymphocytes were prepared, as previously described (33), and resuspended in complete medium (CM) (34). Lymphocytes were cultured in 24-well tissue plates at 5 x 10^6 cells/ml in CM alone or in the presence of MOG_{35-55} (30 μg/ml) in a total volume of 1 ml for three to five days at 37º C. Bead-isolated $CD4^+$, $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells were cultured for the total of 3 days in the presence of 1:1 ratio of T cell-depleted splenic feeder cells with or without Ag re-stimulation (89). The supernatants were collected by centrifugation and stored at -80º C. Capture ELISA was employed to quantify, on at least triplicate sets of samples, the levels of IFN-γ, IL-4, IL-

10, IL-13, IL-17, and TGF-β produced by lymphocytes, as previously described (33). For detection of IL-21 IL- 22 , IL-27, or IL-28 , microtiter wells were coated with 2 μg/ml of purified Abs: goat anti-mouse IL-21 pAb, goat anti-mouse IL-22 pAb, goat anti-mouse IL-27p28 pAb, and anti-mouse IL-28B/IFN-λ3 mAb (clone 244716) (all from R&D Systems, Minneapolis, MN). After blocking with PBS + 1% BSA for 2h at 37º C, washed wells were incubated with cell culture supernatants at 4º C for 24 h. After washing, 0.5 μg/ml biotinylated Abs: goat anti-mouse IL-21 pAb, goat anti-mouse IL-22 pAb, goat anti-mouse IL-27p28 pAb, or rat anti-mouse IL-28 mAb (clone 244707) (R&D Systems) was added, respectively, for 90 min at 37º C. Following washing, 1:500 HRP-goat anti-biotin Ab (Vector Laboratories, Inc., Burlingame, CA) was added for 1h at room temperature (RT). After washing, ABTS peroxidase substrate (Moss, Inc.) was added to develop the reaction.

FACS Analysis

Lymphocytes from the HNLNs, MLNs, and spleens were isolated 18 days after challenge and single cell suspensions were prepared, as described above (33). Cells were stained for FACS analysis using conventional methods. T cell subsets were analyzed using fluorochrome-conjugated mAbs for CD4, CD25, B220, CTLA-4 (all from BD Pharmingen), and PE-conjugated CD73 (eBiosicence), and biotinylated TGF-β (R&D Systems). Purified rabbit polyclonal CD39 Ab was purchased from Santa Cruz Biotechnology and biotinylated in-house. Intracellular staining for FoxP3 was accomplished using FITC-, Cy-, or PE-anti-Foxp3 mAb (clone FJK-16s; eBioscience, San Diego, CA), PE, or APC- anti-IL-10 and anti-IL-4, (all from BD Pharmingen).

Bound fluorescence was analyzed with a FACS Canto (BD Biosciences, Mountain View, CA).

Adoptive Transfer Studies

Following MOG-pσ1 immunization, total $CD4^+$ T cells from spleens, HNLNs, and MLNs were obtained (negative $CD4^+$ T cell isolation kit, Dynal Biotech ASA, Oslo, Norway). $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were isolated from total $CD4^+$ T cells with > 94 % and 97 % purity, respectively, by positive selection using CELLection Biotin Binder Kit (Dynal Biotech; Invitrogen) and biotin-conjugated anti-mouse CD25 mAbs (PC61, eBioscience), according to manufacture's instructions.

B cells were isolated (negative B cell isolation kit, Dynal Biotech) from spleens, HNLNs, and MLNs of C57BL/6 mice 14 days after nasal administration of MOG-pσ1 or PBS. To test the efficacy of MOG-pσ1-primed T and B cells in rescuing diseased mice from EAE, 6×10^5 of $CD25^-CD4^+$ T cells or $CD25^+CD4^+$ T_{reg} cells, or 2×10^7 of B cells were i.v. injected into naïve recipients. Group of control mice was i.v. injected with sPBS.

Statistical Analysis

The ANOVA followed by posthoc Tukey test was applied to show differences in clinical scores in treated vs. PBS mice. The student *t* test was used to evaluate the differences between variations in cytokine level production, and *p*-values < 0.05 are indicated, unless specified otherwise.

Results

Single Nasal Dose of Pσ1-Delivered rMOG, Protects Mice from Clinical Manifestation of EAE via Induction of MOG-Specific Tolerance

Previously, we demonstrated that nasal administration of a single dose of OVA-pσ1 can induce long-lasting and sustainable tolerance to OVA (89). When applied to prevent autoimmunity, pσ1-based delivery system was proven efficient in mediating auto-Ag-specific tolerance and in generating protection against $PLP_{139-151}$-induced relapsing-remitting (RR) EAE in susceptible SJL mice.

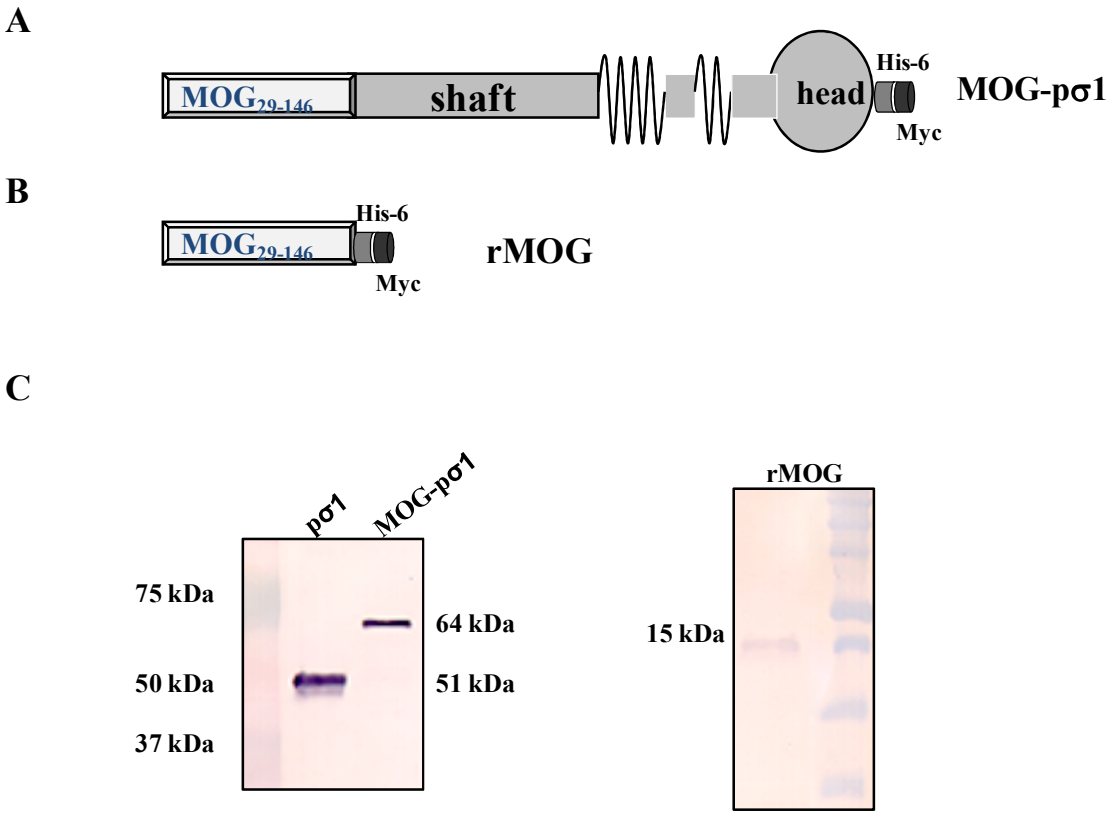
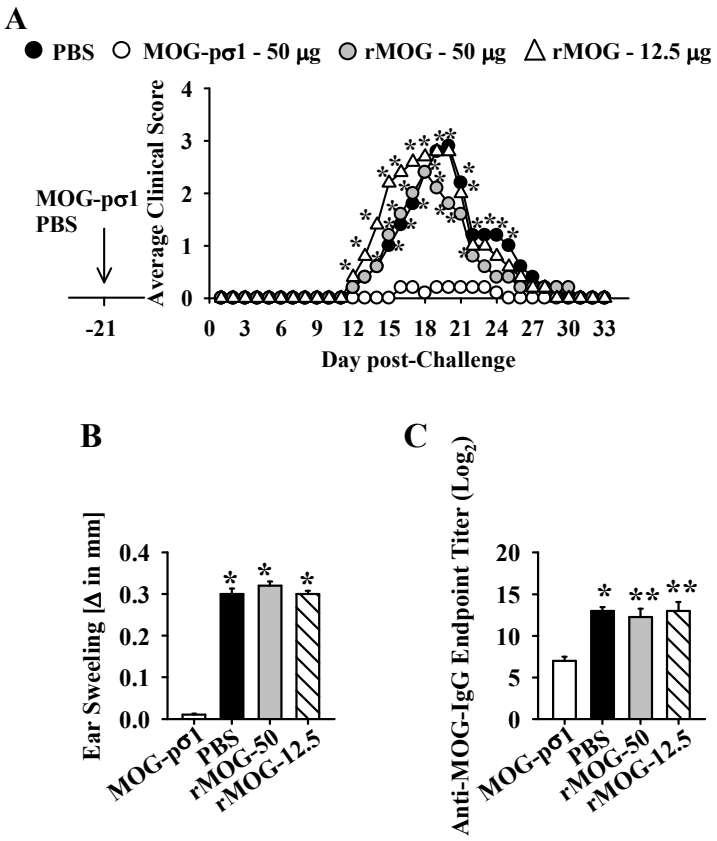


Figure 4.1. Schematic representation of MOG-pσ1 protein and rMOG. (*A*) MOG-pσ1 contains from the N-terminus: MOG_{29-146}, pσ1 (shaft and head), 6 histidine-tag, and Myc Ag-tag.

Figure 4.1. – continued. Recombinant MOG (rMOG) contains from the N-terminus: MOG_{29-146}, 6 histidine-tag, and Myc Ag-tag (components are not drawn to scale) (*B*) Western immunoblot of MOG-pσ1 (64 kDa) and pσ1 (51 kDa) probed with rabbit anti-pσ1 polyclonal Ab (left panel), and rMOG (15 kDa) probed with mouse IgG anti-histidine tag mAb (left panel).

To test the efficacy of pσ1-based vaccine in treating acute EAE, the extracellular domain of myelin oligodenrocyte glycoprotein (MOG_{29-146}) was genetically fused to pσ1, termed MOG-pσ1 (Figure 4.1*A* and *C*-left panel) and tested in susceptible C57BL/6 mice. Mice were nasally dosed with PBS, 50 μg of MOG-pσ1 or recombinant MOG (rMOG) (Figure 4.1*B* and *C*-right panel), or with 12.5 μg rMOG, which represents an equimolar amount of MOG delivered by pσ1. Three weeks later, all mice were challenged with MOG_{35-55} peptide to induce EAE, as previously described (235). In contrast to severe relapsing-remitting onset of EAE in SJL mice, C57BL/6 mice, when challenged with MOG_{35-55} peptide, develop acute disease that is less severe and not characterized by clinical relapses (164, 400). Unlike SJL mice that often succumb at the peak of the acute disease, MOG_{35-55}-challenged C57BL/6 mice usually reach the maximal clinical score of three, and generally all mice recover from the disease. In our experiments, C57BL/6 mice that received PBS prior to EAE induction developed classical onset of the acute EAE. The initiation of clinical symptoms occurred around day 13 post-primary challenge (p.p.ch) in PBS-dosed mice, and progression was observed until the peak (around day 19, p.ch) when mice exhibited the average clinical score of 3 (Figure 4.2*A*, Table 4.1). After day 21, p.ch, clinical signs gradually declined, and all mice entirely recovered from acute disease around day 33, p.ch, (Figure 4.2*A*, Table 4.1). Nasal administration of 50 or 12.5 μg of rMOG did not prevent clinical manifestation of

EAE in mice. C57BL/6 mice dosed with 12.5 μg of rMOG developed EAE around day 12, p.ch, and even though the disease progressed faster in these mice when compared to PBS-dosed mice, both groups reached the peak of the disease and remitted from EAE at the similar time. Ameliorated, although not statistically relevant, was the disease observed in mice dosed with 50 μg of rMOG, when compared to PBS-dosed mice (Figure 4.2*A*, Table 4.1). In contrast to PBS- and rMOG-dosed diseased mice, nasal administration of 50 μg of MOG-pσ1 almost entirely prevented the occurrence of clinical EAE, and in fact only one out of five mice in this group developed the clinical score of 1 on day 16, p.ch, (Figure 4.2*A*, Table 4.1).



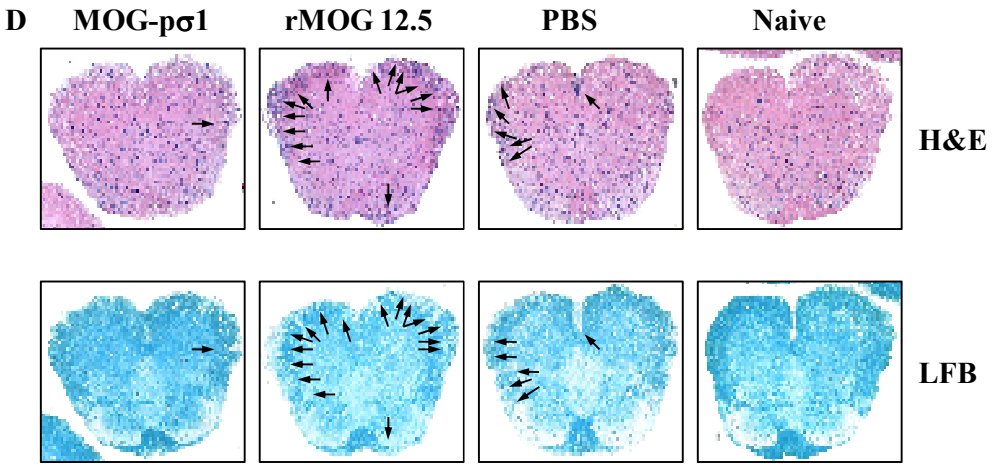


Figure 4.2. Nasal administration of MOG-pσ1 protects mice against MOG_{35-55}-induced acute EAE. C57BL6 mice were dosed with 50 μg of MOG-pσ1 or rMOG, 12.5 μg of rMOG, or sterile PBS on 3 wks prior to EAE induction. Mice were challenged with MOG_{35-55} peptide on day 0 and seven days later, and were monitored for the development of clinical disease. (*A*) Mice dosed with PBS and different doses of rMOG developed fully pronounced EAE and recovered by day 30 p.ch. In contrast to these mice, MOG-pσ1 immunization prior to EAE induction rendered mice resistant to the clinical disease, except one mouse that developed clinical score of 1 on day 16 p.ch. Average of 5-10 mice per group is shown. *, $p < 0.001$ vs. MOG-pσ1-dosed mice. (*B, C*) C57BL/6 mice were dosed with 50 μg of MOG-pσ1 or rMOG, 12.5 μg of rMOG, or with PBS one wk prior to EAE induction with MOG_{35-55} peptide. (*B*) Anti-MOG_{35-55} DTH response measured two weeks after EAE induction confirmed that unlike in PBS- or rMOG-dosed mice, MOG-pσ1-dosed mice showed striking reduction in MOG_{35-55}-specific Th1 cells. Mean ± SEM of 10 mice per group is depicted. *, $p < 0.001$ vs. MOG-pσ1-dosed mice. (*C*) Presence of rMOG-specific Abs in serum of these mice was analyzed by ELISA 28 days after EAE induction. Unlike MOG-pσ1-protected mice that showed significant reduction in rMOG-specific IgG Ab titers in serum, mice dosed with PBS or different doses of rMOG exhibited significant increase in rMOG-specific IgG Abs. Average ± SEM of 5 mice per group is shown. *, $p < 0.001$, **, $p < 0.05$ vs. MOG-pσ1-dosed mice. (*D*) Mice dosed with 50 μg of MOG-pσ1, 12.5 μg of rMOG, or with PBS 7 days before EAE induction were sacrificed at the peak of the disease (day 19, p.ch) and histopathology of their spinal cords was determined by staining with H&E (mononuclear cell infiltration) and LFB (demyelination). Mice dosed with MOG-pσ1 showed significant reduction in the CNS tissue pathology (designated by arrows) comparing to PBS- and rMOG-dosed not protected mice. Representative images of 5 mice/group are depicted. * $p < 0.001$ vs. MOG-pσ1-dosed mice.

Table 4.1. Nasal administration of MOG-pσ1 prior to MOG_{35-55} challenge protects C57BL/6 mice from EAE[a]

Treatment[b]	EAE/Total[c]	Onset[d]	Max. score[e]	CS[f]	Inflamm[g]	Demyelin[h]
PBS	5/5	13.8 ± 1.3*	3.5	21.7	2.2 ± 0.3*	2.7 ± 0.37*
MOG-pσ1	1/5	16 ± 0	1	1.6	0.1 ± 0.03	0.008 ± 0.001
rMOG 50 μg	5/5	13.6 ± 1.1*	3	18.3	2 ± 0.45*	2.5 ± 0.64*
rMOG 12.5 μg	5/5	13 ± 1*	3.5	24.3	2.8 ± 0.54*	3.2 ± 0.6*

[a] C57BL/6 mice were challenged s.c. with 150 μg MOG_{35-55} emulsified in complete Freund's adjuvant on day 0, and with 150 μg MOG_{35-55} emulsified in incomplete Freund's adjuvant on day 7. Mice received also 200 ng PT i.v. on days 0 and 2.
[b] Mice were nasally immunized 21 days prior to challenge with 50 μg of MOG-pσ1 or rMOG, 12.5 μg of rMOG, or with PBS
[c] Number of mice with EAE/total in group.
[d] Mean day ± SD of clinical disease onset.
[e] Maximum (Max.) daily clinical score.
[f] Cumulative scores (CS) were calculated as the sum of all scores from disease onset to day 33 post-challenge, divided by the number of mice in each group.
[g] Mean score ± SEM of inflammation: the infiltration of nucleated cells into spinal cords was scored from 0 to 4 in each mouse separately, and the mean score and SEM were calculated.
[h] Mean score ± SEM of demyelination: the demyelination in spinal cords was scored from 0 to 4 in each mouse separately, and the mean score and SEM were calculated.
*, $p < 0.001$ for PBS vs. MOG-pσ1-dosed mice.

Extracellular domain of MOG present in MOG-pσ1 contains both T and B cell epitopes (399). To test whether MOG-pσ1-mediated protection against EAE is due to the induction of rMOG-specific tolerance, mice nasally dosed with MOG-pσ1, rMOG, or PBS were induced with EAE seven days later. Presence of MOG_{35-55}-specific DTH responses was measured in mice two weeks, p.ch, with MOG_{35-55}. Consistent with protection against EAE, mice that received MOG-pσ1 exhibited virtual lack of MOG_{35-55}-specific memory Th1 cells, whereas the MOG_{35-55}-specific DTH response was very pronounced in unprotected PBS- or rMOG-dosed mice (Figure 4.2*B*). Presence of rMOG-specific Abs in serum was evaluated up to the fourth week p.ch in these mice. In

contrast to diseased PBS- and rMOG-dosed mice that showed significant increases in rMOG-specific IgG Ab titers in serum, MOG-pσ1-protected mice exhibited significant reduction in rMOG-specific IgG Abs even four weeks after challenge (Figure 4.2*C*). rMOG-specific IgA Abs were not detected in serum collected from mice in any experimental group (data not shown). These results indicate that MOG-pσ1-mediated protection against EAE is generated after just a single dose of the vaccine, and this protection is due to the long-lasting MOG-specific tolerance generated after nasal administration of MOG-pσ1.

Nasal MOG-pσ1 Prevents EAE-Associated CNS Pathology

Clinical manifestation of EAE is associated with the destruction of the CNS myelin by infiltrating inflammatory cells. To determine if nasally given MOG-pσ1 can prevent the CNS inflammation, spinal cord sections obtained from MOG-pσ1-protected and rMOG- or PBS-dosed diseased mice were examined for demyelination and mononuclear cell infiltration. Histological analysis revealed that mice dosed with MOG-pσ1 seven days before EAE induction showed virtually no inflammation and demyelination in the CNS when compared to PBS-dosed diseased mice (Figure 4.2*D*, Table 4.1). Interestingly, mice dosed with 12.5 μg of rMOG prior to EAE induction showed more severe infiltration of mononuclear cells and demyelination when compared to PBS-dosed mice, even though there were no significant differences between these two groups in clinical onset of EAE (Figure 4.2*A* and *D*). Mice that received 50 μg of rMOG before EAE induction exhibited similar levels of inflammation and demyelination when

compared to PBS-dosed mice and significantly more pronounced CNS histopathology when compared to MOG-pσ1-protected mice (Table 4.1).

MOG-pσ1-Induced Protection against EAE Is Associated with Suppression of Th1- and Th17-Type Proinflammatory Responses and Induction of Anti-Inflammatory Response

OVA-pσ1-induced tolerance to OVA (89) and PLP:OVA-pσ1-mediated protection against RR EAE were characterized by enhanced production of IL-10 and IL-4, and concomitant reduction in levels of proinflammatory Th1- and Th17-type cytokines. To determine if MOG-pσ1-mediated protection against acute EAE was associated with a similar cytokine response, we evaluated the cytokine profiles of lymphocytes isolated from HNLNs, MLNs, and spleens of mice dosed with MOG-pσ1, rMOG, or with PBS one week prior to EAE induction, and sacrificed at the peak of the clinical EAE (around day 20, p.ch). Lymphocytes obtained from MOG-pσ1-dosed mice did not produce IL-17, or IL-27, but secreted a marginal amount of IL-21 and IFN-γ (Figure 4.3*A*). In contrast to MOG-pσ1-protected mice, cells from PBS-dosed unprotected mice produced predominantly Th17-related cytokines, as evidenced by at least 8-fold enhancement in secretion of IL-17 and almost 2-fold increase in IL-21 when compared to MOG-pσ1-protected mice (Figure 4.3*A*). Negligible amounts of anti-inflammatory cytokines IL-4, IL-10, and also Th1-type cytokines, IFN-γ and IL-27, were secreted by cells isolated from PBS-dosed mice (Figure 4.3*A* and *B*). Mice given various doses of rMOG exhibited the very unique cytokine profile characterized by significant enhancement of Th1-type cytokines, IL-27 and IFN-γ, and concomitant depression of

Th17-type cytokines, IL-17 and IL-21, as well as a very pronounced reduction of anti-inflammatory IL-4 and IL-10 (Figure 4.3*A* and *B*). Consistent with our previous findings, MOG-pσ1-protected mice produced significantly more IL-4 and IL-10 in response to MOG_{35-55} stimulation than any diseased PBS- or rMOG-dosed mice (Figure 4.3*B*). When compared to PBS-dosed mice, lymphocytes isolated from MOG-pσ1-protected mice produced at least 3-fold more of IL-4, and over 3-fold more of IL-10. Minor concentrations of IL-13 were secreted by lymphocytes isolated from any experimental group, and no differences were observed in IL-13 production between protected and diseased mice (Figure 4.3*B*). These results indicate that MOG-pσ1-mediated protection is associated with increased production of IL-10 and IL-4 with concomitant inhibition of Th17- and Th1-type cytokines. Interestingly, what we have learned is, unlike the disease in PBS-dosed mice that is clearly mediated by encephalitogenic Th17 cells, EAE in rMOG-dosed mice is associated with the enhancement of Th1-type immune responses.

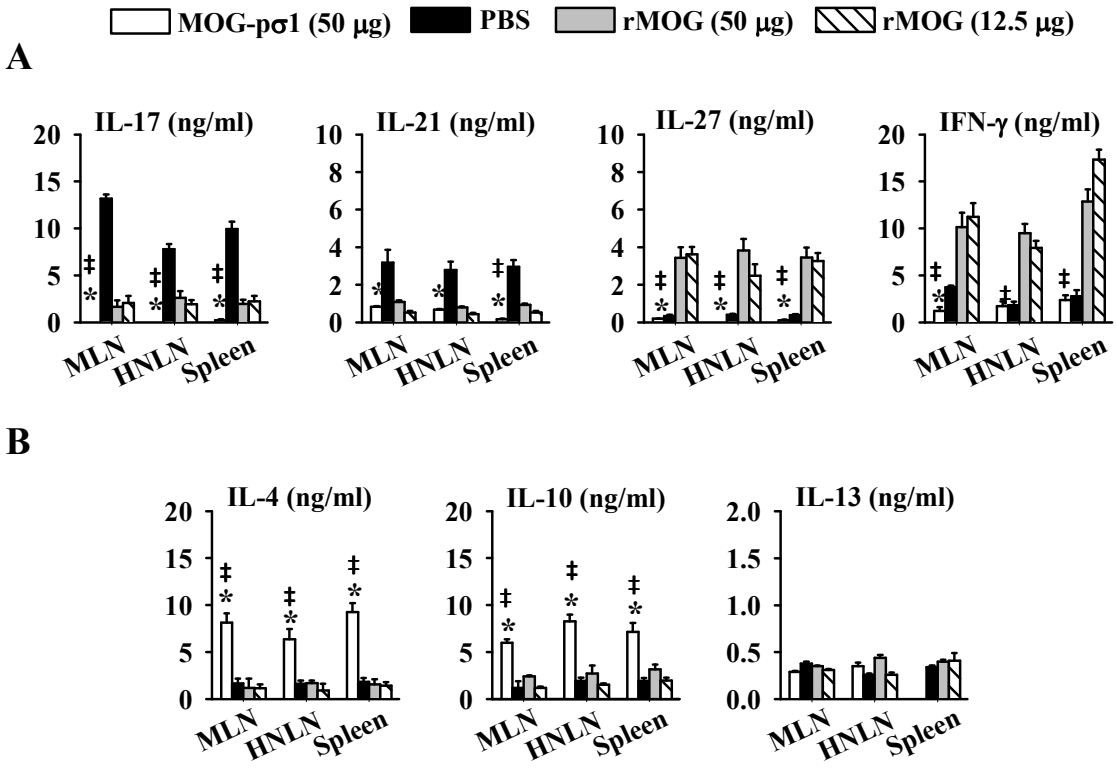


Figure 4.3. MOG-pσ1-mediated protection against EAE is associated with increased production of regulatory cytokines and depressed secretion of proinflammatory cytokines. Mice were immunized and challenged as described in Figure 4.2 *B* and *C* legend. At the peak of clinical disease, mice were sacrificed and lymphocytes from their HNLNs, MLNs, and spleens were isolated, *in vitro* (*A*) proinflammatory and (*B*) anti-inflammatory cytokines. Lymphocytes acquired from PBS-dosed mice produced mostly Th17-associated proinflammatory cytokines (IL-17 and IL-21), whereas cells from mice dosed with rMOG (50 and 12.5 μg) secreted predominantly Th1-type proinflammtory cytokines (IFN-γ and IL-27). In contrast to these mice, lymphocytes obtained from MOG-pσ1-protected mice produced IL-10, IL-4, but no inflammatory cytokines. Results show mean ± SD of 5 mice per group. *, $p < 0.05$ for MOG-pσ1 vs. PBS; ‡, $p < 0.05$ for MOG-pσ1 vs. rMOG (50 μg).

Single Nasal Dose of MOG-pσ1 Provides Immediate Relieve against EAE Clinical Symptoms in an Antigen-Dependent Manner

Our results described in a previous chapter of this manuscript indicated that as little as 100 μg of PLP:OVA-pσ1 given to the mice in the pre-clinical stage of EAE is sufficient to suppress clinical manifestation of the disease. Additionally, we showed here that 50 μg of nasally given MOG-pσ1 can almost entirely prevent clinical manifestation

and the CNS histopathology when given to mice prior to EAE induction. Here we asked whether MOG-pσ1 can be applied to treat ongoing clinical EAE. To answer this question, mice were induced with EAE, and when they reached the peak of the clinical disease, mice were treated with 50 μg of MOG-pσ1 or PBS nasally. Significant, almost 2-fold reduction in clinical symptoms was observed in MOG-pσ1-treated mice 24 h after treatment (Figure 4.4*A*). This immediate and dramatic drop in EAE clinical onset by MOG-pσ1-treated mice was followed by an 8-day recovery phase in which MOG-pσ1-treated mice exhibited an average clinical score of 1. All MOG-pσ1-treated mice recovered by day 30 p.ch. In contrast to MOG-pσ1-dosed mice, nasal administration of PBS did not improve clinical occurrence of EAE, and these mice followed the normal course of disease characterized by a gradual decline that led to complete recovery around 33 days after EAE induction (Figure 4.4*A*).

We previously reported that *in vitro* OVA-pσ1 induced clonal deletion of OVA-Tg $CD4^+$ T cells by apoptosis in a time and dose-dependent manner (89). To determine if this dramatic improvement in clinical disease after MOG-pσ1 treatment was due to the pσ1-induced apoptosis of encephalitogenic MOG-reactive $CD4^+$ T cells, mice challenged with MOG_{35-55} peptide were nasally treated with 50 μg of MOG-pσ1, rMOG, or pσ1, 50 μg of rMOG + 50 μg of pσ1, or with 100 μg of PLP:OVA-pσ1 on day 18 after EAE induction (Figure 4.4*B*). Mice were monitored daily for the development of clinical disease. Nasal treatment with 50 μg of MOG-pσ1 prevented progression of the disease. All MOG-pσ1-treated mice underwent dramatic remission within the first 24 hrs, and by the day 28, p.ch entirely recovered from EAE (Figure 4.4*B*-left panel). In contrast to

MOG-pσ1-rescued mice, mice that received PBS or rMOG on day 18, p.ch developed normal clinical disease and remitted gradually until completely recovered on day 30, p.ch. EAE onset in PLP:OVA-pσ1-treated mice resembled EAE observed in PBS-dosed mice (Figure 4.4*B*-right panel). However, mice that received pσ1 or pσ1 + rMOG on day 18, p.ch developed more severe clinical symptoms than PBS-dosed mice (Figure 4.4*B*-right panel). These results suggest that pσ1-mediated tolerance to the fused protein Ag is Ag-specific, and further confirm that N terminal fusion of protein Ag to pσ1 is critical for induction of pσ1-mediated tolerance. .

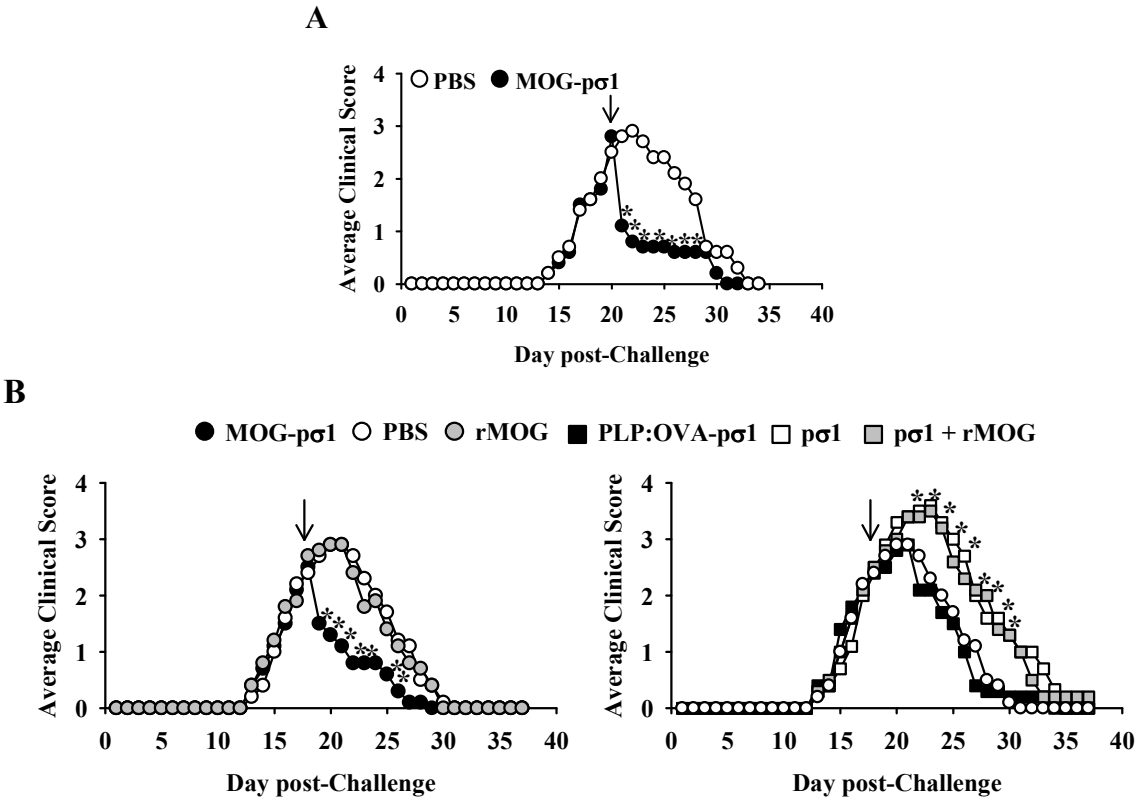


Figure 4.4. Nasal administration of MOG-pσ1 suppresses ongoing EAE. (*A*) C57BL/6 mice were dosed nasally with 50 μg of MOG-pσ1 or PBS at the peak of clinical disease (20 days after challenge with MOG_{35-55} peptide). Mice were monitored daily for the development of clinical EAE. Administration of PBS did not affect acute EAE. In contrast to PBS-treated mice, nasal administration of MOG-pσ1 at the peak of the disease drastically ameliorated clinical manifestation of EAE within 24 h. Average of 10 mice per group is depicted. *, $p < 0.05$ vs. PBS-dosed mice.

Figure 4.4. – continued. (*B*) C57BL/6 mice challenged with MOG_{35-55} were treated 18 days later with one of the following: PBS, 50 μg of MOG-pσ1, pσ1, or rMOG, 50 μg of rMOG + 50 μg of pσ1, or with 100 μg of PLP:OVA-pσ1. Mice were monitored daily for the development of clinical disease. Clinical manifestation of acute EAE was not altered in mice by nasal treatment with PBS, rMOG, or PLP:OVA-pσ1. In contrast to PBS-dosed mice, MOG-pσ1 given nasally to mice significantly reduced the average clinical score within first 24 h after treatment. Treatment with pσ1 or with rMOG + pσ1 induced further progression of EAE, and these mice developed more severe EAE than PBS-dosed unprotected mice. Average of 5 mice per group is depicted. *, $p < 0.05$ vs. PBS-treated mice.

MOG-pσ1-Mediated Protection against EAE Was Subverted in IL-$10^{-/-}$ Mice

Previously we reported that OVA-pσ1-mediated tolerance to OVA could not be induced in IL-$10^{-/-}$ mice (89). Additionally, we have shown that PLP:OVA-pσ1-mediated protection against RR EAE was compromised in the absence of IL-10-producing $CD25^{+}CD4^{+}$ T_{reg} cells. To determine whether IL-10 is essential for MOG-pσ1-mediated protection against acute EAE, we examined if MOG-pσ1 treatment during pre-clinical phase of EAE will suppress the disease in IL-$10^{-/-}$ mice. IL-$10^{-/-}$ and C57BL/6 mice were challenged with MOG_{35-55} and treated with 50 μg of MOG-pσ1 or with PBS on day 11, p.ch. MOG-pσ1 treatment of C57BL/6 mice entirely prevented development of clinical EAE (Figure 4.5*A*, Table 4.2). In contrast, the PBS-dosed C57BL/6 mice developed EAE around day 16, p.ch, and exhibited peak clinical score of 3 and recovered by day 30, p.ch. Clinical manifestation of EAE exhibited by PBS- or MOG-pσ1-treated IL-$10^{-/-}$ mice was more severe than the disease in PBS-dosed C57BL/6 mice (Figure 4.5*A*, Table 4.2). The average peak clinical score in MOG-pσ1- and PBS-dosed IL-$10^{-/-}$ mice was almost 4, and in contrast to PBS-dosed wt mice, MOG-pσ1- and PBS- treated IL-$10^{-/-}$ mice did not recover completely from acute disease (Figure 4.5*A*). On day 21, p.ch serum samples were collected from each group. In contrast to MOG-pσ1-dosed wt mice that showed

significant reduction in rMOG-specific IgG Abs in serum, PBS-dosed wt mice, as well as MOG-pσ1- and PBS-dosed IL-$10^{-/-}$ mice, exhibited significant enhancement in rMOG-specific IgG Ab titers in serum (Figure 4.5*B*). Additionally, in contrast to MOG-pσ1-dosed wt mice that after challenge with MOG_{35-55} remained unresponsive to this peptide, PBS-dosed wt mice, as well as IL-$10^{-/-}$ mice dosed with MOG-pσ1 or PBS, developed MOG_{35-55}-specific DTH response (Figure 4.5*C*). Consistent with the more pronounced EAE observed in IL-$10^{-/-}$ mice, these mice independent of treatment developed significantly higher MOG_{35-55}-specific DTH response than PBS-dosed wt mice (Figure 4.5*C*).

Next we analyzed the cytokine profiles of $CD4^+$ T cells isolated from HNLNs, MLNs, and spleens of MOG-pσ1- or PBS-dosed C57BL/6 and IL-$10^{-/-}$ mice, stimulated *in vitro* with MOG_{35-55}. $CD4^+$ T cells obtained from MOG-pσ1-treated wt mice produced 2-fold more of IL-4, and 4-fold more of IL-10 when compared to the $CD4^+$ T cells acquired from PBS-dosed C57BL/6 mice (Figure 4.5*D*-right column). On the other hand, these $CD4^+$ T cells isolated from MOG-pσ1-treated C57BL/6 mice produced marginal amounts of proinflammatory cytokines, and when compared to $CD4^+$ T cells obtained from the PBS-dosed C57BL/6 mice, MOG-pσ1-derived $CD4^+$ T cells produced at least 5-fold less IL-17, and IFN-γ and significantly less IL-21 (Figure 4.5*D*-left column). There were no significant differences in production of pro- or anti-inflammatory cytokines between IL-$10^{-/-}$ mice treated with MOG-pσ1 or with PBS (Figure 4.5*D*). $CD4^+$ T cells isolated from IL-$10^{-/-}$ mice independent of treatment produced at least 5-fold more IL-17, IL-21, and IFN-γ, when compared to $CD4^+$ T cells acquired from MOG-pσ1-treated

C57BL/6 mice (Figure 4.5*D*). Consistent with the higher average clinical score observed in MOG-pσ1- or PBS-dosed IL-$10^{-/-}$ mice when compared to PBS-dosed C57BL/6 mice, the C57BL/6 $CD4^+$ T cells obtained from PBS-dosed mice produced significantly less of IFN-γ and IL-21, but more IL-4 and IL-10 when compared to $CD4^+$ T cells isolated from PBS-dosed IL-$10^{-/-}$ mice. In contrast to $CD4^+$ T cells isolated from MOG-pσ1-treated C57BL/6 mice, > 4-fold reduction was observed in secretion of IL-4 and IL-10 by the IL-$10^{-/-}$ $CD4^+$ T cells isolated from PBS- or MOG-pσ1-dosed mice (Figure 4.5*D*-right column). Significantly more IL-13 was produced by $CD4^+$ T cells isolated from C57BL/6 mice when compared to IL-$10^{-/-}$ mice, and no difference in production of this cytokine was detected between MOG-pσ1- and PBS-dosed IL-$10^{-/-}$ mice (Figure 4.5*D*-right column). No difference in IL-22 and TGF-β secretion was observed by $CD4^+$ T cells obtained from C57BL/6 or and IL-$10^{-/-}$ mice (data not shown).

Table 4.2. MOG-pσ1-mediated protection against EAE cannot be established in IL-$10^{-/-}$ mice[a]

Treatment[b]	EAE/Total[c]	Onset[d]	Max. score[e]	CS[f]
PBS IL-$10^{+/+}$	10/10	16.3 ± 1.79*	3	20.8
MOG-pσ1 IL-$10^{+/+}$	0/10	0 ± 0	0	0
PBS IL-$10^{-/-}$	11/11	14.64 ± 1.15*	4	33.9
MOG-pσ1 IL-$10^{-/-}$	11/11	14.7 ± 1.88*	4	29.2

[a] WT C57BL/6 (IL-$10^{+/+}$) and IL-$10^{-/-}$ mice were challenged s.c. with 150 μg MOG_{35-55} emulsified in complete Freund's adjuvant on day 0, and with 150 μg MOG_{35-55} emulsified in incomplete Freund's adjuvant on day 7. Mice received also 200 ng PT i.v. on days 0 and 2.
[b] Mice were nasally immunized 11 days after challenge with 50 μg of MOG-pσ1 or with PBS (Figure 4.5).
[c] Number of mice with EAE/total in group.
[d] Mean day ± SD of clinical disease onset.
[e] Maximum (Max.) daily clinical score.
[f] Cumulative scores (CS) were calculated as the sum of all scores from disease onset to day 30 post-challenge, divided by the number of mice in each group. *, $p < 0.001$ for PBS vs. MOG-pσ1-dosed mice.

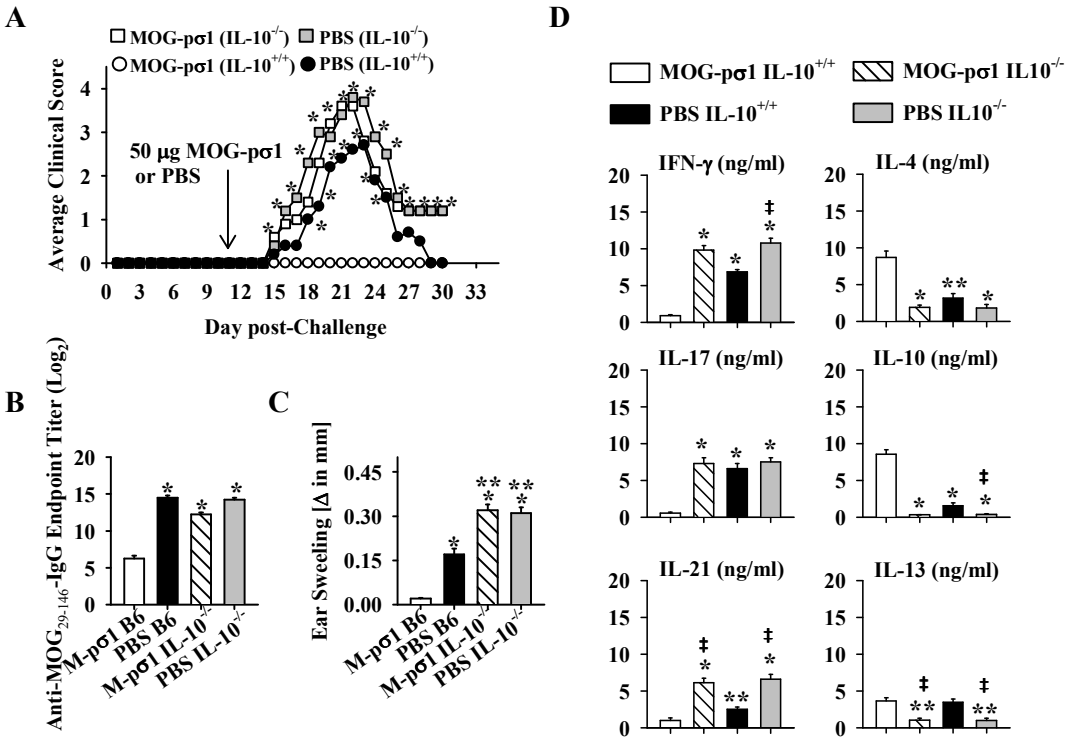


Figure 4.5. MOG-pσ1-mediated protection against EAE cannot be established in IL-10$^{-/-}$ mice. C57BL/6 and IL-10$^{-/-}$ mice were treated with 50 µg of MOG-pσ1 or with PBS 11 days after EAE induction with MOG_{35-55} peptide. (*A*) In contrast to MOG-pσ1-treated C57BL/6 mice that did not develop clinical symptoms of EAE, PBS-dosed IL-10$^{+/+}$ and IL-10$^{-/-}$ mice, as well as MOG-pσ1-treated IL-10$^{-/-}$ mice developed acute disease. Average of 10 mice per group is depicted. *, $p < 0.001$ vs. MOG-pσ1-treated C57BL/6 mice. (*B*) Serum samples were collected from these mice at the peak of the disease (d 21 p.ch), and the presence of rMOG-specific IgG Abs was evaluated by ELISA. MOG-pσ1-dosed C57BL/6 mice exhibited significantly depressed rMOG-specific IgG Abs in serum when compared to PBS-dosed IL-10$^{+/+}$ mice or MOG-pσ1- or PBS-dosed IL-10$^{-/-}$ mice. Mean ± SEM of 5 mice per group is depicted. *, $p < 0.001$ vs. MOG-pσ1-dosed C57BL/6 mice. (*C*) Presence of MOG_{35-55}-specific DTH response was measured in MOG-pσ1- and PBS-dosed and challenged C57BL/6 and IL-10$^{-/-}$ mice on day 28, p.ch. Virtual lack of MOG_{35-55}-specific DTH response was observed in MOG-pσ1-dosed C57BL/6 mice. In contrast to these mice, significantly stronger DTH response to MOG_{35-55} peptide was detected in PBS-dosed IL-10$^{+/+}$ mice. Even more pronounced MOG_{35-55}-specific DTH response was exhibited by MOG-pσ1- and PBS-dosed IL-10$^{-/-}$ mice when compared to PBS-dosed wt mice. Mean ± SEM of 5 mice per group is depicted. *, $p < 0.001$ vs. MOG-pσ1-dosed C57BL/6 mice; **, $p < 0.05$ vs. PBS-dosed C57BL/6 mice. (*D*) At the end of clinical EAE (day 28, p.ch), mice were sacrificed and CD4$^+$ T cells isolated from their HNLNs, MLNs, and spleens were cultured with 1:1 ratio of feeder cells and 30 µg/ml of MOG_{35-55} peptide. Cytokine production was measured from supernatants after 3 days of culture and corrected for cytokine levels produced by unstimulated cells. C57BL/6 CD4$^+$ T cells from mice dosed with MOG-pσ1 produced enhanced IL-13 when compared to CD4$^+$ T cells from MOG-pσ1- or PBS-dosed IL-10$^{-/-}$ mice. Significantly more IL-10, IL-4 was produced by C57BL/6 CD4$^+$ T cells from MOG-pσ1-dosed mice when compared to CD4$^+$ T cells obtained from diseased IL-10$^{+/+}$ and IL-10$^{-/-}$ mice. Proinflammatory cytokines were produced by diseased IL-10$^{-/-}$ and IL-10$^{+/+}$ mice. Mean ± SEM of 5-6 mice per group are depicted. *, $p < 0.001$, **, $p < 0.05$ vs. MOG-pσ1-dosed C57BL/6 mice; ‡, $p < 0.05$ vs. PBS-dosed C57BL/6 mice.

Nasally Given MOG-pσ1 in the Absence
of IL-10 Induces Proinflammatory Cytokines

To further verify cytokine profiles associated with MOG-pσ1-mediated protection against EAE in IL-$10^{+/+}$ mice and lack of this protection in IL-$10^{-/-}$ mice, we examined the production of various pro- and anti-inflammatory cytokines by $CD25^{+}CD4^{+}$ T_{reg} cells and $CD25^{-}CD4^{+}$ T cells obtained from these mice dosed with MOG-pσ1 or PBS one week prior to EAE induction. T_{reg} cells and $CD25^{-}CD4^{+}$ T cells were isolated from HNLNs, MLNs, and spleens of MOG-pσ1- or PBS-dosed C57BL/6 and IL-$10^{-/-}$ mice, and these were cultured for 3 days with equal ratio of feeder cells and MOG_{35-55} peptide. C57BL/6 $CD25^{-}CD4^{+}$ T cells from PBS-dosed mice produced significantly more IFN-γ, IL-17, and IL-21 when compared to $CD25^{-}CD4^{+}$ T cells from MOG-pσ1-dosed mice (Figure 4.6*A*). Consistent with lack of protection in MOG-pσ1-dosed IL-$10^{-/-}$ mice, $CD25^{-}CD4^{+}$ T cells isolated from these mice secreted significantly more of IFN-γ and IL-21 when compared to PBS-dosed C57BL/6 mice, and significantly more IFN-γ, IL-17, and IL-21 when compared to MOG-pσ1-dosed C57BL/6 mice (Figure 4.6*A*). There were no significant differences in production of pro- or anti-inflammatory cytokines by $CD25^{-}CD4^{+}$ T cells from IL-$10^{-/-}$ mice dosed with MOG-pσ1 or PBS. In contrast to proinflammatory cytokines, over 2-fold increase in secretion of IL-4 was observed in $CD25^{-}CD4^{+}$ T cells between MOG-pσ1-dosed and PBS-dosed. Moreover, significantly less IL-4 was obtained in $CD25^{-}CD4^{+}$ T cells isolated from MOG-pσ1-dosed IL-$10^{-/-}$ mice when compared to PBS-dosed C57BL/6 mice. Only a minute amount of IL-10 was derived from $CD25^{-}CD4^{+}$ T cells from either MOG-pσ1- or PBS-dosed C57BL/6 mice. $CD25^{-}$

$CD4^+$ T cells isolated from MOG-pσ1-dosed C57BL/6 mice produced significantly more IL-13 than MOG-pσ1- or PBS-dosed IL-$10^{-/-}$ mice; significantly more TGF-β than PBS-dosed C57BL/6 or IL-$10^{-/-}$ mice; and significantly more IL-28 than $CD25^-CD4^+$ T cells isolated from any other experimental group (Figure 4.6*A*).

T_{reg} cells were also sorted and evaluated for their production of cytokines. T_{reg} cells obtained from MOG-pσ1-protected C57BL/6 mice produced 11-fold more of IL-10 and 3-fold more of IL-28 than T_{reg} cells obtained from their PBS-dosed C57BL/6 mice (Figure 4.6*B*). Very little to no TGF-β, IL-13, nor IL-4 was produced by T_{reg} cells isolated from any experimental group. MOG-pσ1-derived C57BL/6 T_{reg} cells did not secrete IL-17, IL-21, and secreted considerably less IFN-γ when compared to PBS-dosed C57BL/6 mice and IL-$10^{-/-}$ mice dosed with MOG-pσ1 or PBS (Figure 4.6*B*). Equivalent amounts of IFN-γ and IL-17 produced by T_{reg} cells were obtained from PBS-dosed C57BL/6 mice and PBS- or MOG-pσ1-dosed IL-$10^{-/-}$ mice. Additionally, significantly more IL-21 was secreted by T_{reg} cells obtained from MOG-pσ1- or PBS-dosed IL-$10^{-/-}$ mice when compared to PBS-dosed C57BL/6 mice.

These results indicate that MOG-pσ1-mediated protection in C57BL/6 mice is associated with IL-10 production by T_{reg} cells, and IL-4 production by $CD25^-CD4^+$ Th2 cells.

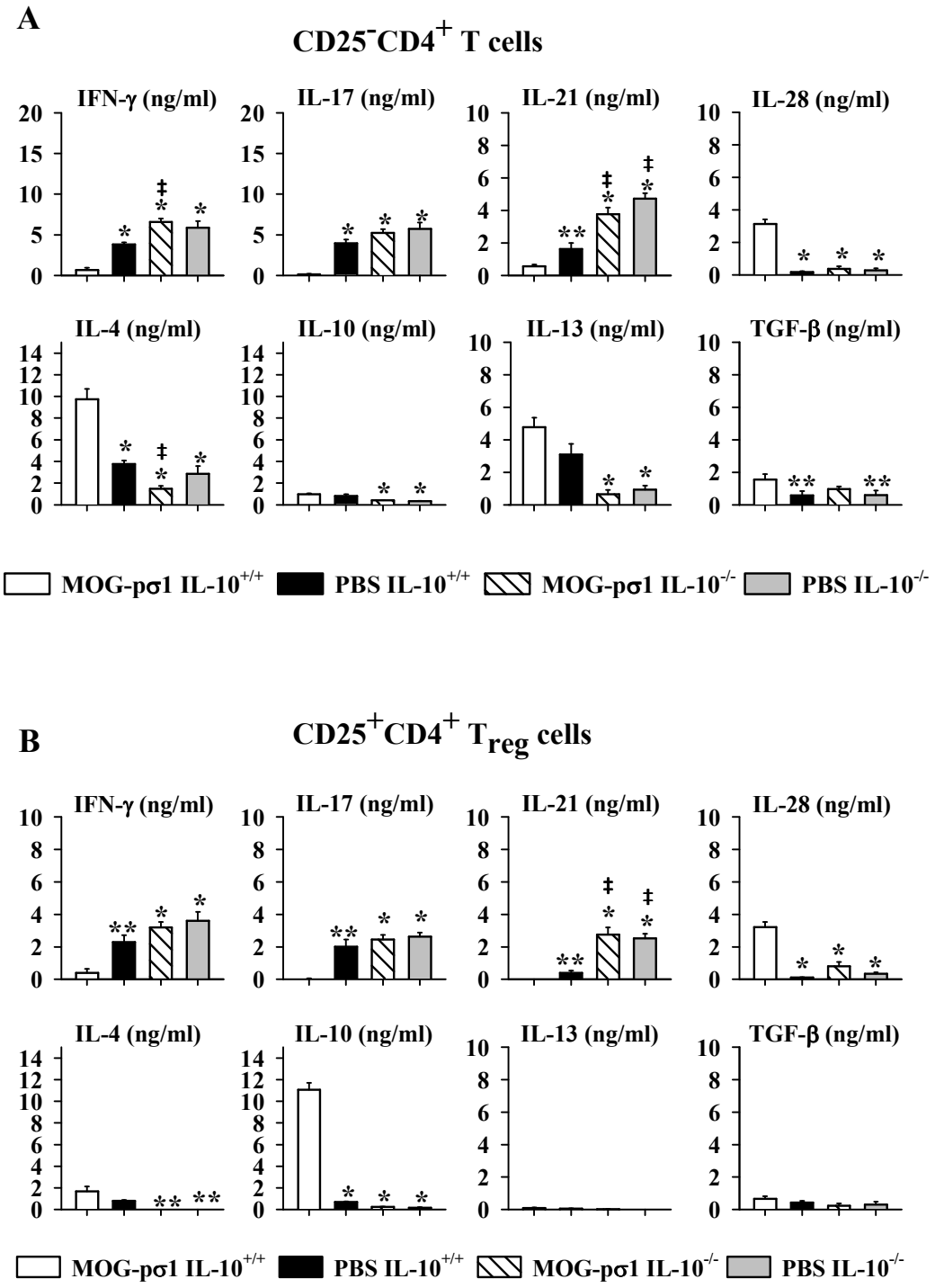


Figure 4.6. MOG-pσ1-mediated protection against EAE in C57BL/6 mice is mediated by IL-10-producing T cells, as evidenced by lack of protection in MOG-pσ1-dosed IL-10$^{-/-}$ mice.

Figure 4.6. – continued. (*A*) $CD25^{-}CD4^{+}$ T cells and (*B*) T_{reg} cells were purified from C57BL/6 and IL-$10^{-/-}$ mice that were dosed with 50 μg of MOG-pσ1 or PBS on day -7, challenged with EAE on day 0, isolated 21 days after EAE challenge, and co-stimulated *in vitro* with 30 μg/ml MOG_{35-55} peptide in the presence of APCs. Cytokine production was measured from supernatants after 3 days of culture and for cytokine produced by unstimmulated cells. (*A*) $CD25^{-}CD4^{+}$ T cells from diseased PBS-dosed C57BL/6 mice produced significantly more of IFN-γ, IL-17, and IL-21, but less of IL-4, IL-28, and TGF-β when compared to $CD25^{-}CD4^{+}$ T cells from MOG-pσ1-protected C57BL/6 mice. C57BL/6 $CD25^{-}CD4^{+}$ T cells from PBS- and MOG-pσ1-dosed mice produced more IL-13 than these cells from MOG-pσ1- and PBS-dosed IL-$10^{-/-}$ mice. $CD25^{-}CD4^{+}$ T cells from diseased IL-$10^{-/-}$ mice produced predominantly IFN-γ, IL-17, and IL-21. (*B*) T_{reg} cells from MOG-pσ1-protected C57BL/6 mice produced predominantly IL-10, but also IL-4 and IL-28. Marginal amounts of these cytokines were produced by T_{reg} cells from diseased IL-$10^{+/+}$ and IL-$10^{-/-}$ mice. IL-13 and TGF-β were not produced by T_{reg} cells from MOG-pσ1- and PBS-dosed IL-$10^{+/+}$ and IL-$10^{-/-}$ mice. T_{reg} cells from diseased MOG-pσ1- and PBS-dosed IL-$10^{-/-}$ mice produced elevated IL-21, IL-17, and IFN-γ when compared to MOG-pσ1-protected IL-$10^{+/+}$ mice. Equivalent amounts of IFN-γ and IL-17 were produced by T_{reg} cells from diseased IL-$10^{+/+}$ and IL-$10^{-/-}$ mice. Results depict mean ± SEM of 4-6 mice per group. *, $p < 0.001$; **, $p < 0.05$ vs. MOG-pσ1-dosed C57BL/6 mice, ‡, $p < 0.05$ vs. PBS-dosed C57BL/6 mice.

Table 4.3. Characterization of lymphocytes from combined LNs and spleens of PBS- and MOG-pσ1-dosed C57BL/6 mice[a]

Treatment[b]	$CD25^+CD4^+$ T_{reg} cells[c]	$CD25^-CD4^+$ Th2 cells[c]	B cells[c]
MOG-pσ1	27.19 ± 1.23*	74.6 ± 3.18	38.7 ± 5.38
$FoxP3^+$	96.28 ± 2.47	23.18 ± 1.58*	2.26 ± 0.37
	$FoxP3^+$ T_{reg} cells	**$FoxP3^+CD25^-$ Th2 cells**	
TGF-β^+	3.69 ± 0.41	9.22 ± 1.08*	0.39 ± 0.54
CTLA-4^+	27.48 ± 1.31	2.56 ± 1.12	8.18 ± 0.93
IL-10^+	77.17 ± 2.17*	7.57 ± 1.78	18.49 ± 1.29*
IL-4^+	15.68 ± 1.29*	76.29 ± 1.84*	7.2 ± 0.78*
$CD73^+$	64.2 ± 2.58*	22.6 ± 1.25*	2.18 ± 0.64
$CD39^+$	3.72 ± 0.48	2.89 ± 1.42	25.5 ± 2.84**
PBS	11.9 ± 1.18	89.73 ± 3.28	41.2 ± 4.18
$FoxP3^+$	89.74 ± 3.98	8.18 ± 0.73	1.07 ± 1.04
	$FoxP3^+$ T_{reg} cells	**$FoxP3^+CD25^-$ Th2 cells**	
TGF-β^+	3.44 ± 0.47	2.02 ± 0.34	0.94 ± 0.78
CTLA-4^+	25.82 ± 0.86	3.07 ± 1.28	9.18 ± 0.71
IL-10^+	11.54 ± 1.09	4.28 ± 2.69	4.16 ± 1.38
IL-4^+	4.53 ± 0.71	5.62 ± 1.12	3.48 ± 0.71
$CD73^+$	33.9 ± 3.29	10.7 ± 0.48	3.4 ± 1.16
$CD39^+$	2.83 ± 0.48	2.18 ± 0.74	16.8 ± 2.78

[a]C57BL/6 mice were challenged s.c. with 150 μg MOG_{35-55} emulsified in complete Freund's adjuvant on day 0, and with 150 μg MOG_{35-55} emulsified in incomplete Freund's adjuvant on day 7. Mice received also 200 ng PT i.v. on days 0 and 2.

[b] Mice were nasally immunized 21 days prior to challenge with 50 μg of MOG-pσ1 or with PBS.

[c]Percentages of $CD25^-CD4^+$ T cells, $CD25^+CD4^+$ T_{reg} cells, and B cells were analyzed by FACS on day 20 p.ch. $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells are presented as a proportion of $CD4^+$ T cells, B cells are presented as a proportion of lymphocytes

Mean ± SEM of combined HNLNs, MLNs, and spleens from 3 mice per group is presented.

*, $p < 0.001$, **, $p < 0.05$ for PBS vs. MOG-pσ1-dosed mice.

B Cells from MOG-pσ1-Treated Mice
Produce IL-10 in Response to EAE Challenge

The presence of IL-10 seems to be critical for MOG-pσ1-mediated protection against EAE, as evidenced by the lack of protection in MOG-pσ1-treated IL-$10^{-/-}$ mice. Moreover, we have shown here that T_{reg} cells isolated from MOG-pσ1-dosed C57BL/6 mice produced predominantly IL-10, whereas $CD25^{-}CD4^{+}$ T cells obtained from these mice produced IL-4. It has been demonstrated that in addition to $CD4^{+}$ T cells, other cell types, including B cells, can produce anti-inflammatory cytokines (98, 241, 401), and IL-10-producing B cells can be important in the recovery from EAE (236). To determine if nasal administration of MOG-pσ1 induces production of anti-inflammatory cytokines by B cells, FACS analysis was performed. C57BL/6 mice were dosed nasally with 50 μg of MOG-pσ1 or with PBS 21 days prior to EAE induction. Lymphocytes isolated at the peak of the clinical disease (day 20, p.ch) from their HNLNs, MLNs, and spleens were cultured for 72 h with MOG_{35-55}, stained for the expression of regulatory cells markers and analyzed by FACS. As expected, MOG-pσ1-protected mice generated significantly more $FoxP3^{+}CD25^{+}CD4^{+}$ T_{reg} cells and $FoxP3^{+}CD25^{-}CD4^{+}$ T cells than diseased PBS-dosed mice ($p < 0.01$; Table 4.3). Moreover, over 75 % of these T_{reg} cells from MOG-pσ1–protected mice produced IL-10, whereas as many $FoxP3^{+}CD25^{-}CD4^{+}$ T cells produced IL-4. Interestingly, we have observed nearly 50 % enhancement in expression of CD73 on $CD25^{+}$ and $CD25^{-}$ $CD4^{+}FoxP3^{+}$ T cells isolated from MOG-pσ1-protected mice when compared with these cells from PBS-dosed diseased mice (Table 4.3).

Analysis of B cells revealed induction of IL-10 and IL-4 expression by these cells obtained from MOG-pσ1-protected mice. MOG-pσ1-derived B cells produced over 4-fold more of IL-10 and 2-fold more of IL-4 when compared to the B cells obtained from PBS-dosed diseased mice (Table 4.3). Interestingly, B cells isolated from MOG-pσ1-protected mice expressed significantly more CD39 activation marker than B cells acquired from PBS-dosed unprotected mice.

B Cells Play Supportive Role in MOG-pσ1-Mediated Protection against EAE

To investigate the role of B cells in MOG-pσ1-mediated protection against EAE, we evaluated if this protection can be established in μMT mice that lack functional B cells. C57BL/6 and μMT mice were challenged with MOG_{35-55} peptide and 10 days later treated with 50 μg of MOG-pσ1 or with PBS. C57BL/6 mice treated with MOG-pσ1 were entirely protected against EAE development, and neither of MOG-pσ1-treated C57BL/6 mice showed clinical manifestation of EAE (Figure 4.7*A*, Table 4.4). In contrast to MOG-pσ1-protected C57BL/6 mice, PBS-dosed mice developed EAE around day 13, p.ch, and exhibited an average clinical score of 3 at the peak of EAE (day 20, p.ch). Unlike previous experiments, one C57BL/6 mice treated with PBS died at the peak of the disease (Table 4.4); however remaining mice from this group remitted entirely from EAE by day 28, p.ch (Figure 4.7*A*, Table 4.4). The μMT mice dosed with PBS during the pre-clinical stage of EAE developed lower average clinical scores (2.5) at the peak of the disease when compared to PBS-dosed C57BL/6 mice. However, these μMT mice did not remitted entirely from acute EAE, and one of the mice in this group

died at the peak of the disease (Figure 4.7*A*, Table 4.4). MOG-pσ1-treated μMT mice developed clinical disease that was significantly more severe when compared to MOG-pσ1-dosed C57BL/6 mice, though considerably less pronounced than EAE seen in PBS-dosed C57BL/6 mice (Figure 4.7*A*, Table 4.4).

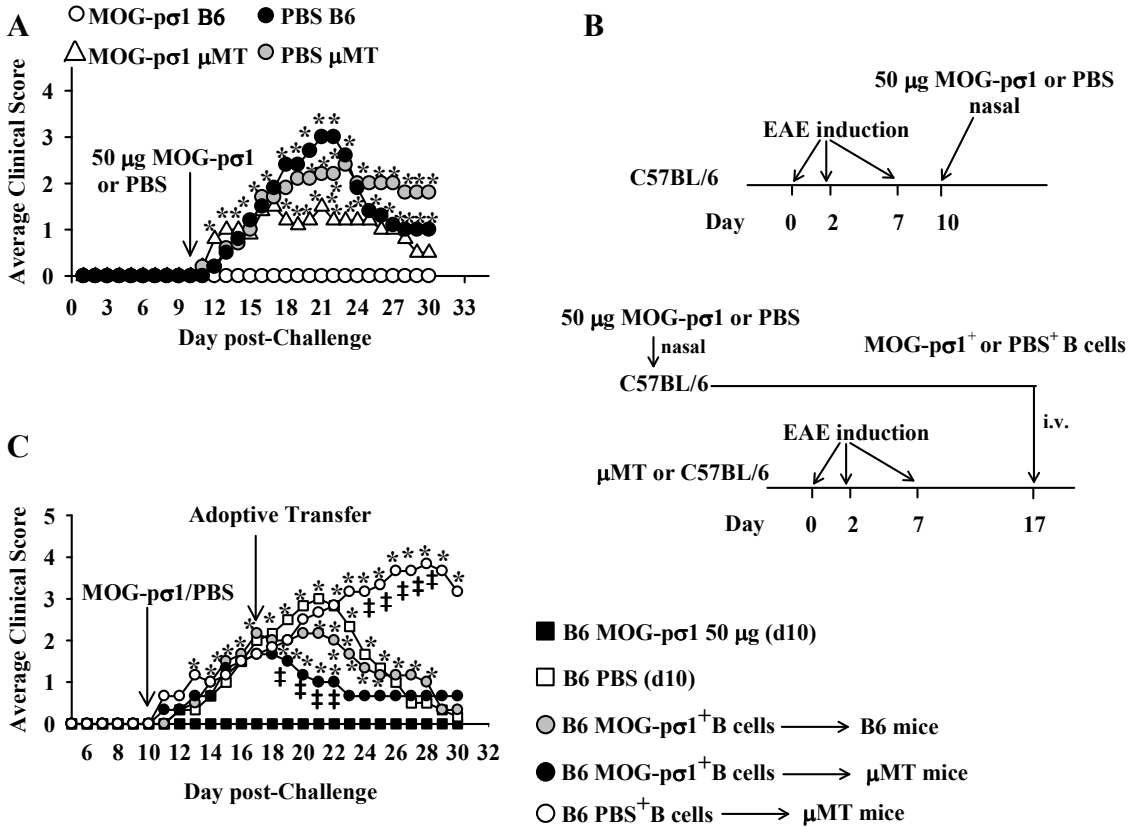


Figure 4.7. B cells play a supportive role in MOG-pσ1-mediated protection against EAE. (*A*) C57BL/6 and μMT mice were challenged with MOG_{35-55} peptide on day 0 and nasally treated with 50 μg of MOG-pσ1 or with PBS on day 10 after EAE induction. C57BL/6 mice treated with MOG-pσ1 did not exhibit clinical symptoms of EAE, whereas PBS-dosed mice developed acute EAE. MOG-pσ1-dosed μMT mice developed acute disease that was ameliorated when compared to PBS-dosed μMT and C57BL/6 mice, but significantly more pronounced when compared to MOG-pσ1-protected C57BL/6 mice. The average of 5 mice per group is shown. *, $p < 0.05$ vs. MOG-pσ1-dosed C57BL/6 mice. (*B*) Schematic presentation of the adoptive transfer experiment. The μMT mice were induced with EAE on day 0 and adoptively transferred with 2 x 10^7 of B cells isolated from MOG-pσ1- or PBS-dosed C57BL/6 mice.

Figure 4.7. – continued. A group of C57BL/6 mice was adoptively transferred with 2 x 10^7 of MOG-pσ1-primed C57BL/6 B cells 17 days after EAE induction with MOG_{35-55} peptide. Control C57BL/6 mice were nasally treated with 50 μg of MOG-pσ1 or with PBS 10 days after challenge with MOG_{35-55} peptide. (*C*) Nasal administration of MOG-pσ1 entirely protected wt mice against EAE, whereas PBS-dosed C57BL/6 mice showed acute EAE. Adoptive transfer of MOG-pσ1-primed C57BL/6 B cells inhibited progression of EAE in μMT mice and accelerated clinical remission; in contrast MOG-pσ1-primed C57BL/6 B cells into C57BL/6 mice inhibited disease progression though clinical remission from EAE was not accelerated in transferred wt mice. Interestingly, μMT mice adoptively transferred with normal (B6) B cells developed more severe and prolonged disease than any other experimental group. Average of 3-5 mice per group is depicted. *, $p < 0.05$ vs MOG-pσ1-dosed wt mice, ‡, $p < 0.05$ vs PBS-dosed wt mice.

Table 4.4. B cells play a supportive role in MOG-pσ1-mediated protection against EAE[a]

Treatment[b]	EAE/Total[c]	Onset[d]	Max. score[e]	CS[f]
PBS B6	5/5	13.6 ± 1.14*	5	30.9
MOG-pσ1 B6	0/5	0 ± 0	0	0
PBS μMT	5/5	14.2 ± 2.17*	5	32.4
MOG-pσ1 μMT	5/5	13.4 ± 2.41*	3	20

[a] WT C57BL/6 (B6) and μMT mice were challenged s.c. with 150 μg MOG_{35-55} emulsified in complete Freund's adjuvant on day 0, and with 150 μg MOG_{35-55} emulsified in incomplete Freund's adjuvant on day 7. Mice received also 200 ng PT i.v. on days 0 and 2.
[b] Mice were nasally immunized 10 days after challenge with 50 μg of MOG-pσ1 or with PBS (Figure 4.7*A*).
[c] Number of mice with EAE/total in group.
[d] Mean day ± SD of clinical disease onset.
[e] Maximum (Max.) daily clinical score.
[f] Cumulative scores (CS) were calculated as the sum of all scores from disease onset to day 30 post-challenge, divided by the number of mice in each group.
*, $p < 0.001$ vs. MOG-pσ1-dosed C57BL/6 mice.

To further investigate the relevance of B cells in MOG-pσ1-mediated protection against EAE, μMT mice were treated with MOG-pσ1- or PBS-primed C57BL/6 B cells (Figure 4.7*B*). Isolated B cells from HNLNs, MLNs, and spleens from MOG-pσ1- or PBS-dosed C57BL/6 mice were adoptively transferred 17 days after EAE induction into diseased μMT mice. Additionally, MOG-pσ1-derived wt B cells were also transferred into diseased C57BL/6 wt mice 17 days after EAE induction (Figure 4.7*B*-lower panel).

Control C57BL/6 mice were treated with MOG-pσ1 or with PBS at the pre-clinical stage of EAE on day 10 after EAE induction (Figure 4.7*B*-upper panel). C57BL/6 mice dosed with PBS in the pre-clinical phase of EAE were unprotected and developed an average clinical score of 3 at the peak of the disease (d 21 p.ch.). In contrast to these mice, nasally given MOG-pσ1 prevented occurrence of clinical EAE onset and all the mice in this group remained protected throughout the study (Figure 4.7*C*, Table 4.5).

Adoptive transfer of MOG-pσ1-primed B6 B cells into diseased wt mice inhibited further progression of EAE and these mice at the peak of clinical manifestation exhibited the average clinical score of 2 (Figure 4.7*C*, Table 4.5). Moreover, the remission from EAE in C57BL/6 mice adoptively transferred with MOG-pσ1^{+} B6 B cells was not significantly accelerated when compared to their PBS-dosed C57BL/6 mice, and both of these groups recovered entirely by day 31, p.ch. Adoptive transfer of MOG-pσ1-primed B6 B cells into diseased μMT mice prevented further progression of the disease, and induced accelerated remission when compared to PBS-dosed C57BL/6 mice (Figure 4.7*C*). On day 21, p.ch (peak of the clinical disease in PBS-dosed mice) MOG-pσ1^{+} B6 B cell-treated μMT mice exhibited the average clinical score of 1 and 60 % of these mice never remitted below this clinical symptom (Figure 4.7*C*). In striking contrast to μMT mice rescued by MOG-pσ1-primed B6 B cells, adoptive transfer of PBS-primed B cells significantly enhanced the disease in μMT mice (Figure 4.7*C*). On day 21 after challenge, these B cells from PBS-dosed B6 mice adoptively transferred into μMT mice developed an average clinical score of almost 3 and the progression of a disease was observed until day 28, p.ch when the average clinical score in this group reached almost 4

(Figure 4.7*C*, Table 4.5). The maximum clinical score observed in the μMT recipient mice given B cells from PBS-treated B6 mice was 4, meaning that none of the mice succumbed to EAE in this experimental group (Table 4.5). Interestingly, none of these mice entirely recovered from the disease, but all mice remitted until they reached the clinical score of 1 on day 40 after EAE induction (Table 4.5; data not shown).

Table 4.5. Adoptive transfer of MOG-pσ1-primed wt B cells ameliorated clinical manifestation of EAE in μMT mice[a]

Treatment[b]	EAE/Total[c]	Onset[d]	Max. score[e]	CS[f]
B6 PBS	3/3	13.61 ± 1.53*	3	27
B6 MOG-pσ1	0/3	0 ± 0	0	0
B6 + MOG-pσ1$^+$ B cells	3/3	13 ± 1*	2.5	25.7
μMT + MOG-pσ1$^+$ B cells	3/3	12.67 ± 1.53*	2	18.2
μMT + PBS$^+$ B cells	3/3	11.67 ± 1.15*	4	47.3

[a] WT C57BL/6 (B6) and μMT mice were challenged s.c. with 150 μg MOG_{35-55} emulsified in complete Freund's adjuvant on day 0, and with 150 μg MOG_{35-55} emulsified in incomplete Freund's adjuvant on day 7. Mice received also 200 ng PT i.v. on days 0 and 2.
[b] Mice were nasally immunized 10 days after challenge with 50 μg of MOG-pσ1 or with PBS or were adoptively transferred with MOG-pσ1- or PBS-primed wt B cells on day 17, p.p.ch as described in Figure 4.7*B* and *C* legend.
[c] Number of mice with EAE/total in group.
[d] Mean day ± SD of clinical disease onset.
[e] Maximum (Max.) daily clinical score.
[f] Cumulative scores (CS) were calculated as the sum of all scores from disease onset to day 30 post-challenge, divided by the number of mice in each group.
*, $p < 0.001$ vs. MOG-pσ1-dosed C57BL/6 mice

IL-10-Production by
MOG-pσ1-Induced $CD25^+CD4^+$ T_{reg} Cells
Is Essential for Protection against EAE

Our results so far have implicated that IL-10 is essential for MOG-pσ1-mediated protection against acute EAE, as evidenced by lack of protection in MOG-pσ1-treated IL-$10^{-/-}$ mice. We have demonstrated that MOG-pσ1-induced $FoxP3^+$ T_{reg} cells and B cells can produce IL-10 and MOG-pσ1-induced $FoxP3^+$ $CD25^-CD4^+$ T cells produce IL-4 in response to stimulation with MOG_{35-55} peptide. Additionally, adoptive transfer of MOG-pσ1-primed B6 B cells ameliorated clinical onset of EAE in μMT mice. To identify which cells are essential for MOG-pσ1-mediated protection against EAE, MOG-pσ1-primed $CD4^+$ T cells were adoptively transferred into IL-$10^{-/-}$ mice which were challenged with MOG_{35-55}. Seventeen days after EAE induction, mice were adoptively transferred with $CD25^+CD4^+$, $CD25^-CD4^+$ T cells or B cells sorted from MOG-pσ1-dosed C57BL/6 mice (Figure 4.8*A*). Control IL-$10^{-/-}$ mice treated i.v. with PBS developed EAE by day 10, p.ch and exhibited average clinical score of 4 at the peak of the disease (d 21 p.p.ch; Figure 4.8*B*). In contrast to PBS-dosed mice, adoptive transfer of MOG-pσ1-primed T_{reg} cells immediately inhibited progression of clinical disease, accelerated remission from EAE, and all these mice recovered by day 27, p.ch. Adoptive transfer of MOG-pσ1-primed $CD25^-CD4^+$ T cells or B cells also suppressed progression of clinical disease. Mice that received MOG-pσ1-primed $CD25^-CD4^+$ T cells started remitting from the disease significantly earlier when compared to PBS-dosed mice, but not as potently as mice adoptively transferred with T_{reg} cells. Mice given $CD25^-CD4^+$ T cells recovered entirely 3 days after those mice rescued with MOG-pσ1-derived T_{reg}

cells. Clinical remission from EAE in mice transferred with MOG-pσ1-primed B cells was just one day delayed when compared to $CD25^{-}CD4^{+}$ T cells-treated mice, though 30 % of these B-cell transferred mice continued to show clinical score of 1 and never entirely recovered from acute EAE (Figure 4.8*B*).

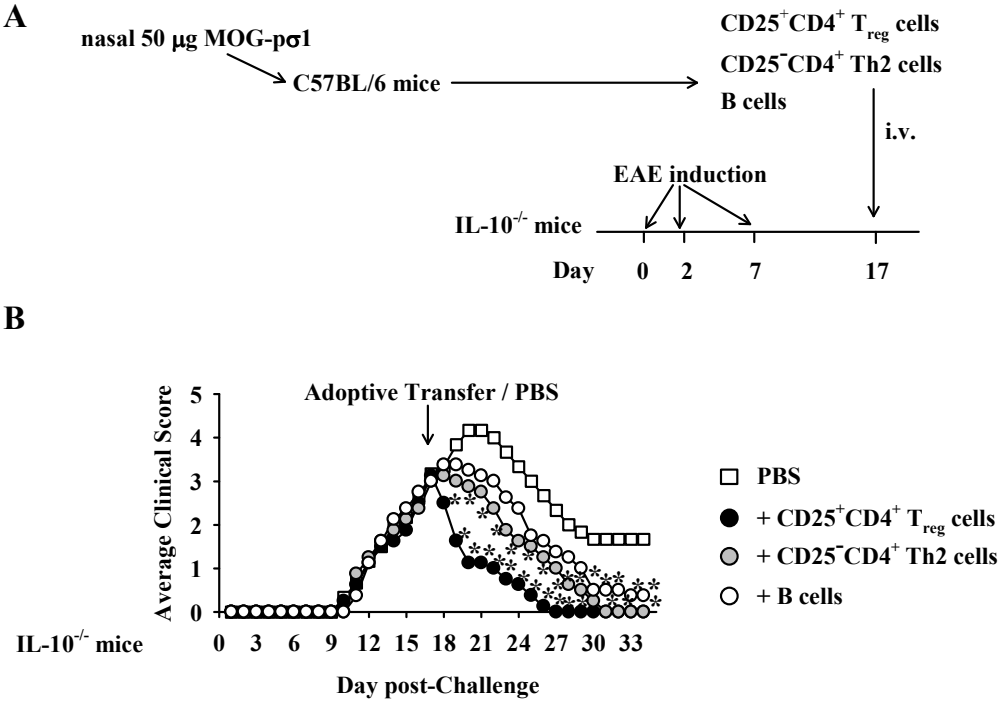


Figure 4.8. IL-10-producing T_{reg} cells are essential for MOG-pσ1-mediated protection against EAE. (*A*) Schematic presentation of adoptive transfer experiment. IL-$10^{-/-}$ mice were challenged with MOG_{35-55} peptide on day 0 and 7 and were adoptively transferred on day 17 with cells obtained from C57BL/6 mice nasally dosed with MOG-pσ1. IL-$10^{-/-}$ mice received one of the following populations of MOG-pσ1-primed wt cells: B cell (2 x 10^7 cell per mouse), $CD25^{-}CD4^{+}$ T cells (6 x 10^5 cells per mouse), or $CD25^{+}CD4^{+}$ T_{reg} cells (6 x 10^5 cells per mouse). A separate group of IL-$10^{-/-}$ mice received i.v. injection of PBS as a control. (*B*) PBS-dosed IL-$10^{-/-}$ mice developed acute EAE at day 10 p.ch, reached the average peak clinical score of 4, and never entirely recovered from the disease. In contrast to these mice, adoptive transfer of MOG-pσ1-derived T and B cells inhibited progression of EAE. IL-$10^{-/-}$ mice that received MOG-pσ1-derived $CD25^{-}CD4^{+}$ T cells or T_{reg} cells recovered entirely from acute disease. However, only IL-$10^{-/-}$ mice transferred with MOG-pσ1-primed T_{reg} cells remitted faster from the disease when compared to PBS-dose mice. Average of 3-4 mice per group is shown. *, $p < 0.05$ vs. PBS-dosed mice.

MOG-pσ1-Mediated Protection
against EAE Could Not Be Established
in CD11b-Deficient (CD11b$^{-/-}$) Mice

Expression of CD11b (Mac-1) on antigen presenting cells (APCs) was shown to be important for generation of low-dose mucosal tolerance (99). To test whether CD11b expression is necessary for MOG-pσ1-mediated protection against EAE, Mac-1$^{-/-}$ and C57BL/6 mice were nasally dosed with 50 μg of MOG-pσ1 or with PBS 21 days prior to the challenge with MOG_{35-55} peptide.

In our experiments, Mac-1$^{-/-}$ mice, independent of treatment, developed more severe acute EAE when compared to PBS-dosed C57BL/6 mice (Figure 4.9*A*). MOG-pσ1- and PBS-dosed Mac-1$^{-/-}$ mice displayed the average clinical score of 4 at the peak of the disease, whereas the average peak clinical score in PBS-dosed C57BL/6 mice was 3 (Figure 4.9*A*). In contrast to PBS-dosed C57BL/6 mice that recovered entirely from the acute disease by day 28, p.ch, MOG-pσ1- and PBS-dosed Mac-1$^{-/-}$ mice never recovered entirely from the acute disease, and exhibited the average score of 1 (mean ± SD for MOG-pσ1-treated Mac-1$^{-/-}$, 1 ± 0.2; PBS-treated Mac-1$^{-/-}$, 1 ± 0.3) until sacrificed. In agreement with our previous data, MOG-pσ1-dosed C57BL/6 mice remained entirely protected against EAE throughout the experiment. Consistent with lack of the disease in MOG-pσ1-dosed C57BL/6 mice, serum samples collected from these mice at the peak of the disease showed very little rMOG-specific IgG Abs, when compared to diseased C57BL/6 or Mac-1$^{-/-}$ mice (Figure 4.9*B*). In contrast to MOG-pσ1-protected C57BL/6 mice, significant increases in rMOG-specific IgG Ab titers were detected in serum

samples from PBS-dosed C57BL/6 mice, as well as MOG-pσ1- and PBS-dosed Mac-1$^{-/-}$ mice (Figure 4.9*B*).

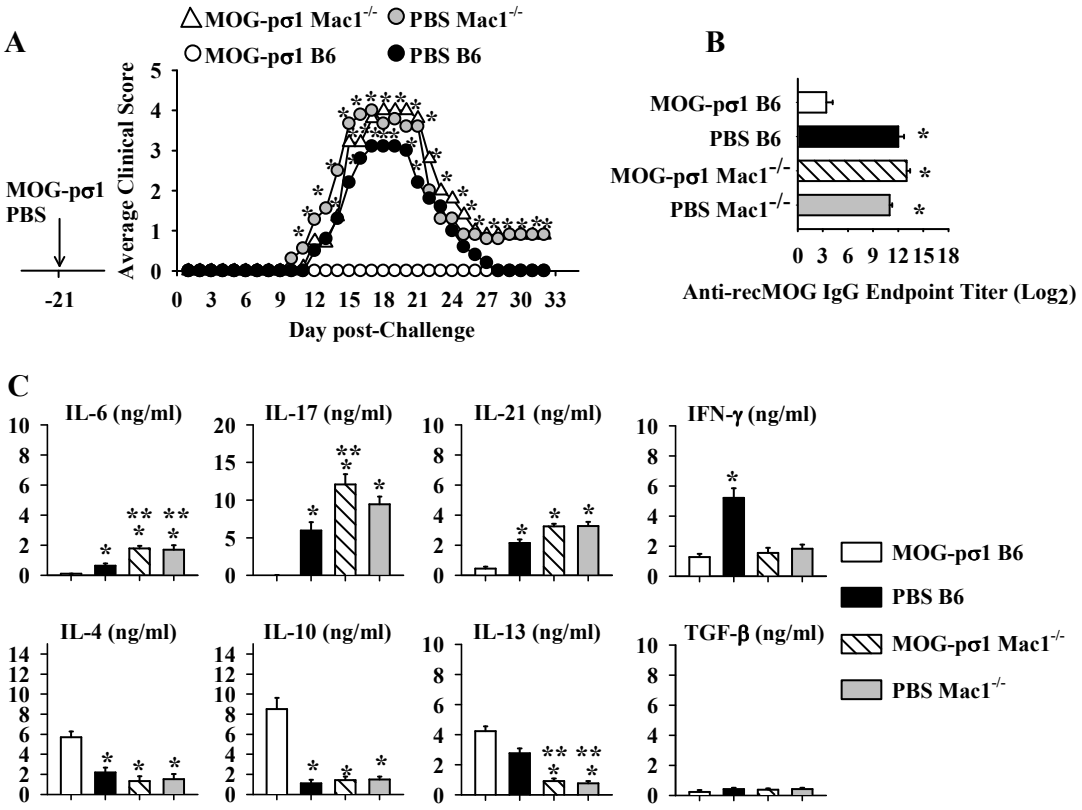


Figure 4.9. CD11b (Mac-1)-deficient mice are not protected against EAE by nasally given MOG-pσ1. C57BL/6 and Mac-1$^{-/-}$ mice were nasally dosed with 50 μg of MOG-pσ1 or PBS three wks prior to EAE induction and were challenge with MOG_{35-55} peptide on days 0 and 7. (*A*) MOG-pσ1-dosed wt mice were protected against EAE development, whereas PBS-dosed C57BL/6 mice developed EAE on day 12 p.ch and exhibited the average clinical score of 3 at the peak of the disease. In contrast to MOG-pσ1-protected C57BL/6 mice, Mac-1$^{-/-}$ mice developed severe EAE independent of treatment. Average of 10 mice per group is shown. *, $p < 0.001$ vs. MOG-pσ1-protected C57BL/6 mice. (*B*) Serum samples were collected from immunized and challenged C57BL/6 and Mac-1$^{-/-}$ mice at the peak of the clinical disease (day 21 after EAE induction) and were analyzed by ELISA for the presence of MOG-specific Abs. MOG-specific IgA Abs were not detected in any experimental group (data not shown). MOG-pσ1-protected C57BL/6 mice showed significant reduction in rMOG-specific serum IgG Abs. When compared to these mice, PBS-dosed wt mice as well as MOG-pσ1- and PBS-dosed Mac-1$^{-/-}$ mice produced significantly more MOG-specific IgG Abs. Average of 10 mice per group is depicted. *, $p < 0.001$ vs. MOG-pσ1-dosed C57BL/6 mice. (*C*) Purified CD4^{+} T cells were isolated from HNLNs, MLNs, and spleens from the immunized and challenged mice on day 28 after EAE induction. Cytokine production was measured from supernatants after 3 days of culture with MOG_{35-55} and feeder cells, and basal cytokine levels produced by unstimulated cells were subtracted from the results. CD4^{+} T cells from diseased PBS-dosed C57BL/6 mice and Mac-1$^{-/-}$ mice dosed with MOG-pσ1 or PBS produced predominantly proinflammatory cytokines.

Figure 4.9. – continued. In contrast to these mice, C57BL/6 $CD4^+$ T cells from MOG-pσ1-protected mice produced mostly anti-inflammatory cytokines (IL-4, IL-10, and IL-13). $CD4^+$ T cells from Mac-$1^{-/-}$ mice independent of treatment produced significantly more of IL-6, IL-17, and IL-21 than $CD4^+$ T cells from MOG-pσ1-protected wt mice. Additionally, $CD4^+$ T cells from PBS-dosed C57BL/6 mice produced more IFN-γ than $CD4^+$ T cells obtained from other experimental groups. Results show mean ± SEM of combined HNLNs, MLNs, and spleens from 8-10 mice per group. *, $p < 0.05$ vs. MOG-pσ1-dosed C57BL/6 mice; **, $p < 0.05$ vs. PBS-dosed C57BL/6 mice.

We have analyzed the cytokine profiles of $CD4^+$ T cells sorted from HNLNs, MLNs, and spleens of C57BL/6 and Mac-$1^{-/-}$ mice dosed with MOG-pσ1 or with PBS. $CD4^+$ T cells isolated from MOG-pσ1-protected wt mice produced very little to no proinflammatory cytokines (Figure 4.9*C*). When compared to PBS-dosed C57BL/6 mice and MOG-pσ1- and PBS-dosed Mac-$1^{-/-}$ mice, $CD4^+$ T cells from MOG-pσ1-protected C57BL/6 mice showed significant reduction in secretion of IL-21, and they did not produce any IL-17 and IL-6. Interestingly, $CD4^+$ T cells from MOG-pσ1-protected C57BL/6 mice produced as much of IFN-γ as $CD4^+$ T cells from diseased MOG-pσ1- or PBS-dosed Mac-$1^{-/-}$ mice, but significantly less when compared to PBS-dosed C57BL/6 mice. $CD4^+$ T cells from Mac-$1^{-/-}$ mice dosed with MOG-pσ1 or PBS produced more of IL-6, than $CD4^+$ T cells from PBS-dosed C57BL/6 mice, and MOG-pσ1-dosed Mac-$1^{-/-}$ $CD4^+$ T cells secreted more IL-17 than PBS-dosed C57BL/6 $CD4^+$ T cells. In agreement with the lack of the clinical disease in MOG-pσ1-protected C57BL/6 mice, $CD4^+$ T cells isolated from these mice showed over 2-fold enhancement in secretion of IL-4, and over 4-fold increase in IL-10 when compared to the $CD4^+$ T cells isolated from their PBS-dosed C57BL/6 mice (Figure 4.9*C*). IL-13 was produced by $CD4^+$ T cells from MOG-pσ1- and PBS-dosed C57BL/6 mice, and a significant depression of IL-13 production was exhibited by $CD4^+$ T cells from MOG-pσ1- or PBS-dosed Mac-$1^{-/-}$ mice. Virtually

no TGF-β was produced by any experimental group. These results indicate that lack of MOG-pσ1-mediated protection in Mac-$1^{-/-}$ mice is associated with increased production of Th17-type cytokines and dramatic suppression of IL-10 and IL-4.

Discussion

EAE is a severe neurological disorder initiated by myelin-reactive encephalitogenic T cells that secrete proinflammatory mediators to activate local CNS cells and recruit peripheral inflammatory cells for destruction of the CNS myelin (3, 6, 22, 56, 58). Inhibition of these autoreactive T cells and suppression of EAE has been achieved by vaccination with altered peptide ligands (APLs) (267, 278), induction of a Th2 cell bias (33, 78), and by treatment with DNA vaccines encoding encephalitogenic peptides (141, 266). Additionally, systemic injection with Ig chimera-coupled with encephalitogenic peptide (144, 146), or adoptive transfer of T_{reg} cells from diseased animals (384) has been successfully used to ameliorate EAE severity.

Significant efforts have been focused on treating EAE by induction of myelin-specific (35, 67), or -nonspecific (33, 34) mucosal tolerance. Additionally, successful down-regulation of autoimmune responses has been reported following multiple mucosal administrations of autoreactive Ags chemically coupled (67, 68), or genetically fused (35) to cholera toxin B subunit (CTB). Along these lines, we have demonstrated in the previous chapter that protection against relapsing-remitting EAE can be achieved by just a single nasal dose of pσ1-based tolerogen (PLP:OVA-pσ1). PLP:OVA-pσ1 prevented

development of fully pronounced EAE by inducing T_{reg} cells that specifically down regulated encephalitogenic $PLP_{139-151}$-activated T cells.

Here we show that similar strategy can be applied to treat acute EAE in C57BL/6 mice. Just a single 50 μg nasal dose of MOG-pσ1 entirely protected susceptible C57BL/6 mice from development and clinical manifestation of MOG_{35-55}-induced EAE. Moreover, these MOG-pσ1-protected mice showed virtually no mononuclear cell infiltration and affiliated CNS demyelination, which are the hallmarks of ongoing EAE. We have determined that MOG-pσ1-mediated protection against EAE induced with MOG_{35-55} peptide is due to generation of MOG-specific tolerance, as evidenced by the striking reduction in rMOG-specific IgG Abs in serum of MOG-pσ1-dosed mice and virtual lack of MOG_{35-55}-specific DTH responses after challenge with encephalitogenic peptide. Consistent with the mechanism of PLP:OVA-pσ1-mediated protection against RR EAE, we have demonstrated that MOG-pσ1-protected mice exhibited increased production of IL-10 and IL-4 and consequent depression of proinflammatory Th1- and Th17- type cytokines.

In agreement with reported apoptosis of OVA-Tg $CD4^+$ T cells induced by OVA-pσ1 *in vitro* (89), and clonal deletion of OVA-specific $CD4^+$ T cells following tolerization with OVA-pσ1 (397), we demonstrated that nasal administration of a single low dose of MOG-pσ1 to mice exhibiting pronounced clinical disease rapidly and significantly improved EAE symptoms. We have reported previously, that even though OVA-pσ1 and pσ1 induce comparable levels of OVA-Tg $CD4^+$ T cell apoptosis *in vitro*, only OVA-pσ1, but not pσ1alone or pσ1 + OVA induces these Tg $CD4^+$ T cells to

produce regulatory cytokines (89). Consistent with this finding, when mice exhibiting pronounced clinical MOG_{35-55}-induced EAE, instead of MOG-pσ1, were treated with pσ1 alone or co-delivered with rMOG, the disease became more severe. These results implicate that fusion of an Ag to pσ1 is pivotal for the induction of Ag-specific tolerance mediated by pσ1, and further confirm the Ag-specificity of pσ1-delivered tolerance and protection against EAE.

OVA-specific nasal tolerance induced by OVA-pσ1 (89), and $PLP_{139-151}$-specific protection against EAE generated by PLP:OVA-pσ1 were dependent on the presence of IL-10 and/or IL-10-producing T_{reg} cells. Consistent with these findings, MOG-pσ1-mediated protection against acute EAE could not be established in IL-$10^{-/-}$ mice, and lack of protection in these IL-$10^{-/-}$ mice was associated with significant production of proinflammatory cytokines and depression of anti-inflammatory cytokines. In agreement with OVA-pσ1-mediated tolerance to OVA, and PLP:OVA-pσ1-dependent protection against $PLP_{139-151}$-induced EAE, we have learned that T_{reg} cells isolated from MOG-pσ1-protected mice produced predominantly IL-10, whereas $CD25^{-}CD4^{+}$ T cells from these mice produced mostly IL-4.

Consistent with the reported ability of B cells to generate anti-inflammatory cytokines (98, 236), nasal MOG-pσ1 induced IL-10- and IL-4-expressing B cells that supported MOG-pσ1-mediated protection against EAE. We have demonstrated that nasally given MOG-pσ1 ameliorates, but fails to entirely prevent clinical manifestation of EAE in mice deficient of functional B cells. Moreover, adoptive transfer of B cells from MOG-pσ1-tolerized C57BL/6 mice only partially rescued diseased μMT or C57BL/6

mice. Adoptive transfer of naive B cells from PBS-dosed C57BL/6 mice exacerbated clinical manifestation of EAE in μMT mice.

To further determine the importance of IL-10 production by T_{reg} cells and/or by B cells for MOG-pσ1-mediated protection against EAE, studies were performed using T_{reg} cells from MOG-pσ1-dosed C57BL/6 mice adoptively transferred into diseased IL-$10^{-/-}$ mice. Even though adoptive transfer of MOG-pσ1-derived C57BL/6 B cells, $CD25^{-}$ $CD4^{+}$ T cells, or T_{reg} cells prevented further progression of EAE in diseased IL-$10^{-/-}$ mice, only MOG-pσ1-derived T_{reg} cells greatly accelerated recovery from the acute disease.

The role of CD11b-expressing antigen presenting cells (APCs) has been well-documented in induction of mucosal tolerance (47, 99), but their role in EAE has been controversial (393-396). In our hands, Mac-$1^{-/-}$ mice developed more severe MOG_{35-55}-induced EAE than C57BL/6 mice, and MOG-pσ1-mediated protection against EAE could not be established in these mice.

Even though suppression of EAE by mucosal administration of encephalitogenic peptides has been achieved, it usually requires multiple and/or large doses of an Ags (24, 56-58). More importantly, although successful in a small open label study, feeding with bovine myelin preparations to patients with RR MS did not significantly improved the disease onset during clinical trial (22). Additionally, in our hands nasal administration of 12.5 or 50 μg of rMOG did not inhibit CNS histopathology and clinical manifestation of MOG_{35-55}-induced EAE. Continued efforts have been focused on improving mucosal delivery for protection against EAE. A strategy of delivering auto-Ags chemically coupled (67, 68) or genetically fused to CTB (35) has been well-explored, and although

successful, multiple dosed of CTB-delivered auto-Ags are required. Additionally, chemical modifications of a carrier molecule are associated with the risk of altering immunogenecity of delivered Ag (discussed in ref. 35, 82, 154). Moreover, CTB can be used as mucosal adjuvant since it is not associated with the toxicity of native CT (64), and mucosally administered CTB-coupled Ag was shown to induce either immunity or tolerance dependent on the presence of preexisting immunity to CTB (73, 74). Mucosal delivery of CTB-coupled auto-Ag produced varied results and induced production of IFN-γ (67), or TGF-β and IL-10 (68). Therefore, treatment of inflammatory disease by CTB-delivered auto-Ags can be unsafe, although the concept of inducing Ag-specific tolerance by mucosa-binding molecule is very attractive. In contrast, just a single nasal dose of pσ1-delivered vaccine can entirely prevent, as well as treat mice against EAE. The genetic modification of pσ1 renders it tolerogenic to both the fused protein Ag and pσ1 (89). Moreover, as demonstrated in a previous chapter, pσ1-induced nasal tolerance to a fused protein Ag can be successfully applied to prevent $PLP_{139-151}$-induced RR EAE in susceptible SJL mice. Here we show that the strategy of generating pσ1-mediated tolerance can be ubiquitously applied to treat other autoimmune diseases, as evidenced by suppression of MOG_{35-55}-induced acute EAE in C57BL/6 mice by nasally administered recombinant MOG-pσ1. Additionally, we determined that pσ1-mediated protection against EAE is specific to the fused Ag, since nasal administration of PLP:OVA-pσ1 had no effect on clinical onset of MOG_{35-55}-induced EAE. Due to a better availability of various mouse knockout models on C57BL/6 genetic background, we further determined the mechanism of pσ1-delivered tolerance and protection against EAE.

Single 50 μg dose of MOG-pσ1 efficiently prevented EAE development in C57BL/6 mice. In contrast to MOG-pσ1-protected mice, nasal administration of various doses of rMOG did not inhibit development of clinical disease and CNS histopathology. Interestingly, in contrast to PBS-dosed diseased mice that developed "classical" EAE mediated by Th17-type cells (168, 169), the disease in rMOG-dosed mice exhibited evident Th1-type bias. When compared to diseased mice dosed with PBS, mice that received rMOG prior to EAE induction produced over 5-fold more of IL-27 and > 3-fold more of IFN-γ, but very little of IL-17 and IL-21. This discovery is intriguing given the controversial role of Th1-type cytokines in EAE pathogenesis. Even though increased production of IFN-γ has been reported during clinical phase of EAE (6, 24, 172), others have demonstrated that neutralization of IFN-γ with anti-IFN-γ mAb (203), or using IFN-$\gamma^{-/-}$ or IFN-$\gamma R^{-/-}$ mice (182, 204) resulted in exacerbation of EAE. Moreover, IL-27 originally described as a proinflammatory cytokine that induces Th1-type cells (186, 202), recently has been implicated in supression of Th17-type cells (202). Therefore, the evident Th1-type bias observed in diseased mice nasally dosed with rMOG and challenged with MOG_{35-55} peptide can provide new insights into the role of Th1-type cells in EAE.

Consistent with our previous findings, we learned that MOG-pσ1-mediated protection against acute EAE is associated with a significant increase of $FoxP3^+CD25^+CD4^+$ T_{reg} cells that produce predominantly IL-10 and IL-4-producing $FoxP3^+CD25^-CD4^+$ Th2 cells. These results are not surprising, since induction of $FoxP3^+$ $CD25^+CD4^+$ (33, 34, 107, 112, 115, 116) and $FoxP3^+$ $CD25^-CD4^+$ (116, 122) regulatory

cells has been associated with suppression of autoimmune responses and/or recovery from EAE. Alternatively, these FoxP3-expressing $CD25^{-}CD4^{+}$ T cells can represent a population of peripheral $CD25^{-}CD4^{+}$ T cells undergoing conversion to the $CD25^{+}$ phenotype (117, 122, 123, 127).

Interestingly, we have learned that MOG-pσ1-induced $FoxP3^{+}$ T_{reg} cells and $FoxP3^{+}CD25^{-}CD4^{+}$ Th2 cells express significantly more CD73 when compared to these respective cell types isolated from PBS-dosed diseased mice. CD73 (ecto-5'-nucelotidase) is an enzyme that catalyzes dephosporylation of purine and pyrimidine ribo- and deoxyribonucleoside monophosphates to their corresponding nucleotides (402). CD73 can mediate costimulatory signals for T cell activation (402, 403), though the predominant function for CD73 is regulation of adenosine availability for interaction with cell surface adenosine receptors, by converting adenosine monophosphate (AMP) into adenosine (402). Extracellular ATP (404) and cAMP (405) have been shown to promote apoptosis. In contrast, adenosine converted from AMP by CD73 is known to exhibit a strong anti-inflammatory and immunomodulatory potential when interacting via adenosine receptors (406-408). Engagement of the high affinity adenosine receptor $A_{2A}R$ expressed on T and B cells has been demonstrated to downregulate production of IL-12 and other proinflammatory cytokines, but stimulates secretion of IL-10 (406, 407). Furthermore, CD73 expression has been demonstrated on suppressive T cells, and CD73-mediated synthesis of adenosine has been linked to the anti-inflammatory function of these cells (408) and induction of anti-inflammatory phenotype of uncommitted primed precursor Thpp cells (407). Interestingly, $CD73^{-/-}$ mice, with the deletion restricted to

their T cells were shown to be resistant to development of EAE and exhibited reduced CNS infiltration when compared to wild-type mice (403). This discovery indicates that expression of CD73 on encephalitogenic $CD4^+$ T cells may be required for the penetration through the blood brain barrier and eventual access into the CNS (403). Co-expression of FoxP3 and CD73 was evident by regulatory T cells ($CD25^+$ and $CD25^-$) isolated from MOG-pσ1-protected mice. In contrast to $FoxP3^+CD25^+CD4^+$ and $FoxP3^+CD25^-CD4^+$ T cells obtained from PBS-dosed mice, over 50 % of $FoxP3^+CD25^+$ T_{reg} and $FoxP3^+CD25^-$ Th2 cells isolated from MOG-pσ1-dosed mice expressed CD73, implicating the role of these cells in suppression of inflammatory response at the pheriphery and possibly also within the CNS via convertion of AMP into anti-inflammatory adenosine.

CD73, together with another ecto-nucleotidase, CD39, has been implicated in the suppressive function of T_{reg} cells (408, 409). The enhanced expression of CD73 by $FoxP3^+CD25^+$ or $CD25^-CD4^+$ T cells from MOG-pσ1-protected mice was not followed by an increase in CD39 expression by these cells. However, we have seen enhanced CD39 expression on B cells derived from MOG-pσ1-protected mice. CD39 has been described as an early activation marker for B cells (410), but it is expressed also by NK cells, DCs, subsets of activated T cells, and monocyte-macrophages (409, 411). The role of CD39 in humoral and cellular responses is still being investigated, and it has been suggested that CD39 may play a role in Ab affinity maturation (409). $CD39^{null}$ mice were shown to spontaneously develop Th1 type autoimmune diseases and exhibit impaired B cell memory responses to T cell-dependent Ags (409). CD39 ATPDase

rapidly hydrolizes ATP and ADP to AMP, a substrat for CD73-mediated synthesis of adenosine (406). It is plausible that induction of CD39 on B cells from MOG-pσ1-protected mice, but not on B cells from diseased PBS-dosed mice, suggests the supportive function of these cells in MOG-pσ1-mediated tolerance. Taken together our results suggest that administration of MOG-pσ1 induces regulatory T and B cells capable of altering the inflammation to anti-inflammatory response, possibly via CD73- and/or CD39-dependent pathway.

For a number of years, the major attention has been focused on the pathogenic role of B cells and Abs in EAE (6, 235, 236, 391). Recently, B regulatory (B_{reg}) cells have been implicated in the recovery from autoimmune diseases (98, 236). B cells isolated from MOG-pσ1-protected mice, but not from PBS-dosed diseased mice, produce significant amounts of IL-10 and IL-4. Production of anti-inflammatory cytokines IL-10 (236) and IL-4 (401) by B_{reg} and Be2 cells, respectively, has been demonstrated. Up regulation of IL-4 by Be2 cells has been shown to rely on the presence of T cell and IL-4 (401). Additionally, the regulatory function of B_{reg} cells during autoimmune inflammation has been associated with production of IL-10 (236), or TGF-β (242), as well as with a direct interaction between B_{reg} cells and pathogenic T cells (98). IL-10-producing B cells have been associated with enhanced recovery from EAE (236), and induction of a low-dose oral tolerance (242). Consistent with the supportive role of B cells in regulating autoimmune inflammation, mice lacking functional B cells (μMT mice) were shown to develop similar (235) or more severe and prolonged onset of EAE

when compared to the wild-type mice (236, 238). On the other hand, one study has reported resistance of H-2^b μMT mice to development of rMOG-induced EAE (240).

Consistent with the majority of reports, μMT mice in our study developed more severe EAE than C57BL/6 mice when challenged with MOG_{35-55} peptide. Additionally, nasal administration of MOG-pσ1 in the pre-clinical phase of EAE did not prevent development of EAE in μMT mice, but when compared to PBS-dosed C57BL/6 or μMT mice, MOG-pσ1-dosed μMT mice exhibited a significant reduction in the severity of clinical symptoms, suggesting a supportive, but not pivotal, role of B cells in MOG-pσ1-mediated protection against EAE. Furthermore, adoptive transfer of MOG-pσ1- but not PBS-primed C57BL/6 B cells to diseased μMT mice prevented further progression of clinical EAE and accelerated remission from the acute disease. Interestingly, when PBS-primed B cells were transferred to diseased μMT mice, exacerbation of clinical EAE was obtained, suggesting that MOG-pσ1-tolerization also induces regulatory properties of B cells. In contrast to these results, Lyons *et al* showed that passive transfer of B cells from MBP-primed or naive mice did not reconstitute the ability to develop EAE in MOG-challenged, but EAE-resistant μMT mice (240). Adoptive transfer of MOG-pσ1-primed B cells into diseased C57BL/6 mice prevented further progression of EAE, but had only a minor effect on recovery from acute disease. The important role of IL-10 in induction of mucosal tolerance (87, 89, 90) and protection against EAE (3, 22, 56, 105) has been well described. IL-10 production has been demonstrated by suppressive Tr1 cells (105), $FoxP3^+$ $CD25^+CD4^+$ T_{reg} cells (89, 114), $FoxP3^-$ $CD25^+CD4^+$ T cells (114, 128), as well as tolerogenic B cells (236), and DCs (248).

In contrast to diseased PBS- or rMOG-dosed mice, lymphocytes from MOG-pσ1-protected mice secreted predominantly IL-10 and IL-4, but virtually no proinflammatory cytokines, IFN-γ, IL-17, IL-21 or IL-27. The pivotal role of IL-10 in MOG-pσ1-mediated protection against EAE was demonstrated by inability to prevent development of EAE in MOG-pσ1-dosed IL-$10^{-/-}$ mice. Consistent with previous reports, IL-$10^{-/-}$ mice induced with EAE by s.c. injection of MOG_{35-55} developed more severe disease than C57BL/6 mice (90, 132). Further evaluation of diseased MOG-pσ1-treated IL-$10^{-/-}$ mice revealed significant enhancement in rMOG-specific IgG Ab titers and MOG_{35-55}-specific DTH response when compared to MOG-pσ1-protected C57BL/6 mice. Secretion of proinflammatory cytokines, IL-17, IL-21 and IFN-γ, and evident depression in IL-4 were observed in these mice when compared to MOG-pσ1-protected C57BL/6 mice. Therefore, reduction of IL-4 secretion by IL-$10^{-/-}$ mice may be related to the activation of proinflammatory Th1 and Th17 cells, which has been demonstrated by others (168, 169, 197).

Additionally, in contrast to MOG-pσ1-protected C57BL/6 mice, unprotected IL-$10^{-/-}$ mice dosed with MOG-pσ1 or PBS, and PBS-dosed C57BL/6 mice produced virtually no IL-28. IL-28 secreted by innate immune cells was reported to display anti-viral activity (223). Moreover, this cytokine exhibits a genetic similarity to IL-10, and structural resemblance to type I IFNs used successfully in MS therapy (223). It has been demonstrated that IL-28-treated DCs acquire a phenotype characterized by the high expression of MHC I and II, but down regulation of co-stimulatory molecules, and these IL-28-treated DCs were shown to promote IL-2-dependent proliferation of

FoxP3$^+$CD25$^+$CD4$^+$ T_{reg} cells (224). Interestingly, increased production of IL-28 was observed by CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells from MOG-pσ1-protected wild-type mice. Additionally, as shown in Chapter 3 of this manuscript, only mice protected against $PLP_{139-151}$-induced EAE by nasally administered PLP:OVA-pσ1 produced IL-28 (see Chapter 3, Figure 3.8). Therefore, our results suggest that IL-28 exhibit anti-encephalitogenic potential and that it can be produced by regulatory (CD25$^+$ and/or CD25$^-$) CD4$^+$ T cells. Consistent with OVA-pσ1-induced tolerance to OVA (89) and PLP:OVA-pσ1-mediated protection against $PLP_{139-151}$-induced RR EAE, MOG-pσ1-induced T_{reg} cells secreted predominantly IL-10, whereas CD25$^-$CD4$^+$ T cells obtained from these MOG-pσ1-protected C57BL/6 mice secreted the majority of IL-4. The further argument for the importance of IL-10$^+$ T_{reg} cells in MOG-pσ1-mediated protection against EAE came from the adoptive transfer studies. We demonstrated that adoptive transfer of T_{reg} cells, but not B or CD25$^-$CD4$^+$ T cells, from MOG-pσ1-dosed C57BL/6 mice significantly ameliorated disease and accelerated recovery from MOG_{35-55}-induced EAE in IL-10-deficient mice, implicating that IL-10 secreted by MOG-pσ1-primed T_{reg} cells is essential for MOG-pσ1-mediated protection against EAE. Even though adoptive transfer of MOG-pσ1-primed C57BL/6 CD25$^-$CD4$^+$ T cells or B cells was not as effective as transfer of MOG-pσ1-primed T_{reg} cells in inducing recovery from EAE, these cells also inhibited progression of EAE and triggered complete recovery of diseased IL-10$^{-/-}$ mice when compared to PBS-dosed IL-10$^{-/-}$ mice. Therefore, IL-10 produced by MOG-pσ1-primed B cells and IL-4-secreted by MOG-pσ1-derived CD25$^-$ CD4$^+$ T cells are supportive in MOG-pσ1-mediated protection against EAE.

The role of IL-4 in mucosal tolerance and EAE has been well described. IL-4 secreted by Th2 cells has been shown to support inhibition of inflammatory response to $PLP_{139-151}$ peptide (33) and OVA (89). Additionally, IL-4-mediated induction of T_{reg} cells from naive peripheral $CD25^{-}CD4^{+}$ T cells has been reported (117). IL-4 being a major Th2 type cytokine can inhibit differentiation of Th17 cells (168, 169) and has been shown to negatively regulate production of IFN-γ (194). Moreover IL-4 can induce B cells to perform the APC function, which has been shown to promote development of anergy (3, 22). Interestingly, $IL\text{-}4^{-/-}$ mice are not more predisposed to develop autoimmune disorders than wild-type mice (6, 130). We have demonstrated IL-4 production by $CD25^{-}CD4^{+}$ T cells isolated from OVA-pσ1-tolerized (89) and PLP:OVA-pσ1-protected mice. Additionally, as reported in the previous chapter, *in vitro* blockade of IL-4 partially restored PLP:OVA-pσ1-mediated suppression of $CD4^{+}$ T cell proliferation, and *in vivo* neutralization of IL-4 partially reversed PLP:OVA-pσ1-mediated protection in $PLP_{139-151}$-challenged SJL mice. Therefore, IL-4 can support IL-10-dependent protection against EAE as observed in MOG-pσ1-dosed C57BL/6 mice via induction of Th2-type bias, which previously was shown to support mucosal tolerance (76, 117). Alternatively, IL-4 secretion induced by IL-10 can enhance conversion of $CD25^{-}CD4^{+}$ T cells into T_{reg} cells (117).

It has been demonstrated that certain APCs, especially DCs, can be involved in mediation of mucosal tolerance (47). $CD11b^{+}$ $CD8\alpha^{-}$ DCs or myeloid DCs were shown to induce regulatory T cells (47), but also to stimulate differentiation, IL-4- and IL-10-production of Th2 cells *in vitro* (248). The supportive role of $CD11b^{+}CD8\alpha^{-}$ DCs in

recovery from EAE has been attributed to IL-10 production by these cells (31, 207). It has been reported that oral tolerance cannot be established in CD11b$^{-/-}$ mice, and a deficiency in CD11b triggers the induction of Th17 cells via IL-6-dependent mechanism (99). In contrast to these findings, Mac-1$^{-/-}$, but not Mac-1$^{+/-}$ mice were shown to develop significantly delayed onset and attenuated EAE, and upon adoptive transfer of MOG_{35-55}-restimulated wild-type T cells into Mac-1$^{-/-}$ mice, disease was ameliorated; transfer of MOG_{35-55}-restimulated T cells from Mac-1$^{-/-}$ mice failed to induce disease in Mac-1$^{+/+}$ mice (396). The crucial role for induction of peripheral tolerance to i.v. injected MOG_{35-55} peptide and protection against passively transferred EAE were recently demonstrated for IL-10-producing myeloid DCs (393). Consistent with this report was another study demonstrating that CD11b^{+}Ly-6C^{hi} monocytes play an important role in suppression of encephalitogenic T cells during EAE (395). In addition to these findings, CD11b^{+} monocytes were shown to inhibit experimental autoimmune myocarditis mediated by Th17 cells via IFN-γ-dependent mechanism (394). In agreement with the established role of CD11b in inhibition of Th17 cell-mediated inflammatory response, in our studies, Mac-1$^{-/-}$ mice developed more severe clinical onset of MOG_{35-55}-induced EAE than C57BL/6 mice. Consistent with reported resistance of Mac-1$^{-/-}$ mice to induction of mucosal tolerance (99), nasal MOG-pσ1 failed to generate MOG-specific tolerance in Mac-1$^{-/-}$ mice, which resulted in lack of protection against EAE in MOG-pσ1-dosed Mac-1$^{-/-}$ mice. It has been reported that T cells from MOG_{35-55}-primed Mac-1$^{-/-}$ mice, which developed a less severe EAE produce elevated levels of TGF-β and IL-10, but reduced levels of IFN-γ, IL-12, and IL-4 (396). CD4^{+} T cells obtained from diseased MOG-pσ1-

and/or PBS-dosed Mac-$1^{-/-}$ mice produce mostly IL-6, IL-17, and IL-21 when compared to diseased PBS-dosed or MOG-pσ1-protected C57BL/6 mice, and displayed reductions in IL-4, IFN-γ, IL-10, and IL-13, when compared to MOG-pσ1-protected C57BL/6 mice. Therefore, our results are consistent with the previous findings that mucosal tolerance in Mac-$1^{-/-}$ mice cannot be established due to induction of Th17 cell response (99).

We have demonstrated that nasal treatment with MOG-pσ1 at the peak of clinical EAE induced with MOG_{35-55} peptide immediately reduces the average clinical score of diseased mice and leads to the accelerated recovery from acute disease. Since activation of elevated numbers of regulatory T cells would likely occur over a number of days, we attributed this drastic and immediate reduction in clinical onset of EAE to apoptosis of autoreactive cells induced by nasal MOG-pσ1. Induction of apoptosis has been associated with modulation of an immune response by pσ1 (375, 376). Apoptosis of OVA-specific $CD4^+$ T cells has been demonstrated *in vivo* following oral tolerization with OVA-pσ1 (397). We have shown that OVA-pσ1, pσ1 and co-administered OVA + pσ1 induce apoptosis of OVA-Tg $CD4^+$ T cells *in vitro* in time- and dose-dependent manner (89). To further determine the Ag specificity of pσ1-induced apoptosis, we treated diseased C57BL/6 mice two days before the peak of clinical MOG_{35-55}-induced EAE with MOG-pσ1, PLP:OVA-pσ1, rMOG, pσ1, pσ1 plus rMOG, or with PBS. Treatment with rMOG did not significantly improve clinical onset of the disease when compared to PBS-treated mice. On the other hand, nasally delivered MOG-pσ1 very rapidly depressed the average clinical score and significantly accelerated recovery from EAE. In contrast to treatment with MOG-pσ1 that significantly ameliorated EAE, nasal

delivery PLP:OVA-pσ1 did not improve the clinical manifestation of EAE when compared to PBS- or rMOG-treated mice. Surprisingly, nasal pσ1 administered alone or in combination with rMOG enhanced the progression and severity of EAE in C57BL/6 mice. Combined evidences suggest that pσ1-induced apoptosis may be mediated by sialic acid binding domain (49, 159, 160). Therefore, it is plausible that nasal administration of pσ1 alone, pσ1 co-delivered with rMOG, or MOG-pσ1 induce apoptosis of effector encephalitogenic $CD4^+$ T cells in mice exhibiting MOG_{35-55}-induced EAE. However, in contrast to nasal administration of pσ1 alone or co-delivered with rMOG that resulted in exacerbation of clinical disease, administration of MOG-pσ1 inhibited disease progression and accelerated recovery from EAE. The disease amelioration in MOG-pσ1-treated mice suggests involvement of Ag-specific mechanism for pσ1 action, like CD73/CD39/adenosine –mediated suppression of inflammation, and/or Ag-specific cell lysis by nasally administered MOG-pσ1 interacting with MOG-specific Abs. This result is consistent with our *in vitro* data that have shown that even though OVA-pσ1, pσ1, and OVA plus pσ1, induced apoptosis of OVA-Tg $CD4^+$ T cells, only OVA-pσ1 induced production of regulatory cytokines by these Tg $CD4^+$ cells (89). Alternatively, the extent of pσ1-mediated apoptosis may be dose-dependent, since mice dosed with pσ1 or pσ1 plus rMOG, received twice the amount of pσ1 molecules when compared to PLP:OVA-pσ1 or MOG-pσ1-dosed mice. The lack of recovery after nasal administration of PLP:OVA-pσ1 suggests the Ag-specificity of pσ1-mediated tolerance, in which pσ1-delivered $PLP_{139-151}$ peptide and OVA are not recognized by MOG_{35-55}-

responsive cells. Since C57BL/6 mice are not susceptible to EAE induction by $PLP_{139-151}$ peptide (178, 412), it is possible that this peptide was simply not recognized by H-2^b restricted immune cells during the acute disease when the epitope spreading is perhaps not advanced enough (380, 413).

In summary, we have demonstrated here that single nasal dose of MOG-pσ1 fusion protein entirely protects mice against development of clinical onset and the CNS histopathology of MOG_{35-55}-induced EAE. In contrast to MOG-pσ1, nasal administration of various low-doses of rMOG alone is unable to prevent EAE development in C57BL/6 mice. This MOG-pσ1-mediated protection against EAE was due to induction of rMOG-specific peripheral tolerance that was mediated by IL-10-secreting $FoxP3^+$ T_{reg} cells and supported by IL-4-producing $CD25^-CD4^+$ Th2 cells, both of which showed enhanced expression of ecto-5'nucleotidase CD73. Nasal MOG-pσ1 markedly inhibited production of proinflammatory Th1- and Th17-type cytokines. Moreover, we have shown here that MOG-pσ1-mediated protection against EAE is also associated with induction of IL-10- and IL-4-producing B cells that express early activation marker CD39. The pivotal role of IL-10 in MOG-pσ1-mediated protection against EAE was evidenced by the inability of MOG-pσ1 to induce protection in IL-$10^{-/-}$ mice. Furthermore, the essential role of MOG-pσ1-induced T_{reg} cells in production of IL-10 was demonstrated in adoptive transfer studies in which only the injection of MOG-pσ1-primed T_{reg} cells, but not $CD25^-CD4^+$ T cells or B cells, significantly enhanced the recovery of IL-$10^{-/-}$ mice from EAE. Additionally, we demonstrated that MOG-pσ1-primed $CD25^-CD4^+$ T cells and B cells support T_{reg} cells in MOG-pσ1-mediated protection against EAE, since adoptive transfer

of these cells prevented further progression of EAE in IL-$10^{-/-}$ mice. Moreover, lack of functional B cells in μMT mice prevented generation of full MOG-pσ1-mediated protection against EAE, and adoptive transfer of wild-type MOG-pσ1-primed B cells into diseased μMT mice resulted in amelioration of EAE, but did not entirely suppressed the disease. Remarkably, treatment of C57BL/6 mice with single nasal dose of MOG-pσ1 resulted in dramatic and immediate remission from MOG_{35-55}-induced EAE, whereas treatment with PLP:OVA-pσ1 had no effect on clinical onset of EAE in these mice. When pσ1 alone was used as a treatment, exacerbation of MOG_{35-55}-induced disease was observed, suggesting Ag-specificity of pσ1-mediated protection against EAE. Future studies need to be performed to determine the mechanism and possible restrictions of pσ1-mediated apoptosis. Nonetheless, the efficacy of a single nasal dose of MOG-pσ1 in recovery from ongoing EAE remains valid and makes a pσ1-based system very attractive for delivery of tolerance, protection and treatment against autoimmune diseases.

NOTE: Histology slides presented in Figure 4.2*D* and incorporated into Table 4.1 were made by Gayle Callis.

SUMMARY

Mucosal tolerance is an intensively studied strategy to suppress autoimmune diseases. The mucosal route of tolerance induction is clinically more attractive than the peripheral route means because of the ease of needle-free application. Even though inhibition of autoiummune diseases has been demonstrated after mucosal administration of auto-Ags, induction of sustainable and long–lasting protection often requires large and/or repeatable doses of an Ag. Administration of much smaller doses of auto-Ags delivered by mucosa-binding molecules is an attractive alternative. In this regard, chemically modified cholera toxin B subunit (CTB) have been described, although potential risks of applying chemically modified molecule and mucosal adjuvant for treatment of proinflammatory autoimmune diseases may circumvent the benefits of this approach.

Previously, we demonstrated that chemically modified pσ1 binds to murine M cells in the NALT and significantly enhances immunity to encoded DNA. Here we show that recombinant pσ1, when genetically modified, can be successfully applied to prevent and treat autoimmune diseases. Efficient tolerance to auto-Ags is induced by administration of a single, very low dose of pσ1-delivered auto-Ag.

As proof of concept, in Chapter 2, we show that recombinant fusion protein, OVA-pσ1, induces OVA-specific and pσ1-specific tolerance when applied nasally to mice. In contrast to OVA-pσ1, nasal administration of OVA alone, which is known to be a poor mucosal immunogen, elicits greater Ag-specific Ab responses. Results presented in Figure 2.7*A* and *B* clearly show that a single nasal administration using ~ 1000-fold

molar less of OVA, when genetically fused to pσ1, was sufficient to induce OVA-specific tolerance. Remarkably, tolerance induced by OVA-pσ1 circumvented the immunostimulatory effect of CT, and even though at this point we cannot provide a clear explanation for this phenomenon, it is plausible that OVA-pσ1 resisted co-treatment with CT due to the pσ1-adhesive properties, which may influence different receptors than CT. Nasal treatment with OVA-pσ1 also resisted peripheral challenge with OVA and IFA, suggesting induction of sustainable mucosal and systemic tolerance to OVA.

Since chemically modified pσ1 stimulates enhanced immunity to itself, whereas genetically altered pσ1 induces tolerance to the fused protein Ag, we investigated if co-administration of OVA-pσ1 with plasmid cDNA would dominate the OVA-specific tolerance. Results presented in Figure 2.7*C* and *D* clearly demonstrate that this was not the case. In contrast to mice that received OVA or OVA plus CpG-ODN, mice dosed with OVA-pσ1 with or without CpG-ODN exhibited significant reduction in OVA-specific IgG Ab titers in serum and OVA-specific DTH response. CpG-ODNs are known to activate an innate immune system to produce proinflammatory cytokines, and when administered mucosally, CpG-ODNs induce Th1-type immune responses. Remarkably, lymphocytes isolated from OVA-pσ1 plus CpG-ODN-dosed mice produced significantly less Th1-type cytokines when compared to those obtained from OVA plus CpG-ODN-dosed mice (Figure 2.8*B*). Of interest, Th cells from OVA-pσ1 plus CpG-ODN-dosed mice produced enhanced amounts of IL-13 and TGF-β, when compared to Th cells from OVA-pσ1-dosed mice, which were more IL-4- and IL-10-dependant (Figure 2.8*A*). This observation offers an exciting new direction of research to investigate how mucosal

adjuvants can augment or change the type of T_{reg} cells induced. These results clearly indicate that genetically modified pσ1 is a very efficient tolerance vehicle and suggest that tolerogenic effect of OVA-pσ1 is clearly dependent on genetic modification of pσ1.

Next we investigated if mucosal administration of pσ1 is required for tolerance induction. In Chapter 2, we show that recombinant pσ1 efficiently binds to L cells *in vitro* (Figure 2.1*C*). Previous reports have demonstrated that recombinant pσ1 binds M cells in NALT and interacts with murine L929 (L) cells, Caco-2 cells, RFL-6 cells, mammalian erythrocytes and intestinal epithelial cells. Since pσ1 is capable of acting upon various cell types, we questioned if mucosal delivery of OVA-pσ1 would be required for induction of OVA-specific tolerance. To address this question, we first evaluated the presence of OVA-specific immune responses in mice i.m. dosed with OVA-pσ1. In contrast to mice i.m. administered with OVA or OVA plus CT, systemic administration of OVA-pσ1 or OVA-pσ1 plus CT did not induce OVA-specific IgG Abs in serum before peripheral challenge with OVA and IFA (Figure 2.9*A*, and *B*). However, one week after challenge with OVA/IFA, OVA-pσ1- and OVA-pσ1 plus CT-dosed mice exhibited induced OVA-specific IgG titers. Therefore, we concluded that mucosal delivery of OVA-pσ1 is required for induction of systemic tolerance to OVA. Further confirmation that mucosal delivery is pivotal for pσ1-mediated tolerance came from the experiments in PPs-deficient $LT\alpha^{-/-}$ mice that lacked PP-associated M cells. Results shown in Figure 2.9*C*, *D* and *E* clearly demonstrate that $LT\alpha^{-/-}$ mice are resistant to pσ1-mediated tolerance. When fed with OVA-pσ1, $LT\alpha^{-/-}$ mice develop significantly

elevated OVA-specific IgG and IgA Ab titers in serum and OVA-specific DTH responses when compared to C57BL/6 mice orally given OVA-pσ1 (Figure 2.9*C*, *D* and *E*). Therefore, M cells are essential for OVA-pσ1-mediated tolerance, and together these results suggest that induction of pσ1-mediated tolerance is limited to mucosal delivery. As demonstrated in our proposed model for pσ1 modulation of an immune reponse (Figure 5.1), our results implicate that nasally administered OVA-pσ1 is uptaken by M cells in NALT and transported into mucosal sites, where OVA-pσ1 encounters APCs.

In an attempt to determine the mechanism of pσ1-mediated tolerance to OVA, we learned, that OVA-pσ1-induced tolerance is $CD4^+$ T cell-dependent. OVA-specific tolerance could be adoptively transferred, as shown when $CD4^+$ T cells isolated from CLNs of OVA-pσ1-tolerized mice were adoptively transferred into naïve mice (Figure 2.2*A*). Since induction of a low-dose tolerance has been associated with activation of regulatory T cells, results presented in Figure 2.2*D* and *E* show that nasal administration of OVA-pσ1 induces more than a 40 % increase in $FoxP3^+CD25^+CD4^+$ and over a 60 % increase in $FoxP3^+CD25^-CD4^+$ T cells, when compared to naïve mice. $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells isolated from OVA-pσ1-tolerized mice were suppressive (Figure 2.2*B*), and adoptive transfer of OVA-pσ1-primed $CD25^+CD4^+$ T cells very effectively suppressed proliferation of OVA-Tg $CD4^+$ T cells *in vivo*, as evidence by only 15 % of these cells proliferating. Moreover, almost equally effective suppression of Tg $CD4^+$ T cells proliferation was also achieved by adoptively transferred $CD25^-CD4^+$ T cells obtained from OVA-pσ1-tolerized mice.

To understand the mechanism by which OVA-pσ1-derived $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells suppress OVA-specific immune responses, results shown in Figure 2.3 demonstrate that adoptive transfer of $CD25^+CD4^+$ T cells induces IL-10-producing $CD4^+$ T cells in OVA-Tg recipient mice. Additionally, adoptive transfer of OVA-pσ1-derived $CD25^-CD4^+$ T cells induces production of IL-4 and IL-10 by $CD4^+$ T cells isolated from recipient mice. Importantly, secretion of proinflammatory cytokines was depressed in OVA-Tg mice that received OVA-pσ1-primed $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells. Cytokine analyses performed on $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells obtained from OVA-pσ1-tolerized mice revealed pronounced polarization of cytokine responses. As presented in Figure 2.4, $CD25^+CD4^+$ $FoxP3^+$ T_{reg} cells obtained from OVA-pσ1-tolerized mice secrete predominantly IL-10, whereas $CD25^-CD4^+FoxP3^+$ T cells from these mice primarily secrete IL-4. IL-10 production by T_{reg} cells from nasally tolerized mice is well-documented, whereas IL-4 is considered one of the major cytokines that characterizes the Th2-type anti-inflammatory cells. Consistent with these findings, IL-10-producing T_{reg} cells and IL-4-producing $CD25^-CD4^+$ Th2 cells derived from OVA-pσ1-tolerized mice were potent suppressors of OVA-specific proliferation of $CD4^+$ T cells and significantly inhibited inflammation induced by systemic challenge with OVA in IFA. Another regulatory cytokine, TGF-β, was only transiently expressed by OVA-pσ1-derived regulatory T cells ($CD25^+$ and $CD25^-$) (Figure 2.4*E*), which may implicate this cytokine in conversion of $CD25^-CD4^+$ $FoxP3^+$ T cells to $CD25^+$ phenotype (122, 123).

Processing and presentation of OVA-pσ1 by professional APCs, like DCs, can result in various outcomes (Figure 5.1). It is now well established that certain subsets of mucosal DCs promote induction of tolerance over immunity. Processing and presentation of OVA-pσ1 by these tolerogenic DCs may lead to induction of OVA-pσ1-specific regulatory T cells ($CD25^+$ and $CD25^-$ $CD4^+$), that express FoxP3 and secrete anti-inflammatory cytokines (IL-10 and IL-4). Alternatively, presentation of processed epitopes of OVA-pσ1 by DCs may result in T cell anergy. After nasal administration of pσ1-delivered protein Ag, limited numbers of naïve $CD4^+$ T cells may undergo activation to proinflammatory phenotypes; however, we believe that these effector cells are effectively suppressed by OVA-pσ1-specific regulatory cells, resulting in induction of Ag-specific tolerance. Along these lines, proinflamamtory immune response induced by systemic challenge of mice with OVA in IFA is efficiently inhibited by these OVA-pσ1-induced regulatory T cells that spread to the systemic compartment after expansion in LNs (Figure 5.1).

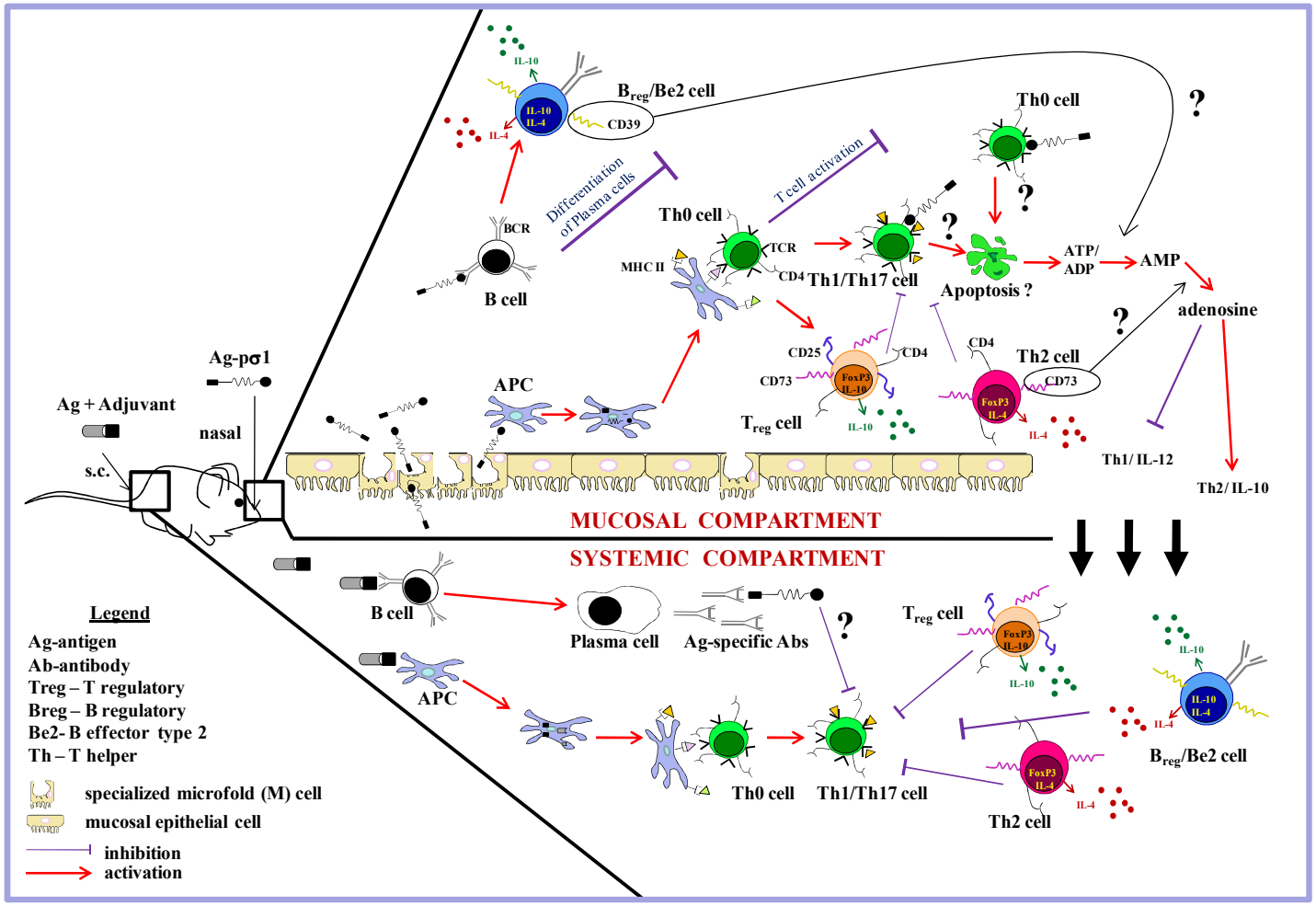


Figure 5.1. Proposed mechanism for the pσ1 action as a mucosal delivery vehicle. Nasally administered Ag-pσ1 fusion protein is internalized by M cells and translocated into the mucosal sites.

Figure 5.1. – continued. Tolerogenic APCs (presumably DCs) process Ag-pσ1 and present its epitoped on MHC II to $CD4^+$ T cells. Due to the tolerogenic properties of Ag-pσ1 and/or DCs, induction of T regulatory cells occurs, whereas Ag-activated $CD4^+$ T cells undergo anergy, and/or active suppression by Ag-pσ1-induced regulatory T cells, and/or pσ1-mediated apoptosis. Additionally, via direct interaction with B cell receptor (BCR), Ag-pσ1 can induce regulatory characteristics of B cells, and suppress B cell immune response. ATP/ADP released by apoptotic cells is hydrolized by CD39 expresed on B regulatory cells to AMP that is rapidly converted into anti-inflammatory adenosine byT_{reg}/FoxP3 Th2 cell-associated CD73. Regulatory T and/or B cells spread on the pheriphery, and suppress the immune response induced by systemic administration of Ag emulsified in adjuvant.

Since IL-10 was predominantly produced by OVA-pσ1-primed T_{reg} cells (Figure 2.4) and these cells were the most effective in suppressing OVA-specific proliferation of OVA-Tg $CD4^+$ T cell *in vivo* (Figure 2.2*B*), we next investigated the requirement for IL-10 in OVA-pσ1-mediated tolerance to OVA. Results presented in Figure 2.5 clearly indicate that IL-10 is essential for OVA-pσ1-mediated tolerance. We show that OVA-pσ1-mediated tolerance to OVA cannot be induced in IL-$10^{-/-}$ mice, which is consistent with another report showing that early production of IL-10 by $CD4^+$ T cells is important for induction of a high-dose oral tolerance (370). Moreover, we show that nasal administration of OVA-pσ1 does not induce T_{reg} cells in IL-$10^{-/-}$ mice, further suggesting the importance of IL-10-secreting T_{reg} cells is OVA-pσ1-induced tolerance.

It is well-established that induction of mucosal tolerance can occur by active suppression (33, 34, 78, 89), generation of anergy (22, 24), and/or clonal deletion of effector cells (22, 24, 397). Results presented in Chapter 2 clearly show active suppression of OVA-reactive $CD4^+$ T cells by OVA-pσ1-induced T regulatory cells. When investigating if pσ1-mediated tolerance utilizes additional inhibitory mechanism, results in Table 2.3 and 2.4 showed that OVA-pσ1 induces apoptosis of OVA-reactive Tg $CD4^+$ T cells *in vitro*. This discovery is in agreement with the well established ability of

reoviral and recombinant pσ1 to induce apoptosis (375, 376). Additionally, apoptosis of OVA-reactive $CD4^+$ T cells has recently been implicated in induction of oral tolerance by OVA-pσ1 (397). Therefore, in addition to an active suppression, clonal deletion of effector $CD4^+$ T cells is likely to contribute to nasally delivered OVA-pσ1. Following M cell-mediated translocation, OVA-pσ1 encouters also T and B lymphocytes. Based on our *in vitro* results presented in Chapter 2 (Table 2.3 and 2.4), our *in vivo* data presented in Chapter 4 (Figure 4.4), and results published by others (397), as well as established role of pσ1 in inducing apoptosis (375, 376), we speculate that pσ1 can induce apoptosis of OVA-reactive effector Th17 and Th1 cells, as well as other activated Th cells *in vivo* (Figure 5.1).

Ability of pσ1 and OVA-pσ1to induced apoptosis *in vitro* and *in vivo* triggered a question about the specificity of pσ1-mediated tolerance. In Chapters 3 and 4, we showed that pσ1-mediated tolerance is specific to delivered protein Ag. To fully address this question, we developed another pσ1-based fusion protein, which in addition to OVA, also carried the encephalitogenic $PLP_{130-151}$ epitope (Figure 3.1). In Figure 3.2, we show that nasal administration of PLP:OVA-pσ1 induces PLP-specific tolerance and protects mice against $PLP_{139-151}$-induced EAE. In contrast to PLP:OVA- pσ1-dosed mice, mice that received OVA-pσ1 developed EAE and showed significant increase of $PLP_{139-151}$-specific DTH response. $CD4^+$ T cells obtained from PLP:OVA-pσ1-protected mice produced only anti-inflammatory and regulatory cytokines and no proinflammatory cytokines after *in vitro* re-stimulation with OVA or with $PLP_{139-151}$, implicating that pσ1-mediated tolerance is limited to pσ1-delivered Ag. Importantly, even though OVA-pσ1-

dosed mice were not protected against EAE, these mice remained tolerant to OVA (Figure 3.2*D*). Analysis of their $CD4^+$ T cells *in vitro* re-stimulated with OVA exhibited elevated production of anti-inflammatory and regulatory cytokines but not proinflammatory cytokines; however, when these cells were re-stimulation with $PLP_{139-151}$ peptide, they produced predominantly pro-inflammatory cytokines, further indicating Ag-specificity of pσ1-delivered tolerance.

We showed in Chapter 2 that efficient tolerance to OVA can be accomplished using 1000-fold less OVA-pσ1 than OVA alone. Consequently, we queried if protection against EAE can also be established after a single nasal dose using PLP:OVA-pσ1. Results presented in Figure 3.3*B* and *C* clearly show that a 100 μg dose of PLP:OVA-pσ1 administered to mice before the disease induction or during its pre-clinical stage is sufficient to significantly reduce clinical manifestation of EAE. This discovery is important because the ability to establish protection after a single administration of an Ag reduces the risk for development of allergies which is a serious concern if an Ag needs to be administered multiple times.

It is believed that the CNS histopathology in EAE translates directly to the clinical manifestation of the disease, and we sought to determine if nasal administration of PLP:OVA-pσ1 prevents the CNS histopathology in mice. Results presented in Figure 3.3*D* clearly demonstrate that mice given a single dose of PLP:OVA-pσ1 exhibit profound reduction of mononuclear cells' infiltration and associated demyelination of the CNS when compared to PBS-dosed diseased mice.

In Chapter 2, we provided evidence that OVA-specific tolerance induced by nasally administered OVA-pσ1 is mediated by IL-10-producing $CD25^+CD4^+$ T_{reg} cells and IL-4-secreting $CD25^-CD4^+$ T cells. Induction of IL-10-producing regulatory T cells has been linked with suppression of EAE (90, 95), and introduction of Th2 cell bias has been suggested as a supportive mechanism for EAE inhibition (33, 78). Thus, we queried if enhanced activation of regulatory T cells protects mice against EAE when PLP:OVA-pσ1 is nasally administered. FACS analysis depicted in Figure 3.4*A* clearly demonstrates that nasally administered PLP:OVA-pσ1 induces significant enrichment in the percentages of $FoxP3^+CD25^+CD4^+$ and $FoxP3^+CD25^-CD4^+$ T cells. Consistent with induction of IL-10-producing T_{reg} cells and IL-4-producing $FoxP3^+$ Th2 cells after OVA-pσ1 tolerization, we show that > 85 % of T_{reg} cells from protected PLP:OVA-pσ1-dosed mice produce IL-10, and almost as many $CD25^-CD4^+FoxP3^+$ Th2 cells from these mice produce IL-4 (Figure 3.4*A* and *B*). In agreement with the well-established role of IL-10 in protection against EAE (90, 95), results in Figure 3.5*A* show that *in vitro* blockade of IL-10 reverses the PLP:OVA-pσ1-mediated suppression of $CD4^+$ T cell proliferation in response to *in vitro* stimulation with $PLP_{139-151}$ peptide.

To investigate the relevance of IL-4 for PLP:OVA-pσ1-mediated protection against EAE, in Figure 3.5*A, in vitro* inactivation of IL-4 partially abolishes PLP:OVA-pσ1-induced suppression of $PLP_{139-151}$-specific proliferation of $CD4^+$ T cells. Consistent with reported by others supportive role of Th2 cell bias in suppression of EAE (266-270), we determined that *in vivo* inhibition of IL-4 by anti-IL-4 mAb results in partial restoration of EAE in PLP:OVA-pσ1-dosed mice (Figure 3.5*B*). Moreover, $CD4^+$ T cells

from anti-IL-4 mAb-treated PLP:OVA-pσ1-dosed mice produced 2-fold less IL-10 and 3-fold more IFN-γ when compared to PLP:OVA-pσ1-protected mice treated with isotype control Ab (Figure 3.5*C*). This result is not surprising since it is well-established that Th1 and Th2-type cytokines counter-regulate each other (195-197) and is consistent with the supportive role of IL-4 in IL-10-mediated protection against EAE observed in PLP:OVA-pσ1-dosed mice.

In an effort to further evaluate the mechanism of PLP:OVA-pσ1-mediated protection against EAE, we next investigated if PLP:OVA-pσ1-primed T_{reg} and/or $CD25^-$ $CD4^+$ Th2 cell could prevent EAE development when adoptively transferred into naïve mice. Results depicted in Figure 3.6*A* clearly show the importance of PLP:OVA-pσ1-primed T_{reg} cells in protection against EAE. Adoptive transfer of these T_{reg} cells provided complete protection against the development of clinical disease in recipient mice. Interestingly, consistent with the supportive role of Th2 cells in suppression of EAE, adoptive transfer of PLP:OVA-pσ1-derived $CD25^-CD4^+$ Th2 cells significantly delayed and ameliorated disease in mice. To determine if PLP:OVA-pσ1-mediated protection against EAE could be established in the absence of functional $CD25^+CD4^+FoxP3^+$ T_{reg} cells, mice were nasally dosed with PLP:OVA-pσ1 and treated with anti-CD25 mAb concurrently. These mice developed very severe clinical disease and all succumbed to peak clinical symptoms (Figure 3.6*B*). Further investigation revealed that induction of Th17-type cells is responsible for the aggressive EAE observed in anti-CD25 mAb-treated PLP:OVA-pσ1-dosed mice. Interestingly, we show that $CD4^+$ T cells from severely diseased PLP:OVA-pσ1-dosed, anti-CD25 mAb-treated mice produce

significantly more of proinflammatory cytokines and at least 2-fold more TGF-β than PLP:OVA-pσ1-protected mice (Figure 3.6*C*).

The anti-inflammatory and anti-encephalitogenic potential of TGF-β has been well-established (33, 78, 104, 120, 267). On the other hand, this cytokine has been also implicated in induction of Th17-type cells, which are now considered the major encephalitogenic cell population in EAE. Thus, we questioned whether the increased production of TGF-β by cells isolated from severely diseased mice dosed with PLP:OVA-pσ1 plus treatment with anti-CD25 mAb is related to the lack of protection against EAE and to the strong proinflammatory phenotype observed in these mice. We learned that simultaneous inhibition of TGF-β and inactivation of CD25 result in restored protection against EAE in mice nasally dosed with PLP:OVA-pσ1 (Figure 2.7). Interestingly, analysis of cytokines produced by lymphocytes isolated from these rescued mice revealed strong Th2 cells bias, as evidenced by > 6-fold increase in IL-4, 2-fold increase in IL-13, and almost 7-fold increase in IL-28, as well as the concomitant reduction in Th1 and Th17-type cytokines when compared to diseased mice dosed with PLP:OVA-pσ1 plus anti-CD25 mAb treatment (Figure 3.8). When compared to anti-CD25 mAb-treated PLP:OVA-pσ1-dosed mice, > 8-fold reduction in IL-6, 5-fold reduction in IL-17 and IL-23, and 3-fold reduction in IL-22 and IL-21 were observed in mice dosed with PLP:OVA-pσ1 plus treated with anti-CD25 and anti-TGF-β mAbs (Figure 3.8).

In addition to TGF-β, other cytokines, including IL-23, can induce Th17-type cells. To evaluate if induction of Th17 cells and abrogation of PLP:OVA-pσ1-mediated

protection against EAE in mice treated with anti-CD25 mAb are dependent on IL-23, we queried what happens if IL-23 is neutralized. Results shown in Figure 3.9 clearly suggest that IL-23 is not essential for induction of Th17-type cells in these mice, as evidenced by the lack of effect of IL-23 neutralization on the disease course in mice dosed with PLP:OVA-pσ1 and treated with anti-CD25 mAb.

EAE induced by $PLP_{139-151}$ peptide in SJL mice mimics relapsing-remitting MS observed in human patients, whereas MOG_{35-55}-induced EAE resembles the secondary progressive MS (6, 172). PLP:OVA-pσ1 when nasally administered to mice prevents development of $PLP_{139-151}$-induced EAE. When investigating if MOG-pσ1 fusion protein would efficiently suppress MOG_{35-55}-induced acute EAE in C57BL/6 mice, results presented in Chapter 4 clearly demonstrated that MOG-pσ1 is very effective in preventing MOG_{35-55}-induced EAE. As little as 50 μg of MOG-pσ1 prevented any clinical manifestation of acute EAE and the CNS histopathology (Figure 4.2). Importantly, nasal administration of $rMOG_{(29-146)}$ alone did not have the same effect and did not prevent EAE development. Results of cytokine analysis presented in Figure 4.3 clearly demonstrate that cells obtained from MOG-pσ1-protected mice produced > 8-fold less of IL-17, 3-fold less of IL-21, but displayed > 3-fold increase in IL-4, and 6-fold increase in IL-10 when compared to PBS-dosed diseased mice (Figure 4.3). Interestingly, we learned that rMOG- and PBS-dosed mice develop similar clinical EAE, although the disease mechanism is dramatically different between these groups. Consistent with the general opinion of EAE being mediated predominantly by Th17-type cells (168, 169, 187, 190), PBS-dosed diseased mice produce predominantly IL-17 and

IL-21, and almost no IL-4 or IL-10. On the other hand, disease in rMOG-dosed mice exhibits a strong Th1 cell bias, as evidenced by prominent production of IFN-γ and IL-27 and concomitant reduction in Th17- and Th2-type cytokines (Figure 4.3). This discovery is especially interesting given the controversial role of IFN-γ and other Th1-type cytokines in the progression of EAE, and may provide a new insight into the mechanism of EAE pathogenesis. In agreement with our results is a recent finding by Kroenke *et al*, demonstrating that either IL-12p70-polarized Th1 cells or IL-23-polarized Th17 cells can induce clinical EAE when adoptively transferred into mice (414). Even though no clinical differences were exhibited by mice transferred with $PLP_{139-151}$-specific Th1 or Th17 cells, these mice displayed different patterns and composition of CNS infiltrates. EAE caused by adoptively transferred encephalitogenic Th1 cell was associated with the CNS infiltration by macrophages/monocytes, whereas neutrophiles predominantly penetrated the CNS in mice that received encephalotogenic Th17 cells (414).

T_{reg} cells induced in mice tolerized with OVA-pσ1, or protected against EAE with PLP:OVA-pσ1, produce predominantly IL-10. Moreover, results shown in Chapter 2 clearly demonstrate that OVA-pσ1-mediated tolerance cannot be established in IL-$10^{-/-}$ mice (Figure 2.5). Furthermore, as reported in Chapter 3, *in vitro* inactivation of IL-10 abolishes PLP:OVA-pσ1-mediated suppression of $PLP_{139-151}$-specific proliferation of $CD4^+$ T cells. Upon determining if MOG-pσ1-mediated protection against acute EAE cannot be established in IL-$10^{-/-}$ mice (Figure 4.5), it was quickly learned that in EAE-induced IL-$10^{-/-}$ mice, MOG-pσ1 was not protective. In fact, it was associated with an increased production of proinflammatory cytokines IFN-γ, IL-17, and IL-21 and

concomitant reductions of IL-4 and IL-13, when compared to MOG-pσ1-dosed C57BL/6 mice.

To analyze potential phenotypic traits for MOG-pσ1-induced regulatory cells, in agreement with our results presented in Chapters 2 and 3, MOG-pσ1-induced T_{reg} cells produce predominantly IL-10, and $FoxP3^{+}CD25^{-}CD4^{+}$ T cells secrete IL-4 (Figure 4.6). Moreover, we have determined that FoxP3-expressing regulatory T cells ($CD25^{+}$ and $CD25^{-}$) exhibit ~ 50 % increase in expression of CD73 (Table 4.3). Interestingly, B cells isolated from MOG-pσ1-dosed mice produce increased amounts of IL-10 and IL-4 and express over 30 % more of CD39, when compared to B cells from PBS-dosed mice (Table 4.3). CD39 (ATPDase) hydrolizes extracellular pro-apoptotic ATP and ADP (404, 405) to AMP, which is then converted to anti-inflammatory immunemodulator, adenosine, by CD73 (ecto-5'-nucleotidase) (406). Expression of CD73 and CD39 has been associated with the suppressive / anti-inflammatory potential of regulatory T cells (409). Production of IL-10 and IL-4 by anti-inflammatory B cells has been established (98, 236, 241, 401), although expression of CD39 has not yet been associated with regulatory B cells phenotype. The importance of this discovery for our model has been presented in Figure 5.1. We propose that ATP/ADP released by activated $CD4^{+}$ T cells directed to undergo apoptosis by pσ1, may be hydrolyzed to AMP by CD39 expressed by regulatory B cells. Furthermore, CD73 expressed by regulatory $CD4^{+}$ T cells activated by nasal administration of MOG-pσ1 can plausibly convert AMP into anti-inflammatory adenosine, providing anti-inflammatory environment, and further supporting activation of T_{reg} and anti-inflammatory Th2 cells.

To further investigate the role of B cells in MOG-pσ1-induced protection against EAE, consistent with the reported regulatory function of IL-10-producing B cells (98), our results show in Figure 4.7*A* that B cells play a supportive role in MOG-pσ1-mediated protection against EAE. Mice lacking functional B cells (μMT mice) are only partially protected against EAE when nasally dosed with MOG-pσ1 (Figure 4.7*A*). Moreover, adoptive transfer of B cells from MOG-pσ1-dosed C57BL/6 mice to diseased C57BL/6 mice prevents further progression of the disease but does not accelerate the recovery from EAE (Figure 4.7*B*). When B cells obtained from MOG-pσ1-dosed C57BL/6 mice were adoptively transferred to diseased μMT mice, further progression of the disease was ameliorated, and these mice remitted faster from the disease, though did not recover completely (Figure 4.7*B*). Interestingly, when diseased μMT mice received B cells from PBS-primed C57BL/6 mice, the disease progression was accelerated, and these mice developed significantly more severe EAE when compared to PBS-dosed C57BL/6 mice. In agreement with the work of others suggesting supportive role of regulatory B cells against inflammatory diseases, we determined that nasally administered MOG-pσ1 induces regulatory potential of B cells exhibited by production of anti-inflammatory cytokines and/or expression of CD39 (Figure 5.1).

Results described in Chapter 4 indicate that MOG-pσ1-induced T_{reg} cells produce predominantly IL-10, and MOG-pσ1-mediated protection against EAE cannot be established in IL-$10^{-/-}$ mice. Moreover, as determined in Chapter 3, PLP:OVA-pσ1-mediated protection against RR EAE is abolished when mice are depleted of functional T_{reg} cells at the time of the disease induction. Therefore, IL-10 plays a crucial role in

pσ1-mediated tolerance to delivered Ag. B cells from MOG-pσ1-protected mice produce elevated amounts of IL-10 when compared to B cells obtained from diseased PBS-dosed mice. Thus, we questioned whether the source of IL-10 is important for MOG-pσ1-mediated protection against EAE and, if so, which IL-10-producing cells are pivotal for this protection. The answer to this question was provided by the results of another adoptive transfer experiment. As depicted in Figure 4.8, adoptive transfer of T_{reg} cells from MOG-pσ1-primed C57BL/6 mice to diseased IL-$10^{-/-}$ mice inhibits progression and significantly accelerates recovery from EAE. Even though adoptive transfer of B cells from MOG-pσ1-primed C57BL/6 mice also prevents further progression of EAE, IL-$10^{-/-}$ mice that received MOG-pσ1-primed B cells did not recover from EAE significantly earlier when compared to PBS-dosed control mice. Therefore, IL-10-producing T_{reg} cells are pivotal for pσ1-mediatd tolerance, but B cells and Th2 cells can play a supportive anti-inflammatory role.

Induction of a low-dose mucosal tolerance is not restricted to T cells. There is a growing body of evidence suggesting that APCs, especially certain subset of DCs, are necessary for providing a "regulatory" signal to T cells (31, 47). We took advantage of the available CD11$b^{-/-}$ mouse model to determine if APCs play a role in pσ1-mediated tolerance to the fused protein Ag and MOG-pσ1-mediated protection against acute EAE. Results shown in Figure 4.9 suggest that CD11b expression is required for pσ1-mediated tolerance, and mice lacking this molecule are not tolerized to MOG and are not protected against EAE by nasally administered MOG- pσ1. This discovery is consistent with the reported inability to induce tolerance in CD11$b^{-/-}$ mice by mucosally applied Ag (99). In

agreement with the clinical disease exhibited by CD11b$^{-/-}$ mice nasally dosed with PBS or with MOG-pσ1, these mice produced significantly more of proinflammatory cytokines, but showed major depression in anti-inflammatory cytokines when compared to protected MOG-pσ1-dosed C57BL/6 mice (Figure 4.9*C*). These results further support our hypothesis, that nasally administered MOG-pσ1, after being transported by M cells to mucosal sites, is processed by "tolerogenic" subpopulation of DCs, resulting in activation of regulatory T cells that suppress MOG-specific effector Th17 and Th1 cells via production of regulatory cytokines (Figure 5.1).

In Chapter 3, we reported that single dose of PLP:OVA-pσ1 administered to mice before EAE induction or during its pre-clinical phase significantly ameliorates $PLP_{139-151}$-indcued EAE (Figure 3.3). As little as 50 μg of MOG-pσ1 entirely prevented clinical manifestation of MOG_{35-55}-induced acute EAE (Chapter 4). To investigate if MOG-pσ1 can suppress ongoing clinical EAE, the results presented in Figure 4.4*A* clearly show that 50 μg of MOG-pσ1 nasally administered to mice at the peak of clinical disease drastically improve EAE manifestation within the first 24 h after treatment. This instant remission from the acute disease was attributed to the well-established ability of pσ1 to induce apoptosis. We reported in Chapter 2 that OVA-pσ1 induces apoptosis of OVA-Tg $CD4^+$ T cells *in vitro*. In agreement with our results are findings of others suggesting that pσ1-induced apoptosis can be mediated by sialic acid binding domain on pσ1. To verify the specificity of pσ1-mediated apoptosis, we treated mice that developed acute EAE with MOG-pσ1, PLP:OVA-pσ1, rMOG, pσ1, or with rMOG plus pσ1 (Figure 4.9*B*). Our results clearly indicate that immediate improvement in clinical manifestation of EAE

is achieved only after nasal administration of MOG-pσ1. In contrast to MOG-pσ1-mediated recovery, nasal treatments with PLP:OVA-pσ1, rMOG, or PBS have no effect on recovery from EAE. Significantly, mice that received pσ1 or rMOG plus pσ1 developed more restricted disease after treatment indicating unspecific deletion of $CD4^+$ T cells by pσ1 not fused with encephalitogenic peptide. Therefore, our results suggest that the mechanism of pσ1-delivered tolerance to the fused protein Ag relies on induction of active suppression and clonal deletion of effector cells. Systemic administration of encephalitogenic MOG_{35-55} peptide emulsified in a potent adjuvant (CFA), results in activation of strong systemic proinflammatory response mediated by MOG_{35-55}-reactive Th17 and Th1 cells (Figure 5.1). Additionally, presence of activated $CD4^+$ T cells and Ag, activates clonal expansion of B cells that differentiate into plasma cells and produce MOG_{35-55}-specific Abs. Since induction of MOG-specific suppression of the immune reponse by regulatory T cells would be unlikely after just 24 hours following nasal administration of MOG-pσ1, we hypothesize that this immediate amelioration of the disease in MOG-pσ1- but not PLP:OVA-pσ1-, pσ1-, or rMOG + pσ1-treated mice may be attributed to specific lysis of MOG-reactive effector $CD4^+$ T cells by MOG-pσ1-Ab complexes (Figure 5.1). The hypothesis proposing that MOG-specific Abs may play a role in MOG-pσ1-mediated recovery from EAE need to be confirmed; nonetheless our discovery opens an exciting new area of studies to determine other potential mechanisms of pσ1-mediated tolerance, and provides an argument that the type of protein Ag delivered by pσ1 can influence the type regulatory response mediated by pσ1.

Although our results indicate that a pσ1-based delivery vehicle can be successfully applied to prevent and/or treat autoimmune diseases, several questions remain to be answered. First, the potential toxicity of pσ1 needs to be evaluated *in vivo* in order to potentially test pσ1-based vaccines in primates. Secondly, the direct effect of pσ1 on cells and tissues in the CNS has not been evaluated and is of importance given the pσ1's ability to induce apoptosis. As demonstrated in Chapter 4, MOG-pσ1-induced protection against EAE could not be established in $CD11b^{-/-}$ mice, suggesting the importance of APCs for generation of pσ1-mediated tolerance. Future studies should address the influence of pσ1 on innate cells, especially DCs, since a growing body of evidence suggests the importance of these cells in induction of low-dose mucosal tolerance. Nasal administration of MOG-pσ1 drastically improves MOG_{35-55}-induced EAE. On the other hand, nasally given PLP:OVA-pσ1 has no effect on clinical manifestation of EAE, and administration of not fused pσ1 exacerbates the disease. Future studies are needed to confirm that these effects are due to the pσ1-mediated apoptosis and to investigate the conditions under which pσ1-mediated apoptosis is restricted to effector $CD4^+$ T cells. Additionally, the potential role of B cells and auto-Ags in pσ1-mediated tolerance need to be investigated, since our results in Chapter 4 suggested the potential involvement of Ab-mediated lysis in pσ1-mediated recovery from EAE. Finally, the initial interaction with mucosal cells can determine the fate of pσ1-based mucosal vaccines. Therefore, it is important to investigate if tolerogenic effect is

restricted to pσ1 interaction with M cells or if additional cell types are important for tolerogenic outcome of pσ1-based vaccine platform.

In summary, our results have demonstrated the feasibility of pσ1-based protein vaccines to induce single-dose mucosal tolerance, which is restricted to the pσ1-fused protein Ag and can be utilized as a treatment and/or preventive drug against autoimmune inflammatory diseases and allergies. Furthermore, the requirement for just a single-dose nasal administration overcomes the serious potential for health issues, such as the development of allergies.

Aside of the great practical relevance of a pσ1-based delivery system, that provides a tool for single low-dose nasal tolerization, we have learned that genetically modified pσ1 can induce tolerance to fused protein Ag and to itself by at least two distinct mechanisms, namely active suppression of Ag-specific effector $CD4^+$ T cells and/or clonal deletion of effector cells. The proposed pro-apoptotic function of pσ1 needs to be characterized in more details, to determine under what conditions pσ1-mediated apoptosis is Ag-specific, and to investigate the possible involvement of Abs and their supportive role in pσ1-mediated tolerance. We have identified new phenotype for regulatory B cells, the surface expressed CD39 ATPDase that may be linked to the suppressive function of these cells. Moreover, we provided an argument that pσ1-induced tolerance can be mediated by various regulatory T cells, depending on the experimental conditions.

Furtheremore, as determined in Chapter 2, tolerance delivered by OVA-pσ1 in the presence or absence of mucosal adjuvant CpG-ODN, was mediated by potentially

different types of regulatory cells, that secreted wither IL-13 and TGF-β or IL-10 and IL-4, respectively. This discovery provides an exciting new direction in research on the role of mucosal adjuvants in alteration between different types of regulatory mechanisms. Likewise, in Chapter 4 we reported that in contrats to PBS-dosed mice that developed Th17 cell-mediated EAE, nasal administration of rMOG, resulted in induction of Th1 type cytokines in response to MOG_{35-55} challenge, providing the new insights into the mechanism of EAE pathogenesis.

REFERENCES

1. Kappler, J. W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49: 273-280

2. Mondino, A., A. Khoruts, and M. K. Jenkins. 1996. The anatomy of T-cell activation and tolerance. *PNAS* 93: 2245-2252

3. Harber, M., A. Sundstedt, and D. Wraith. 2000. The role of cytokines in immunological tolerance: potential for therapy. *Expert Rev Mol Med* 1-20

4. McFarland, H. F. 1996. Complexities in the treatment of autoimmune disease. *Science* 274: 2037-2038

5. Hickey W. F., B. L. Hsu, and H. Kimura. 1991. T-lymphocyte entry into the rat central nervous system. *J Neurosci Res* 28: 254-260

6. Steinman, L., R. Martin, C. Bernard, P. Conlon, and J. R. Oksenberg. 2002. Multiple sclerosis: Deeper understanding of its pathogenesis reveals new targets for therapy. *Annu Rev Neurosci* 25: 491-505

7. Mathisen, P. M., S. Pease, J. Garvey, L. Hood, and C. Readhead. 1993. Identification of an embryonic isoform of myelin basic protein that is expressed widely in the mouse embryo. *PNAS* 90: 10125-10129

8. Khoury, S. J., M. H. Sayegh, W. W. Hancock, L. Gallon, C. B. Carpenter, and H. L. Weiner. 1993. Acquired tolerance to experimental autoimmune encephalomyelitis by intrathymic injection of myelin basic protein or its major encephalitogenic peptide. *J Exp Med* 178: 559-566

9. Song, F., Z. Guan, I. E. Gienapp, T. Shawler, J. Benson, and C. C. Whitacre. 2006. The thymus plays a role in oral tolerance in experimental autoimmune encephalomyelitis. *J Immunol* 177: 1500-1509

10. Borghans, J. A. M., R. L. De Boer, E. Sercarz, and V. Kumar. 1998. T cell vaccination in experimental autoimmune encephalomyelitis: a mathematical model. *J Immunol* 161: 1087-1093

11. Ohashi, P. S., S. Oehen, K. Buerki, H. Pircher, C. T. Ohashi, B. Odermatt, B. Malissen, R. M. Zinkernagel, and H. Hengartner. 1991. Ablation of “tolerance” and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 65(2): 305-317

12. Dittel, B. N., R. M. Merchant, and C. A. Janeway, Jr. 1999. Evidence for Fas-dependent and Fas-independent mechanism in the pathogenesis of experimental autoimmune encpehalomyelitis. *J Immunol* 162: 6392-6400

13. Suvannavejh, G. C., M. C. Dal Canti, L. A. Matis, and S. D. Miller. 2000. Fas-mediated apoptosis in clinical remissions of relapsing experimental autoimmune encephalomyelitis. *J Clin Invest* 105(2): 223-231

14. Singer, G. G., A. C. Carrera, A. Marshak-Rothestein, C. Martinez, and A. K. Abbas. 1994. Apoptosis, Fas and systemic autoimmunity: the MRL-lpr/lpr model. *Curr Opin Immunol* 6(6): 913-929

15. Okuda, Y., M. Okuda, and C. C. A. Bernard. 2002. The suppression of T cell apoptosis influences the severity of disease during the chronic phase but not the recovery from the acute phase of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 131: 115-125

16. Voll, R. E., M. Herrmann, E. A. Roth, C. Stach, J. R. Kalden, and I. Girkontaite. 1997. Immunosuppressive effects of apoptotic cells. *Nature* 390(6658): 350-361

17. Windhagen, A., J. Newcombe, F. Dangond, C. Strand, M. N. Woodroofe, M. L. Cuzner, and D. A. Hafler. 1995. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J Exp Med* 182: 1985-1996

18. Steinman R. M., S. Turley, I. Mellaman, K. Inaba. 2000. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J Exp Med* 191: 411-416

19. Miller, S. D., C. L. Vanderlugt, D. J. Lenschow, J. G. Pope, N. J. Karandikar, M. C. Del Canto, and J. A. Bluestone. 1995. Blockade of CD28/B7-1 interaction prevents epitope spreading and clinical relapses of murine EAE. *Immunity* 3: 739-745

20. Markovic-Plese, S., I. Cortese, K. Wandinger, H. McFarland, and R. Martin. 2001. CD4+CD28-costimulation-independet T cells in multiple sclerosis. *J Clin Invest* 108: 1185-1194

21. Gajewski T. F., D. W. Lancki, R. Stack, and F. W. Fitch. 1994. “Anergy” of TH0 helper T lymphocytes induces downregulation of Th1 characteristics and a transition to a Th2-like phenotype. *J Exp Med* 179(2): 481-491

22. Faria, A., and H. L. Weiner. 2006. Oral tolerance: therapeutical implications for autoimmune disease. *Clin Dev Immunol* 13 (2-4): 143-157

23. Faria, A. M., and H. L. Weiner. 2006. Oral tolerance and TGF-beta-producing cells. *Inflamm Allergy Drug Targets* 5(3): 179-190

24. Weiner, L. H. 1997. Oral tolerance: immune mechanisms and treatment of autoimmune diseases. *Rev Immunol* 18(7): 335-343

25. Miyamoto K., Ch. I. Kingsley, X. Zhang, C. Jabs, L. Izikson, R. A. Sobel, H. L. Weiner, V. K. Kuchroo, and A. H. Sharpe. 2005. The ICOS molecule plays a crucial role in the development of mucosal tolerance. *J Immunol* 175: 7341-7347

26. Mucida, D., N. Kutchkhidze, A. Erazo, M. Russo, J. J. Lafaille, and M. Curotto de Lafaille. 2005. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest* 115(7): 1923-1933

27. Elson, C. O., and W. Ealding. 1984. Cholera toxin feeding did not induce oral tolerance in mice and abrogated oral tolerance to an unrelated protein antigen. *J Immunol* 133(6): 2892-2897

28. Elson, C. O. 1985. Induction and control of gastrointestinal immune system. *Scand J Gastroenterol Suppl* 114: 1-15

29. Weiner, H. L. 2000. Oral tolerance, an active immunologic process mediated by multiple mechanisms. *J Clin Invest* 106(8): 935-937

30. Bouvet, J. P., N. Decroix, and P. Pamonsinlapatham. 2002. Stimulation of local antibody production: parenteral or mucosal vaccination? *Trends Immunol* 23(4): 209-231

31. Legge, K. L., R. K. Gregg, R. Maldonado-Lopez, L. Li, J. C. Caprio, M. Moser, and H. Zaghouani. 2002. On the role of dendritic cells in peripheral T cell tolerance and modulation of autoimmunity. *J Exp Med* 196(2): 217-227

32. Anderton, S. M., and D. C. Wraith. 1998. Hierarchy in the ability of T cell epitopes to induce peripheral tolerance to antigens from myelin. *Eur J Immunol* 28: 1251-1261

33. Ochoa-Repáraz, J., C. Riccardi, A. Rynda, S. Jun, G. Callis, and D. W. Pascual. 2007. Regulatory T cell vaccination without autoantigen protects against experimental autoimmune encephalomyelitis. *J Immunol* 178: 1791-1799

34. Ochoa-Repáraz, J., A. Rynda, M. A. Ascón, X. Yang, I. Kochetkova, C. Riccardi, G. Callis, T. Trunkle, and D. W. Pascual. 2008. IL-13 production by regulatory T cells protects against experimental autoimmune encephalomyeleitis independently of autoantigen. *J Immunol* 181: 954-968

35. Yuki, Y., Y. Byun, M. Fujita, W. Izutani, T. Suzuki, S. Udaka, K. Fujihashi, J. R. McGhee, and H. Kiyono. 2001. Production of a recombinant hybrid molecule of cholera toxin -B subunit and proteolipid-protein-peptide for the treatment of experimental encephalomyelitis. *Biotechnol Bioeng* 72(1): 62-69

36. Friedman, A., and H. L. Weiner. 1994. Induction of anergy or active suppression following oral tolerance is determined by antigen dosage *PNAS* 91: 6688-6692

37. Faria, A., and H. L. Weiner. 1999. Oral tolerance: mechanisms and therapeutic applications. *Adv Immunol* 73: 153-264

38. Kato, T., and R. L. Owen. 2005. Structure and function of intestinal mucosal epithelium. Chap. 8. In *Mucosal Immunology*, Mestecky, J., M. E. Lamm, W. Strober, J. Bienenstock, J. R. McGhee, and L. Mayer, eds. Elsevier-Academic Press, San Diego, CA. 131-151

39. Dubois, B., A. Goubier, G. Joubert, and D. Kaiserlian. 2005. Oral tolerance and regulation of mucosal immunity. *CMLS Cell Mol Life Sci* 62: 1322-1332

40. Neutra, M. R. 1998. Current concepts in mucosal immunity V. Role of M cells in transepithelial transport of antigens and pathogens to the mucosal immune system. *American Physiological Society* G785-G791

41. Mowat A. M., J. J. Thomas, and D. M. V. Parrott. 1986. Divergent effects of bacterial lipopolisascharide on immunity to orally administered protein and particular antigens in mice. *Immunol* 58: 677-684

42. Kelsall, B. L., and F. Leon. 2005. Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease. *Immunol Rev* 206: 132-148

43. Fujihashi, K. T. Dohi, P. D. Rennert, M. Yamamoto, T. Koga, H. Kiyono, and J. R. McGhee. 2001. Peyer's patches are required for oral tolerance to proteins. *PNAS* 98(6): 3310-3315

44. Fujihashi, K., H. Kato, F. W. van Ginkel, T. Koga, P. N. Boyaka, R. J. Jackson, R. Kato, Y. Hagiwara, Y. Etani, I. Goma, K. Fujihashi, H. Kiyono, and J. R. McGhee. 2001. A revisit of mucosal IgA immunity and oral tolerance. *Acta Odontol Scand* 59: 301-308

45. Gonnella, P. A. Y. Chen. J. Komagata, M. Quartulli, and H. L. Weiner. 1998. In situ immune response in gut-associated lymphoid tissues (GALT) following oral antigen in TCR-transgenic mice. *J Immunol* 160(10): 4708-4718

46. Kato, H., K. Fujihashi, R. Kato, Y. Yuki, and J. R. McGhee. 2001. Oral tolerance revisited: prior oral tolerization abrogates cholera toxin-induced mucosal IgA responses. *J Immunol* 166: 3114-3121

47. Fleeton, M., N. Contractor, F. Leon, J. He, D. Wetzel, T. Dermody, A. Iwasaki, and B. Kelsall. 2004. Involvement of dendritic cell subsets in the induction of oral tolerance and immunity. *Ann NY Acad Sci* 1029: 60-65

48. Johansson, C. and B. L. Kelsall. 2005. Phenotype and function of intestinal dendritic cells. *Semin Immunol* 2005. 17(4): 284-294

49. Fleeton, M., N. Contractor, F. Leon, J. D. Wetzel, T. S. Dermody, and B. L. Kelsall. 2004. Peyer's patch dendritic cells process viral antigen from apoptotic epithelial cells in the intestine of reovirus-infected mice. *J Exp Med* 200: 235-245

50. Kelsall, B. L. 2008. Innate and adaptive mechanisms to control of pathological intestinal inflammation. *J Pathol* 214(2): 242-259

51. Strober, W., B. Kelsall, and T. Marth. 1998. Oral tolerance. *J Clin Invest* 18(1): 1-30

52. Benson, J. M., K. A. Campbell. Z. Guan, I. E. Gienapp, S. S. Stuckman, T. Forsthuber, and C. C. Whitacre. 2000. T-cell activation and receptor down modulation precede deletion induced by mucosally administered antigen. *J Clin Invest* 106(8): 1031-1038

53. Schmidt, J., J. M. Metselaar, M. H. M. Wauben, K. V. Toyka, G. Storm, and R. Gold. 2000. T-cell apoptosis *in situ* in experimental autoimmune encephalomyelitis following methylprednisolone pulse therapy. *Brian* 123(7): 1431-1441

54. Chen, Y., V. K. Kuchroo, J. Inobe, D. A. Hafler, and H. L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 256: 1237-1240

55. Reddy, J., H. Waldner, X. Zhang, Z. Illes, K. W. Wucherpfennig, R. A. Sobel, and V. K. Kuchroo. 2005. CD4+CD25+ regulatory T cells contribute to gender differences in susceptibility to experimental autoimmune encephalomyelitis. *J Immunol* 175: 5591-5595

56. Faria, A. M. C., R. Maron, S. M. Ficker, A. J. Slavin, T. Spahn, and H. L. Weiner. 2003. Oral tolerance induced by continuous feeding: enhanced up-regulation of transforming growth factor–β/interleukin-10 and suppression of experimental autoimmune encephalomyelitis. *J Autoimmun* 20: 135-145

57. Salvin, J. A., R. Maron, and H. L. Weiner. 2001. Mucosal administration of IL-10 enhances oral tolerance in autoimmune encephalomyelitis and diabetes. *Int Immunol* 13(6): 825-833

58. Kelly, K. A., and C. C. Whitacre. 1996. Oral tolerance in EAE: reversal of tolerance by T helper cell cytokines. *J Neuroimmunol* 66: 77-84

59. Fukaura, H., S. C. Kent, M. J. Pietrusewicz, S. J. Khoury, H. L. Weiner, and D. A. Hafler. 1996. Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-β1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients. *J Clin Invest* 98: 70-77

60. Olivares-Villagómez, D., Y. Wang, and J. J. Lafaille. 1998. Regulatory CD4+ T cells expressing endogenous T cell receptor chains protect myelin basic protein-specific transgenic mice from spontaneous autoimmune encephalomyelitis. *J Exp Med* 188(10): 1883-1894

61. Weiner, H. L. 2001. Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes Infection* 3: 947-954

62. Holmgren, J., J. Adamson, F. Anjuere, J. Clemens, C. Czerkinsky, K. Erriksson, C. F. Flach, A. George-Chandy, A. M. Harandi, M. Lebens, T. Lehrerm M. Lindblad, E. Nygren, S. Raghavan, J, Sanchez, M. Stanford, J. B. Sun, A. M. Svennerholm, and S. Tengvall. 2005. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin B subunit and CpG DNA. *Immunol Lett* 97(2): 181-188

63. Revillard, J. P., G. Cozon and C. Czerkinsky. 1992. Oral administration of immunomodulators and the mucosal system. *Dev Biol Stand* 77: 31-37

64. Lycke, N. 2004. ADP-ribosylating bacterial enzymes for the targeted control of mucosal tolerance and immunity. *Ann N Y Acad Sci* 1029: 193-208

65. Xu-Amano, J., W. K. Aicher, T. Taguchi, H, Kiono, and J. R. McGhee. 1992. Selective induction of Th2 cells in murine Payer's patches by oral immunization. *Int Immunol* 2: 433-445

66. Fujihashi, K., J. McGhee, M. Yamamoto, T. Hiroi, and H. Kiyono. 1996. The role of lambda-delta T cells in the regulation of mucosal IgA response and oral tolerance. In Oral Tolerance, H. Weiner, L. Mayer (eds) *NY Acad Sci* 55-63

67. Sun, J.-B., C. Rask, T. Olsson, J. Holmgren, and C. Czerkinsky. 1996. Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. *PNAS* 93: 7196-7201

68. Aspord, C., and C. Thivolet. 2002. Nasal administration of CTB-insulin induces active tolerization against autoimmune diabetes in non-obese (NOD) mice. *Clin Exp Immunol* 130: 402-211

69. Sun, J.-B., S. Raghavan, Å. Sjöling, S. Lundin, and J. Holmgren. 2006. Oral tolerance induction with antigen-conjugated to cholera toxin B subunit generates both FoxP3+CD25+ and FoxP3-CD25-CD4+ regulatory T cells. *J Immunol* 177: 7634-7644

70. Chandy, A. G., S. Hultkrantz, S. Raghavan, C. Czerkinsky, M. Lebens, E. Telemo, and J. Holmgren. 2006. Oral tolerance induction by mucosal administration of cholera toxin B-coupled antigen involves T-cell proliferation *in vivo* and is not affected by depletion of CD25+ T cells. *Immunol* 118: 311-320

71. Sun J. B., C. Czerkinsky, and J. Holmgren. 2007. Sublingual "oral tolerance" induction with antigen conjugated to cholera toxin B subunit generates regulatory T cells that induce apoptosis and depletion of effector T cells. *Scand J Immunol* 66(2-3): 278-286

72. Sun, J. B., N. Cuburu, M. Blomquist, B. L. Li, C. Czerkinsky, and J. Holmgren. 2006. Sublingual tolerance induction with antigen conjugated to cholera toxin B subunit induce FoxP3+CD25+CD4+ regulatory T cells and suppresses delayed-type hypersensitivity reactions. *Scand J Immunol* 64(3): 251-259

73. Bergquist, C., T. Lagergard, M. Lindblad, and J. Hilmgren. 1995. Local and systemic antibody response to dextran-cholera toxin B subunit conjugates. *Infect Immun* 63(5): 2021-2025

74. Bergquist, C., E. L. Johansson, T. Lagergars, J. Holmgren, and A. Rudin. 1997. Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina. *Infect Immun* 65: 2676-2684

75. Miller, A., O. Lider, and H. L. Weiner. 1991. Antigen-driven bystander suppression after oral administration of antigens. *J Exp Med* 174: 791-798

76. Al-Sabbagh, A., A. Miller, L. M. Santos, and H. L. Weiner. 1994. Antigen derived tissue-specific suppression following oral tolerance: orally administered myelin basic protein suppresses proteolipid protein–induced experimental autoimmune encephalomyelitis in the SJL mouse. *Eur J Immunol* 24: 2104-2109

77. Markovic-Plese, S., A. K. Singh, and I Singh. 2008. Therapeutic potential of statins in multiple sclerosis: immune modulation, neuroprotection and neurorepair. *Future Neurol* 3(2): 153-167

78. Jun, S., W. Glimore, G. Callis, A. Rynda, A. Haddah, and D. W. Pascual. 2005. A live diarrheal vaccine imprints a Th2 cell bias and acts as an anti-inflammatory vaccine. *J Immunol* 175: 6733-6740

79. Conde A. A., B. Stransky, A. M. Faria, and N. M. Vaz. 1998. Interruption of recently induced immune response by oral administration of antigen. *Braz J Med Biol Res* 31(3):377-380

80. Ostroukhova, M., C. Seguin-Devaux, T. B. Oriss, B. Dixon-McCarthy, L. Yang, B. T. Ameredes, T. E. Corcoran, and A. Ray. 2004. Tolerance induced by inhalated antigen involves CD4+ T cells expressing membrane-bound TGF-β and FoxP3. *J Clin Invest* 114(1): 28-38

81. Huber, S., C. Schramm, H. A. Lehr, A. Mann, S. Schmitt, C. Becker, M. Protschka, P. R. Galle, M. R. Neutra, and M. Blessing. 2004. Cutting edge: TGF-β signaling is required for the *in vivo* expansion and immunosuppressive capacity of regulatory CD4+CD25+ T cells. *J Immunol* 173: 6526-6531

82. Wu, Y., X. Wang, K. L. Csencsits, A. Haddad, N. Walters, and D. W. Pascual. 2001. M cell–targeted DNA vaccination. *PNAS* 98(16): 9319-9323

83. Tamura, S., T. Iwasaki, A. H. Tomphson, H. Asanuma, Z. Chen, Y. Suzuki, C. Aziwa, and T. Kurata. 1998. Antibody-forming cells in the nasal-associated lymphoid tissue during primary influenza virus infection. *J Gen Virol* 79: 291-299

84. Metzler, B., and D. C. Wraith. 1992. Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalitogenic peptide: influence of MHC binding affinity. *Int Immunol* 5: 1159-1165

85. Tamura, S., Y. Ito, H. Asanuma, Y. Hirabayashi, Y. Suzuki, T. Nagamine, C. Aizawa, and T. Kurata. 1992. Cross-protection against influenza virus infection afforded by trivalent inactivated vaccines inoculated intranasally with cholera toxin B subunit. *J Immunol* 149: 981–988

86. Mutsch, M., W. Zhou, P. Rhodes, M. Bopp, R. T. Chen, T. Linder, C. Spyr, and R. Steffen. 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N Engl J Med* 350: 896-903

87. Unger, W. W., F. Hauet-Broere, W. Jansen, L. A. van Berkel, G. Kraal, and J. N. Samsom. 2003. Early events in peripheral regulatory T cell induction via the nasal mucosa. *J Immunol* 171: 4592-4603

88. Samson, J. N., L. A. van Berkel, J. M. van Helvoort, W. W. Unger, W. Jansen, T. Thepen, R. E. Mebius, S. S. Verbeek, and G. Kraal. 2005. FcγRIIB regulates nasal and oral tolerance: a role for dendritic cells. *J Immunol* 174: 5279-5287

89. Rynda, A., M. Maddaloni, D. Mierzejewska, J. Ochoa-Repáraz, T. Maślanka, K. Crist, C. Riccardi, B. Barszczewska, K. Fujihashi, J. R. McGhee, and D. W. Pascual. 2008. Low-dose tolerance is mediated by microfold cell ligand, reovirus protein sigma 1. *J Immunol* 180: 5187-5200

90. O'Neill, E. J., M. J. Day, and D. C. Wraith. 2006. IL-10 is essential for disease protection following intranasal peptide administration in the C57BL/6 model of EAE. *J Neuroimmunol* 178: 1-8

91. Myers, L. K., J. M. Seyer, J. M. Stuart, and A. H. Kang. 1997. Suppression of murine collagen-induced arthritis by nasal administration of collagen. *Immunol* 90(2): 161-164

92. Xiao, B.-G., and H. Link. 1997. Mucosal tolerance: A two-edged sword to prevent and treat autoimmune diseases. *Clin Immunol Immunopathol* 85: 119-128

93. Evavold, B. D., L. I. Sloan, and P. M. Allen. 1993. Tickling the TCR: selective T-cell function stimulated by altered peptide ligands. *Immunol Today* 14: 602-609

94. Kappos, L., G. Comi, H. Panitch, J. Orer, J. Antel, and the APL relapsing MS Study Group. 2000. Induction of non-encephalitogenic Th2 autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo controlled, randomized phase II trial. *Nature Med*. 6(10): 1176-1182

95. Burkhart, C., G. Y. Liu, S. M. Anderton, B. Metzler, and D. C. Wraith. 1999. Peptide-induced T cell regulation of experimental autoimmune encephalomyelitis: a role for IL-10. *Int Immunol* 11: 1625-1634

96. Harrison, L. C., M. Dempsey-Collier, D. R. Kramer, K. Takahashi. 1996. Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes. *J Exp Med* 184(6): 2167-2174

97. Wolvers, D. A. W., C. J. J. Coenen-de Roo, R. E. Mebius, M. J. F. van der Cammen, F. Tirion, A. M. M. Miltenburg, and G. Kraal. 1999. Intranasally induced immunological tolerance is determined by characteristics of the draining lymph nodes: studies with OVA and human cartilage gp-39. *J Immunol* 162L 1994

98. Mizoguchi, A., and A. K. Bnad. 2006. Brief reviews: A case for regulatory B cells. *J Immunol* 176: 705-710

99. Ehirchiou, D., Y. Xioung, G. Xu, W. Chen, Y. Shi, and L. Zhang. 2007. CD11b facilitates the development of peripheral tolerance by suppressing Th17 differentiation. *J Exp Med* 294(7): 1519-1524

100. Endharti, A. T., M. Rifa'I, Z. Shi, Y. Fukuoka, Y. Nakahara, Y. Kawamoto, K. Takeda, K. Isobe, and H. Suzuki. 2005. CD8+CD122+ regulatory T cells produce IL-10 to suppress IFN-γ production and proliferation of CD8+ T cells. *J Immunol* 175: 7093-7097

101. Lee, Y. H., Y. Ishida, M. Rifa'I, Z. Shi, K. Isobe, and H. Suzuki. 2008. Essential role of CD8+CD122+ regulatory T cells in the recovery from experimental autoimmune encephalomyelitis. *J Immunol* 180: 825-832

102. Chen, D., A. E. Juedes, U.-A. Temann, S. Shresta, J. P. Allison, N. H. Ruddle, and R. A. Flavell. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409: 97-101

103. Chen, N., Q. Gao, and E. H. Field. 1996. Prevention of Th1 response is critical for tolerance. *Transplant* 61: 1076-1983

104. Prud'homme G. J., and C. A. Piccirillo. 2000. The inhibitory effects of transforming growth factor –beta-1 (TGF-beta-1) in autoimmune diseases. *J Autoimmun* 14(1): 23-42

105. Wildbaum, G., N. Netzer, and N. Karin. 2002. Tr1-dependent active tolerance blunts the pathogenic effects of determinant spreading. *J Clin Invest* 110(5): 701-710

106. Singh, A. K., M. T. Wilson, S. Hong, D. Ovlivares-Villagómez, C, Du, A, K. Stanic, S. Joyce, S. Sriram, Y. Koezuka, and L. Van Kaer. 2001. Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* 194 (12): 1801-1811

107. Suri-Payer, E., A. Z. Amar, A. M. Thornton, and E. Shevach. 1998. CD4+CD25+ T cells inhibit both induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J Immunol* 60: 1212-1218

108. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. *J Exp Med* 188 (2): 287-296

109. Lim, H. W., P. Hillsamer, A. H. Banham, and C. H. Kim. 2005. Cutting edge: direct suppression of B cells by CD4+CD25+ regulatory T cells. *J Immunol* 175: 4180-4183

110. Marski, M., A. Kandula, J. R. Turner, and C. Abraham. 2005. CD18 is required for optimal development of functional CD4+CD25+ T regulatory cells. *J Immunol* 175: 7889-7897

111. Ageegbe, D., A. L. Bayer, R. B. Levy, and T. R. Malek. 2006. Cutting edge: allogenic CD4+CD25+FoxP3+ T regulatory cells suppress autoimmunity while establishing transplantation tolerance. *J Immunol* 176: 7149-7153

112. Shimizu, J. 2002. Stimulation of CD4+CD25+ regulatory T cells through GITR breaks immunological self-tolerance. *Nature Immunol* 3: 135-142

113. Liu, H., B. Hu, and F. Y. Liew. 2003. CD4+CD25+ regulatory T cells cure murine colitis: the role of IL-10, TGF-beta, and CTLA-4. *J Immunol* 171: 5912-5017

114. Vieira, P. L., J. R. Christensen, S. Minaee, E. J. O'Neill, F. J. Barrat, A. Boonstra, T. Barthloot, B. Stockinger, D. C. Wraith, and A. O'Garra. 2004. IL-10-secreting regulatory T cells do not express FoxP3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells. *J Immunol* 172: 5986-5993

115. Ramsdell. F. 2003. FoxP3 and natural regulatory T cells: key to a cell lineage? *Immunity* 19: 165

116. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. FoxP3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunol* 4: 330

117. Skapenko, A., J. R. Kalden, P. E. Lipsky, and H. Schulze-Koops. 2005. The IL-4 receptor α-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors. *J Immunol* 175: 6107-6116

118. Oida, T., L. Xu, H. L. Weiner, A. Kitani, and W. Strober. 2006. TGF-beta-mediated suppression by CD4+CD25+ T cells is facilitated by CTLA-4 signaling. *J Immunol* 177: 2331-2339

119. Kleinwietfield, M., F. Puentes, G. Borsellino, L. Battistini, O. Rötzschke, and K. Falk. 2005. CCR6 expression defines regulatory effector/memory-like cells within CD25+CD4+ T-cell subset. *Blood* 105(7): 2877-2886

120. Chen, M.-L., B.-S. Yan, Y. Bando, V. K. Kuchroo, and H. L. Weiner. 2008. Latency-associated peptide identifies a novel CD4+CD25+ regulatory T cell subset with TGF-beta-mediated function and enhances suppression of experimental autoimmune encephalomyelitis. *J Immunol* 180: 7327-7337

121. Zheng, S. G., J. Wang, and D., A, Horwitz. 2008. Cutting edge: FoxP3+CD4+CD25+ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6. *J Immunol* 180: 7112-7116

122. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells into CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor FoxP3. *J Exp Med* 198: 1875-1886

123. Peng, Y., Y. Laouar, M. O. Li, A. Green, and R. A. Flavell. 2004. TGF-beta regulates *in vivo* expansion of FoxP3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *PNAS* 101(13): 4572-4577

124. Mangan, P. R., L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb, and C. T. Weaver. 2006. Transforming growth factor-β induces development of the T(H)17 lineage. *Nature* 441: 231-234

125. Stockinger, B., and M. Veldhoen. 2007. Th17 T cells: linking innate and adaptive immunity. *Semin Immunol* 19(6): 353-361

126. Kimura, A., T. Naka, and T. Kishimoto. 2007. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *PNAS* 104(29): 12099-12104

127. Wang, Z., J. Hong, W. Sun, G. Xu, N. Li, X. Chen, A. Liu, L. Xu, B. Sun, and J. Z. Zhang. 2006. Role of IFN-gamma in induction of FoxP3 and conversion of CD4+CD25- T cells to CD4+ Tregs. *J Clin Invest* 116(9): 2434-2441

128. Zhang, X., D. N. Koldzic, L. Izikson, J. Reddy, R. F. Nazareno, S. Sakaguchi, V. J. Kuchroo, and H. L. Weiner. 2004. IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25+CD4+ regulatory T cells. *Int Immunol* 16(2): 249-256

129. Stohlman, S. A., L. Pei, D. J. Cua, Z. Li, and D. R. Hinton. 1999. Activation of regulatory cells suppresses experimental allergic encephalomyelitis via secretion of IL-10. *J Immunol* 163: 6338-6344

130. Kleinschek, M. A., A. M. Owyang, B. Joyce-Shaikh, C. L. Langrish, Y. Chen, D. M. Gorman, W. M. Blimenschein, T. McClanahan, F. Brombacher, S. D. Hurst, R. A. Kastelein, and D. J. Cua. 2007. IL-25 regulates IL-17 function in autoimmune inflammation. *J Exp Med* 204(1): 161-170

131. Moore, K. W., A. O'Garra, R. de Waal Malefyt, P. Vieira, and T. R. Mosmann. 1993. Interleukin-10. *Ann Rev Immunol* 11: 165-190

132. Bettelli, E., M. P. Das, E. D. Howard, H. L. Weiner, R. A. Sobel and V. K. Kuchroo. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J Immunol* 161(7): 3299-3306

133. Harrison, L. C., and D. A. Hafler. 2000. Antigen-specific therapy for autoimmune disease. *Curr Opinion Immunol* 12: 704-711

134. Schrempf, W. and T. Ziemssen. 2007. Glatiramer acetate: mechanism of action in multiple sclerosis. *Autoimmun Rev* 7: 469-475

135. Correale, J., M. Farez, and W. Gilmore. 2008. Vaccines for multiple sclerosis: progress to date. *CNS Drugs* 22(3): 175-198

136. Cohen, I. R., F. J. Quintana, and A. Mimran. 2004. Tregs in T cell vaccination: exploring the regulation of regulation. *J Clin Invest* 114(9): 1227-1232

137. Hellings, N., J. Raus, and P. Stinissen. 2004. T-cell vaccination in multiple sclerosis: update on clinical application and mode of action. *Autoimmun Rev* 3(4): 267-275

138. Ben Nun, A., H. Werkle, and I. R. Cohen. 1981. Vaccination against autoimmune encephalomyelitis with T-lymphocyte line cells reactive against myelin basic protein. *Nature* 292: 60-61

139. Medaer, R., P. Stinissen, L. Truyen, J. Raus, and J. Zhang. 1995. Depletion of myelin-basic protein autoreactive T cells by T-cell vaccination: pilot trial in multiple sclerosis. *Lancet* 346: 807-808

140. Koniaras, C., F. R. Carbone, W. R. Health, and A. M. Lew. 1999. Inhibition of naïve class I –restricted T cells by altered peptide ligands. *Immunol Cell Biol* 77: 318-323

141. Ho, P. P., P. Fontoura, P. J. Ruiz, L. Steinman, and H. Garren. 2003. An immunomodulatory CpG oligonucleotide for the treatment of autoimmunity via the innate and adaptive immune system. *J Immunol* 171: 4920-4926

142. Ho, P. P., P. Fontoura, M. Platten, R. A. Sobel, J. J. DeVoss, L. Y. Lee, B. A. Kidd, B. H. Tomooka, J. Capers, A. Agrawal, R. Gupta, J. Zernik, M. K. Yee. B. J. Lee, H. Garren, W. H. Robinson, and L. Steinman. 2005. A suppressive

oligodeoxynucelotide enhances the efficacy of myelin cocktail/IL-4-tolerizing DNA vaccination and treats autoimmune disease. *J Immunol* 175: 6226-6234

143. Alderuccio, F., B. H. Toh, S. S. Tan, P. A. Gleeson, and I. R. van Driel. 1993. An autoimmune disease with multiple molecular targets abrogated by the transgenic expression of a single autoantigen in a thymus. *J Exp Med* 178: 419-426

144. Legge, K. L., B. Min, N. T. Potter, and H. Zaghouani. 1997. Presentation of a T cell receptor antagonist peptide by immunoglobulins ablates activation of T cells by a synthetic peptide or proteins requiring endocytic processing. *J Exp Med* 185: 1043-1053

145. Min, B., K. L. Legge, C. Pack, and H. Zaghouani. 1998. Neonatal exposure to a self-peptide-immunoglobulin chimera circumvents the use of adjuvant and confers resistance to autoimmune disease by a novel mechanism involving interleukin 4 lymph node deviation and interferon gamma-mediated splenic anergy. *J Exp Med* 188 (11): 2007-2017

146. Legge, K. L., B. Min, J. J. Bell, J. C. Caprio, L. Li, R. K. Gregg, and H. Zaghouani. 2000. Coupling of peripheral tolerance to endogenous interleukin 10 promotes effective modulation of myelin-activated T cells and meliorates experimental allergic encephalomyelitis. *J Exp Med* 191(12): 2039-2051

147. Pascual, D. W., J. Ochoa-Repáraz, A. Rynda, and X. Yang. 2007. Tolerance in the absence of auto-antigen. *Endocrine Metabol Immune Disorders Drug Targ* 7: 203-210

148. Kim, W. U., W. K. Lee, J. W. Ryoo, S. H. Kim, J. Kim, J. Youn, S. Y. Min, E. Y. Bae, S. Y. Hwang, S. H. Park, C. S. Cho, J. S. Park, and H. Y. Kim. 2002. Suppression of collagen-induced arthrtis by single administration of poly(lactic-co-glycolic acid) nanoparticles entrapping type II collagen: A novel treatment strategy for induction of oral tolerance. *Arthritid Reum* 46: 1109-1120

149. Masuda, K., K. Horie, R. Suzuki, T. Yoshikawa, K. Hirano. 2002. Oral delivery of antigens in liposomes with some lipid compositions modulates oral tolerance to the antigens. *Microbiol Immunol* 46: 55-58

150. Sun, J.-B., J. Holmgren, and C. Czerkinsky. 1994. Cholera toxin B subunit: An efficient transmucosal carrier-delivery system for induction of peripheral immunological tolerance. *PNAS* 91: 10795-10799

151. Bergerot, I., C. Ploix, J. Petersen, V. Moulin, C. Rask, N. Fabien, M. Lindblad, A. Mayer, C. Czerkinsky, J. Holmgren, and C. Thivolet. 1997. A cholera toxin-insulin

conjugate as an oral vaccine against spontaneous autoimmune diabetes. *PNAS* 94: 4610-4614

152. Lycke, N. 2005. From toxin to adjuvant: basic mechanisms for the control of mucosal IgA immunity and tolerance. *Immunol Lett* 97: 193-198

153. Dermody, T. S., M. L. Nibert, R. Bassel-Duby, and B. N. Fields. 1990. A σ1 region important for hemagglutination by serotype 3 reovirus strains. *J Virol* 64: 5173-5176

154. Wu, Y., M. J. Boysun, K. L. Csencsits, and D. W. Pascual. 2000. Gene transfer facilitated by a cellular targeting molecule, reovirus protein σ1. *Gene Ther* 7: 61-69

155. Kang, Y., L. Xu, B. Wang, A. Chen, and G. Zheng. 2008. Cutting edge: Immunosuppressant as adjuvant for tolerogenic immunization. *J Immunol* 180 5172-5176

156. Khoury, S. J., W. W. Hancock, and H. L. Weiner. 1992. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis and associated with down-regulation of inflammatory cytokines and differential upregulation of transforming growth factor beta, interleukin 4, and prostaglandin E expression in the brain. *J Exp Med* 176: 1355-1364

157. Barton, E. S., J. D. Chappeli, J. L. Connolly, J. C. Forrest, and T. S. Dermody. 2001. Reovirus receptors and apoptosis. *Virol* 290: 173-180

158. Weiner, H. L., D. Drayna, D. R. Averill Jr., and B. N. Fields. 1977. Molecular basis of reovirus virulence: role of the S1 gene. *PNAS* 74: 5744-5748

159. Nibert, M. L., T. S. Dermody, and B. N. Fields. 1990. Structure of the reovirus cell-attachment protein: a model for the domain organization of σ1. *J Virol* 64: 2990-3000

160. Rodgers, S. E., E. S. Barton, S. M. Oberhaus, B. Pike, C. A. Gibson, K. L. Tyler, and T. S. Dermody. 1997. Reovirus-induced apoptosis of MDCK cells is not linked to viral yield and is blocked by Bcl-2. *J Virol* 71: 2540-2546

161. Rivers T. M., D. H. Sprunt, and G. P. Berry. 1933. Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. *J Exp Med* 58: 39-53

162. Baker, D., J. K. O'Neill, S. E. Gschmeissner, C. E. Wilcox, C. Butter, and J. L. Turk. 1990. Induction of chronic relapsing experimental allergic encephalomyelitis in Bozzi mice. *J Neuroimmunol* 28: 261-270

163. Shaw, M. K., C. Kim, H. W. Hao, F. Chen, and H. Y. Tse. 1996. Induction of myelin basic protein-specific experimental autoimmune encephalomyelitis in C57BL/6 mice: mapping of T cell epitopes and T cell receptor Vβ gene segment usage. *J Neurosci Res* 45: 690-699

164. Adelmann, M., J. Wood, I. Benzel, P. Fiori, H. Lasseman, J. M. Matthieu, M. V. Gardiner, K. Dornmair, and C. Linington. 1995. The N-terminal domain of the myelin oligodendrocyte glycoprotein (MOG) induces acute demyelinating experimental autoimmune encephalomyelitis in the Lewis rat. *J Neuroimmunol* 63: 17-27

165. Karpus, W. J., and R. M. Ransohoff. 1998. Chemokine regulation of experimental autoimmune encephalomyelitis: temporal and spatial expression patterns govern disease pathogenesis. *J Immunol* 161: 2667-2671

166. Leonard, J. P., K. E. Waldenburger, R. G. Schaub, T. Smith, A. K. Hewson, M. L. Cuzner, and S. J. Goldman. 1997. Regulation of the inflammatory response in animal models of multiple sclerosis by interleukin-12. *Crit Rev Immunol* 17: 545-553

167. Touil, T., D. Fitzgerald, G. X. Zhang, A. M. Rostami, and B. Gran. 2006. Pathophysiology of interleukin-23 in experimental autoimmune encephalomyelitis. *Drug News Perspect* 19(2): 77-83

168. Park H., Z. Li, X. O. Yang, S. H. Chang, R. Nurieva, Y. H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and Ch. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing IL-17. *Nature Immunol* 6: 1133-1141

169. Harrington L. E., R. D. Hatton, P. R. Mangan, H. Turner, T. L .Murphy, K. M. Murphy, and C. T. Weaver. 2005. IL-17 producing CD4+ effector T cells develop via lineage distinct from T helper type 1 and type 2 lineages. *Nature Immunol* 6: 1123-1132

170. Fife, B. T., G. B. Huffnagle, W. A. Kuziel, and W. J. Karpus. 2000. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *J Exp Med* 192(6): 899-905

171. Olsson, T. 1996. Cytokine-producing cells in experimental autoimmune encephalomyelitis and multiple sclerosis. *Neurology* 45: S11-S15

172. Hafler, D. A., Multiple sclerosis. 2004. *J Clin Invest* 113 (6): 788-794

173. Skulina, C., S. Schmidt, K. Dornmair, H. Babbe, A. Roers, K. Rajewsky, H. Werkle, R. Hohlfeld, and N. Goebels. 2004. Multiple sclerosis: brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood. *PNAS* 101(8): 2428-2433

174. Friese, M. A. and L. Fugger. 2005. Autoreactive CD8+T cells in multiple sclerosis: a new target for therapy? *Brain* 128: 1747-1763

175. Tuohy V. K., R. A. Sobel, and M. B. Lees. 1988. Myelin proteolipod protein-induced experimental allergic encephalomyelitis. Variations of disease expression in different strains of mice. *J Immunol* 140: 1868-1873

176. O'Neill, J. K., D. Baker, A. N. Davidson, S. J. Allen, C. Butter, H. Waldmann, and J. L. Turk. 1993. Control of immune-mediated disease of the central nervous system with monoclonal (CD4-specific) antibodies. *J Neuroimmunol* 45: 1-14

177. Kuchroo, V. K., M. P. Das, J. A. Brown, A. M. Ranger, S. S. Zamvil, R. A. Soel, H. L. Weiner, N. Nabavi, and L. H. Glimcher. 1996. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* 80: 707-718

178. Maron, R., W. W. Hancock, A. Salvin. M. Hattori, V. Kuchroo, and H. L. Weiner. 1999. Genetic susceptibility or resistance to autoimmune encephalomyelitis in MHC congenic mice is associated with differential production of pro- and anti-inflammatory cytokines. *Int Immunol* 11(9): 1573-1580

179. Lehmann, P. V., T. Forsthuber, A. Miller, and E. E. Sercarz. 1992. Spreading of T–cell autoimmunity to cryptic determinants of an autoantigen. *Nature* 358: 155-157

180. Ercolini, A. M., and S. D. Miller. 2006. Mechanisms of immunopathology in murine models of central nervous system demyelinating disease. *J Immunol* 176: 3293-3298

181. Zhang, G.-X., B. Gran, S. Yu, J. Li, I. Siglienti, X. Chen., M. Kamoun, and A. Rostami. 2003. Induction of experimental autoimmune encephalomyelitis is IL-12 receptor-beta2-deficient mice: IL-12 responsiveness in not required in the pathogenesis of inflammatory demyelination in the central nervous system. *J Immunol* 170: 2153-2160

182. Willenborg, D. O., S. Fordham, C. C. Bernard, W. B. Cowden, and I. A. Ramshaw. 1996. IFN-gamma plays a critical down-regulatory role in the induction and effector phase of myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *J Immunol* 157: 3223-3227

183. Krakowski, M., and T. Owens. 1996. Interferon gamma confers resistance to experimental autoimmune encephalomyelitis. *Eur J Immunol* 26: 1641-1646

184. Xiao, B. G., C. G. Ma, L. Y. Xu, H. Link, and C. Z. Lu. 2008. IL-12/IFN-gamma/NO axis plays critical role in development of Th1-mediated experimental autoimmune encephalomyelitis. *Mol Immunol* 45(4): 1191-1196

185. Suen, W. E., C. M. Bergman, P. Hjelmstrom, and N. H. Ruddle. 1997. A critical role for lymphotoxin in experimental allergic encephalomyelitis. *J Exp Med* 186(8): 1233-1240

186. Furuzawa-Caballeda, J., M. I. Vargas-Rojas, and A. R. Cabral. 2006. Autoimmune inflammation from the Th17 perspective. *Autoimmun Rev* 6: 169-175

187. Aranami, T, and T. Yamamura. 2008. Th17 cells and autoimmune encephalomyelitis (EAE/MS). *Allergol Int* 57(2): 115-120

188. Zhang, X., J. Jin, X. Peng, V. S. Ramgolam, and S. Markovic-Plese. 2008. Simvastatin inhibits IL-17 secretion by targeting multiple IL-17-regulatory cytokines and inhibiting the expression of IL-17 transcription factor RORC in CD4+ lymphocytes. *J Immunol* 180: 6988-6996

189. Monteleone, G., F. Pallone, and T. T. Macdonald. 2008. Interleukin-21: a critical regulator of the balance between effector and regulatory T cell response. *Trends Immunol* 29(6): 290-294

190. Coquet, J. M., S. Chakravarti, M. J. Smyth, and D. I. Godfrey. 2008. Cutting edge: IL-21 is not essential for Th17 differentiation or experimental autoimmune encephalomyeleitis. *J Immunol* 180: 7097-7101

191. Thakker, P., M. W. Leach, W. Kuang, S. E. Benoit, J. P. Leonard, and S. Marusic. 2007. IL-23 is critical in the induction but not in the effector phase of experimental autoimmune encephalomyelitis. *J Immunol* 178: 2589-2598

192. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201(2): 233-240

193. Becher, B., B. G. Durell, and R. J. Noelle. 2003. IL-23 produced by CNS-resident cells controls T cell encephalitogenicity during the effector phase of experimental autoimmune encephalomyelitis. *J Clin Invest* 12: 1186-1191

194. Mosmann, T. R., and R. L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7: 145-173

195. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cells. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med* 170: 2081-2095

196. Allen, J. E., and R. M. Maizels. 1997. Th1-Th2: reliable paradigm or dangerous dogma? *Immunol Today* 18: 387-392

197. Kelso, A. 1995. Th1 and Th2 subsets: paradigms lost? *Immunol Today* 16: 374-379

198. Liblou, R. S., S. M. Singer and H. O. McDevitt. 1995. Th1 and Th2 $CD4^+$ T cells in the pathogenesis of organ–specific autoimmune diseases. *Immunol Today* 16: 34-38

199. Arnett, H., J. Manson, K. Suzuk, G. Matsushima, and J. Ting. 2001. TNF alpha promotes proliferation of oligodendrocyte progenitors and re-myelination. *Nature Neurosci* 4: 1116-1122

200. Chabas, D., S. Baranzini, D. Mitchell, C. C. A. Bernard, and S. Rittling. 2001. The influence of the pro-inflammatory cytokine, osteoponin, on autoimmune demyelinating disease. *Science* 294: 1731-1735

201. Fitzgerald, D.C., B. Ciric, T. Touil, H. Harle, J. Grammatikopolou, J. DasSarma, B. Gran, G. X. Zhang, and A. Rostami. 2007. Suppressive effect of IL-27 on encephalitogenic Th17 cells and the effector phase of experimental autoimmune encephalomyelitis. *J Immunol* 179(5): 3268-3275

202. Batten, M. J., Li, S. Yi, N. M. Kljavin, D. M. Danilenko, S. Lucas, J. Lee,F. J. de Sauvage, and N. Ghilardi. 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. *Nature Immunol* 9: 929-936

203. Billau, A. H., H. Heremas, F. Vandekerckhove, R. Kijkmans, I. Sobis, E. Meulepas, and H. Carton. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol* 140: 1506-1510

204. Chu, C, Q., S. Wittmer, and D. K. Dalton. 2000. Failure to support the expansion of the activated CD4 T cells population in interferon gamma deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J Exp Med* 192: 123-128

205. Cua, D. J., J. Sherlock, Y. Chen, C. A. Murphy, B. Joyce, B. Seymour, L. Lucian, W. To, S. Kwan, T. Churakova, S. Zurawski, M. Wlekowski, S. A. Lira, D. Gorman,

R. A. Kastelein, and J. D. Sedgwick. 2003. Interleukin -23 rather than interleukin 12 is the crucial cytokine for autoimmune inflammation of the brain. *Nature* 421: 744-748

206. King, I. L., and B. M. Segal. 2005. Cutting edge: IL-12 induces CD4+CD25- T cells activation in the presence of T regulatory cells. *J Immunol* 175: 641-645

207. Vaknin-Dembinsky, A., K. Balashov, and H. L. Weiner. 2006. IL-23 increased in dendritic cells in multiple sclerosis and down-regulation of IL-23 by antisense oligos increases dendritic cell IL-10 production. *J Immunol* 176: 7768-7774

208. Sutton, C., C. Braraton, B. Keogh, K. H. G. Mills, and E. C. Lavelle. 2006. A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med* 203: 1685-1691

209. Eugster, H. P., K. Frei, M. Kopf, H. Lassmann, and A. Fontana. 1998. IL-6-deficient mice resist myelin oligodendrocyte glycoprotein-induced autoimmune encephalomyelitis. *Eur J Immunol* 28(7): 2178-2187

210. Fantini, M. C., A. Rizzo, D. Fina, R. Caruso, C. Becker, M. F. Neurath, T. T. MacDonald, F. Pallone, and G. Monteleone. 2007. IL-21 regulates experimental colitis by modulating the balance between Treg and Th17 cells. *Eur J Immunol* 37: 3155-3164

211. Vollmer, T. L., R. Liu, M, Price, S. Rhodes, A. La Cava, and F. D. Shi. 2005. Differential effects of IL-21 during initiation and progression of autoimmunity against neuroantigen. *J Immunol* 174(5): 2696-701

212. Piao W. H., Y. H. Jee, R. L. Liu, S. W. Coons, M. Kala, M. Collins, D. A. Young, D. I. Campagnolo, T. L. Vollmer, X. F. Bai, A. LaCava and F. D. Shi. 2008. IL-21 modulates CD4+ CD25+ regulatory T-cell homeostasis in experimental autoimmune encephalomyelitis. *Scan J Immunol* 67(1):37-46

213. Bettelli, E., M. Oukka, and V. K. Kuchroo. 2007. Th-17 cells in the circle of immunity and autoimmunity. *Nature Immunol* 8: 345-350

214. Kreymborg, K., R. Etzensperger, L. Dumoutier, S. Haak, A. Rebollo, T. Buch, F. L. Heppner, J. C. Renauld, and B. Becher. 2007. IL-22 is expressed by Th17 cells in an IL-23-dependent fashion, but not required for the development of autoimmune encephalomyelitis. *J Immunol* 179(12): 8098-104

215. Zenowicz, L. A., G. D. Yancopoulos, D. M. Valenzuela, A. J. Murphy, M. Karow, and R. A. Flavell. 2007. Interleukin-22 but not interleukin-17 provides protection to hepetocytes during acute liver inflammation. *Immunity* 27: 647-659

216. Fort, M. M., J. Cheung, D. Yen, J. Li, S. M. Zurawski, S. Lo, S. Menom, T. Cliffird, B. Hunte, R. Lesley et al. 2001. IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies *in vivo*. *Immunity* 15: 985-995

217. Offner, H., S. Subramanian, C. Wang, M. Afentoulis, A. A. Vandenbark, J. Huan, and G. G. Burrows. 2005. Treatment of passive experimental autoimmune excephalomyelitis in SJL mice with the recombinant TCR ligand induces IL-13 and prevents axonal injury. *J Immunol* 175: 4103-4111

218. Cash, E., A. Minty, P. Ferrera, D. Caput, D. Fradelizi, and O. Rott. 1994. Macrophage-inactivating IL-13 suppresses experimental autoimmune encephalomyelitis in rats. *J Immunol* 153(9): 4258-4267

219. Sinha, S., L. J. Keler, T. M. Proctor, C. Teuscher, A. A. Vandenbark, and H. Offner. 2008. IL-13-mediated gender difference in susceptibility to autoimmune encephalomyelitis. *J Immunol* 180(4): 2679-2685

220. Selvaraj, R. K., and T. L. Geiger. 2007. A kinetic and dynamic analysis of FoxP3 induced in T cells by TGF-β. *J Immunol* 178: 7667-7677

221. Gregory, G. D., S. S. Raju, S. Winandy, and M. A. Brown. 2006. Mast cell IL-4 expression is regulated by Ikaros and influences encephalitogenic Th1 response in EAE. *J Clin Invest* 116(5): 1327-1336

222. Stumhofer, J. S., A. Laurence, E. H. Wilson, E. Huang, C. M. Tato, L. M. Johnson et al. 2006. Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. *Nature Immunol* 7: 937-945

223. Sommereyns, C., S. Paul, P. Staeheli, and T. Michiels. 2008. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells *in vivo*. *PLOS Pathog* 4(3): e1000017

224. Mennechet, F. J., and G. Uzé. 2006. Interferon lambda treated dendritic cells specifically induce proliferation of FoxP3-expressing suppressor T cells. *Blood* 107(11): 4417-23

225. Markovic-Plese, S., B. Hammer, Y. Zhao, R. Simon, C. Pinilla, and R. Martin. 2005. High level of cross-reactivity in influenza virus hemaglutinin–specific $CD4^+$ T cell response: Implications for the initiation of autoimmune response in multiple sclerosis. *J Neuroimmunol* 169: 31-38

226. Niedbala, W., X. Wei, B. Cai, A. J. Hueber, B. P. Leung, I. B. MxInnes, and F. Y. Liew. 2007. IL-35 is a novel cytokine with therapeutic effects against collagen-

induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol* 37: 3021-3029

227. Collison, L. W., C. J. Workman, T. T. Kuo, K. Boyd, Y. Wang, K. M. Vignali, R. Cross, D. Sehy, R. S. Blumberg, and D. A. Vignali. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450(7169): 566-569

228. Huseby, E. S., D. Liggitt, T. Brabb, B. Schnabel, C. Ohlen, and J. Goverman. 2001. A pathogenic role for myelin-specific CD8+ T cells in a model of multiple sclerosis. *J Exp Med* 194: 669-676

229. Ford, M. L., and B. D. Evavold. 2005. Specificity, magnitude, and kinetics of MOG-specific CD8+ T cell responses during experimental autoimmune encephalomyelitis. *Eur J Immunol* 35: 76-85

230. Ji, Q., and J. Goverman. 2007. Virial, antigen, or cell-induced models experimental autoimmune encephalomyelitis mediated by CD8+ T cells. *Ann N Y Acad Sci* 1103: 157-166

231. Abdul-Majid, K. B., J. Wefer, C. Stadelmann, A. Stefferl, H. Lassmann, T. Olson et al. 2003. Comparing the pathogenesis of experimental autoimmune encephalomyelitis in CD4-/- and CD8-/- DBA/I mice defines qualitative roles of different T cell subsets. *J Neuroimmunol* 141: 10-19

232. Neumann H., H. Schmidt, A. Cavalie, D. Jenne, and H. Werkle. 2002. Major histocompatibility compelx (MHC) class I gene expression in single neurons of the central nervous system: differential regulation by interferon gamma and tumor necrosis factor alpha. *J Exp Med* 185: 305-316

233. Koh, D. R., W. P. Fung-Leung, A. Ho, D. Gray, H. Acha-Orbea, and T. W. Mark. 1992. Less nortality but more relapses in experimental allergic encephalomyelitis in CD8-/- mice. *Science* 256: 1210-1213

234. Sonobe, Y., S. Jin, J. Wang, J. Kawanokuchi, H. Takeuchi, T. Mizuno, and A. Suzumura. 2007. Chronological changes of CD4+ and CD8+ T cells subsets in the experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis. *Tohoku J Exp Med* 213: 329-339

235. Hjelmstrom, P., A. E. Juedes, J. Fjell, and N. H. Ruddle. 1998. Cutting edge: B cell-deficient mice develop experimental allergic encephalomyelitis with demyelination after myelin oligodendrocyte glycoprotein sensitization. *J Immunol* 161: 4480-4483

236. Fillatreau, S., C. H. Sweenie, M. J. McGeachy, D. Gray, and S. M. Anderton, 2002. B cells regulate autoimmunity by provision of IL-10. *Nature Immunol* 3: 944-950

237. Chang, T. T., C. Jabs, R. A. Sobel, V. K. Kuchroo, and A. H. Sharpe. 1999. Studies in B7-deficient mice reveal a critical role for B7 co-stimulation in both induction and effector phases of experimental autoimmune encephalomyelitis. *Science* 273: 733-740

238. Mann, M. K. Maresz, L. P. Shriver, T. Yanping, B. N. Dittel. 2007. B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis. *J Immunol* 178: 3447-3456

239. Dittel, B. N. T. H. Urbania, and C. A. Janeway Jr. 2000. Relapsing and remitting experimental autoimmune encephalomyelitis in B cell deficient mice. *J Autoimmun* 14: 311-318

240. Lyons, J.-A., M. J. Ramsbottom, and A. H. Cross. 2002. Critical role of antigen-specific antibody in experimental autoimmune encephalomyelitis induced by recombinant myelin oligodendrocyte glycoprotein. *Eur J Immunol* 32: 1905-1913

241. Harris, D. P., L. Haynes, P. C. Sayles, D. K. Duso, S. M. Eaton, N. M. Lepak, L. L. Johnson, S. L. Swain, and F. E. Lund. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nature Immunol* 1: 475-482

242. Gonnella, P. A., H. P. Waldner, and H. L. Weiner. 2001. B cell-deficient (miuMT) mice have alterations in the cytokine microenvironment of the gut-associated lymphoid tissue (GALT) and defects in the low dose mechanism of oral tolerance. *J Immunol* 166: 4456-4464

243. Seamons, A., A. Perchellet, and J. Goverman. 2006. Endogenous myelin basic protein is presented in the periphery by both dendritic cells and resting B cells with different functional consequences. *J Immunol* 177: 2097-2106

244. Lampropoulou, V., K. Hoehlig, T. Roch, P. Neves, S. C. Gómez, S. H. Sweenie, Y. Hao, A. A. Freitas, U. Steinhoff, S. M. Anderton, and S. Fillatreau. 2008. TLR-activated B cells suppress T cell-mediated autoimmunity *J Immunol* 180: 4763-4773

245. O'Brien, K. D., C. Futzgerald, K. Naiken, K. R. Alugupalli, A. M. Ristami, and B. Gran. 2008. Role of the innate immune system in autoimmune inflammatory demyelination. *Curr Med Chem* 15(11): 1105-1115

246. Schreibelt, G., R. J. P. Musters, A. Reijerkerk, L. R. deGroot, S. M. A. van der Pol, E. M. L. Hendrikx, E. D. Döpp, C. D. Dijkstra, B. Drukarch, and H. E. de Vries. 2006. Lipoic acid affects cellular migration into central nervous system and stabilizes blood-brain barrier integrity. *J Immunol* 177: 2630-2637

247. Zehntner, S. P., C. Brickman, L. Bourbonnière, L. Remington, M. Caruso, and T. Owens. 2005. Neutrophils that infiltrate the central nervous system regulate T cell responses. *J Immunol* 174: 5124-5131

248. Iwasaki, A., and B. L. Kelsall. 2001. Unique function of CD11b+, CD8 alpha+, and double negative Peyer's patch dendritic cells. *J Immunol* 166: 4884-4890

249. Chen, M., Y.-H. Wang, Y. Wang, L. Huang H. Sandoval, Y.–J. Liu, J. Wang. 2006. Dendritic cell apoptosis in the maintenance of immune tolerance. *Science* 311: 1160-1164

250. Bailey-Bucktrout, S. L., S. C. Caulkins, G. Goings, J. A. A. Fischer, A. Dzionek, and S. D. Miller. 2008. Cutting edge: Central nervous system plasmocytoid dendritic cells regulate the severity of relapsing experimental autoimmune encephalomyelitis. *J Immunol* 180: 64457-6461

251. Karni, A., M. Abraham, A. Monsonego, G. Cai, G. J. Freeman, D. Hafler, S. J. Khoury, and H. L. Weiner. 2006. Innate immunity in multiple sclerosis: myeloid dendritic cells in secondary progressive multiple sclerosis are activated and drive a proinflammatory immune response. *J Immunol* 177: 4196-4202

252. Zhang, B., T. Yamamura, T. Kondo, M. Fujiwara, and T. Tabira. 1997. Regulation of experimental autoimmune encephalomyelitis by natural killer (NK) cells. *J Exp Med* 186(10): 1677-1687

253. Xu. W., G. Fazekas, H. Hara, and T. Tabira. 2005. Mechanism of natural killer (NK) cell regulatory role in experimental autoimmune encephalomyelitis. *J Neuroimmunol* 163(1-2): 24-30

254. Galazka, G, M. Stasiolek, A. Walczak, A, Jurewicz, A. Zylicz, C. F. Brosnan, C. S. Raine, and K. W. Selmaj. 2006. Brain-derived heat shock protein-70-peptide complex induce NK cell-dependent tolerance to experimental autoimmune encephalomyelitis. *J Immunol* 176(3): 1588-1599

255. Winkler-Pickett, R., H. A. Young, J. M. Chery, J. Diehl, J. Wine, T. Back, W. E. Bere, A. T. Mason, and J. R. Ortaldo. 2008. *In vivo* regulation of experimental autoimmune encephalomyelitis by NK cells: alteration of primary adaptive response. *J Immunol* 180: 4495-4506

256. Ponomarev, E. D., and B. N. Dittel. 2005. Gamma delta T cells regulate the extent and duration of inflammation in the central nervous system by Fas ligand-dependent mechanism. *J Immunol* 174(8): 4678-4687

257. Odyniec, A., M. Szczepnik, M. P. Mycko, M. Stasiolek, C. S. Raine, and K. W. Selmaj. 2004. Gamma delta T cells enhance the expression of experimental autoimmune encephalomyelitis by promoting antigen presentation and IL-12 production. *J Immunol* 173: 682-694

258. Rajan, A. J., J. D. Klein, and C. F. Brosnan. 1998. The effect of gamma delta T cell depletion on cytokine gene expression in experimental autoimmune encephalomyelitis. *J Immunol* 160(12): 5955-5962

259. Rajan, A. J., V. C. Asensio, I. L. Campbell, and C. F. Brosnan. 2000. Experimental autoimmune encephalomyelitis on the SJL mouse: effect of gamma delta T cell depletion on chemokine and chemokine receptor expression in the central nervous system. *J Immunol* 164(4): 2120-2130

260. Hovelmeyer, N., Z. Hao, K. Kranidioti, G, Kassiotis, T. Buch, F. Frommer, L. von Hoch, D. Kramer, L. Minichiello, G. Kollias, H. Lessmann, and A. Waisman. 2005. Apoptosis of oligidendrocytes via Fas and TNF-R1 is a key event in the induction of experimental autoimmune encephalomyelitis. *J Immunol* 175: 5875-5884

261. Sun, J., B. Hillard, L. Xu, Y. H. Chen. 2005. Essential roles of the Fas-associated death domain in autoimmune encephalomyelitis. *J Immunol* 175(7): 4783-4788

262. Crudici, C., T. Niculescu, F. Niculescu, M. L. Shin, and H. Rus. 2006. Oligodendrocyte cell death in pathogenesis of multiple sclerosis: Protection of oligodendrocytes from apoptosis by complement. *J Rehabil Res Dev* 43(1): 123-32

263. Schuster, N., and K. Krieglstein. 2002. Mechanism of TGF-beta-mediated apoptosis. *Cell Tissue Res* 307(1): 1-14

264. Georgescu, R. K., Vakkalanka, K. B. Elkon, and M. K. Crow 1997. Interleukin 10 promoted activation-induced cell death of SLE lymphocytes mediated by Fas ligand. *J Clin Invest* 100(10): 2622-2633

265. Lee Y. W., H. Kühn, B. Hennig, and M. Toborek. 2000. IL-4 induces apoptosis of endothelial cells through the caspase-3-dependent pathway. *FEBS Letters* 485(102): 122-126

266. Garren H., P. J. Ruiz, T. A. Watkins, P. Fontoura, L. T. Nguyen, E. R. Estline, D. L. Hirschberg, and L. Steinman. 2001. Combination of gene delivery and DNA

vaccination to protect from and reverse Th1 autoimmune disease via deviation to the Th2 pathway. *Immunity* 15(1): 15-22

267. Young, D. A., L. D. Lowe, S. S. Booth, M. J. Whitters, L. Nicholson, V. K. Kuchroo, and M. Collins. 2000. IL-4, IL-10, IL-13, and TGF-beta from an altered peptide ligand-specific Th2 cell clone down-regulate adoptive transfer of experimental autoimmune encephalomyelitis. *J Immunol* 164: 3563-3572

268. Jee, Y., R. Liu, X.-F. Bai, D. I. Campagnolo, F.-D. Shi, and T. L. Vollmer. 2006. Do Th2 cells mediate the effect of glatiramer acetate in experimental autoimmune encephalomyelitis? *Intern Immunol* 18(4): 537-544

269. Ramirez, F., and D. Mason. 2000. Induction of resistance to active experimental allergic encephalomyelitis by myelin basic protein-specific Th2 cell lines generated in the presence of glucocorticoids and IL-4. *Eur J Immunol* 30(3): 747-758

270. Falcone, M., and B. R. Bloom. 1997. A T helper cell 2 (Th2) immune response against non-self antigens modifies the cytokine profile of autoimmune T cells and protects against experimental allergic encephalomyelitis. *J Exp Med* 185(5): 901-907

271. Zheng, X., X. Hu, G. Zhou, Z. Lu, W. Qiu, J. Bao, and Y. Dai. 2008. Soluble egg antigen if *Schistosoma japonicum* modulates the progression of chronic progressive experimental autoimmune encephalomyelitis via Th2-shift response. *J Neuroimmunol* 194(1-2): 107-114

272. Bielakova, B., B. Goodwin, N. Richert, J. Cortese, and T. Kondo. 2000. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nature Med* 6: 1167-1175

273. Muller, U., B. Gimsa, N. A. Mitchinson, A. Radbruch, J. Sieper, and Z. Yin. 1998. Modulation the Th1/ Th2 balance in inflammatory arthritis. *Springer Semin Immunopathol* 20(1-2): 181-196

274. Kleemann, R., F. W. Scott, U. Worz-Pagenstert, W. M. Nimal Ratnayake, and H. Kolb. 1998. Impact of dietary fat on Th1/Th2 cytokine gene expression in the pancreas ad gut of diabetes-prone BB rats. *J Autoimmun* 11(1): 97-103

275. Min, K., W. K. Yoon, S. K. Kim, and B. H. Kim. 2007. Immunosuppressive effect of silibinin in experimental autoimmune encephalomyelitis. 2007. *Arch Pharm Res* 10: 1265-1272

276. Lee, J. S., S. G. Kim, T. H. Lee, Y. I. Jeong, C. M. Lee, M. S. Yoon, Y. J. Na, D. S. Suh, N. C. Park, I. H. Choi, G. Y. Kim, Y. H. Choi, H. Y. Chung, and Y. M. Park.

2007. Sibilinin polarizes Th1/Th2 immune response through the inhibition of immuostimualtory function of dendritic cells. *J Cell Physion* 210(2): 385-397

277. Brod, S. A., and Z. Hood. 2007. Ingested (oral) SIRS peptide 1-21 inhibits acute EAE by inducing Th2-like cytokines. *J Neuroimmunol* 183(102): 89-95

278. Kuchroo, V. K., J. M. Greer, D. Kaul, G. Ishioka, A. Franco, A. Sette, R. A. Sobel, and M. B. Lees. 1994. A single TCR antagonist peptide inhibits experimental allergic encephalomyelitis mediated by a diverse T cell repertoire. *J Immunol* 153: 3326-3336

279. Jegou J. F., P. Chan, M. T. Schouft, P. Gaseque, H. Vaudry, and M. Fontaine. 2007. Protective DNA vaccination against myelin oligodendrocyte glycoprotein is overcome by C3d in experimental autoimmune encephalomyelitis. *Mol Immunol* 44(15): 3691-3701

280. Segal, B. M., J. T. Chang, and E. M. Shevach. 2000. CpG oligonucleotides are potent adjuvants for the activation of autoreactive encephalitogenic T cells *in vivo. J Immunol* 164: 5683

281. Boccaccio, G. L., F. Mor, and L. Steinman. 1999. Non-coding plasmid DNA induces IFN-gamma *in vivo* and suppresses autoimmune encephalomyelitis. *Int Immunol* 11: 289

282. Stuve, O., P. D. Cravens, and T. N. Eagar. 2008. DNA-based vaccines: the future of multiple sclerosis therapy? *Expert Rev Neurother* 8(3): 351-360

283. Prat, E, and R. Martin. 2002. The immunopathogenesis of multiple sclerosis. *J Rehab Res Develop* 39(2): 187-200

284. Tennakoon D. K., R. S. Mehta, S. B. Ortega, V. Bhoj, M. K. Racke, and N. J. Karandikar. 2006. Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis. *J Immunol* 176: 7119-7129

285. Inglese, M. 2006. Multiple Sclerosis: New Insights and trends. *Am J neuroradiol* 27: 954-957

286. Smith, M. E., N. L. Eller, H. F. McFarland, M. K. Racke, and C. S. Raine. 1999. Age dependence of clinical and pathological manifestations of autoimmune demyelination: implications for multiple sclerosis. *Am J Pathol* 155: 1147-1161

287. Amerongen, H. M., G. A. Wilson, B. N. Fields, and M. R. Neutra. 1994. Proteolytic processing of a reovirus is required for adherence to intestinal M cells. *J Virol* 68(12): 8424-8432

288. Lublin F. D., S. C. Reingold. 1996. Defining the clinical course of multiple sclerosis: results of an international survey. National multiple sclerosis society (USA) advisory committee on clinical trials of new agents in multiple sclerosis. *Neurol* 46: 907-911

289. Trapp, D., J. Peterson, R. M. Ransohoff, R. Rudick, S. Mork, and L. Bo. 1998. Axonal transaction in MS lesions. *N Engl J Med* 338: 278-285

290. Prineas, J. W., and R. G. Wright. 1978. Macrophages, lymphocytes and plasma cells in the perivascular compartments in chronic multiple sclerosis. *Lab Invest* 38: 409-421

291. Lucchinetti, C. F., W. Bruck, M. Rodriguez, and H. Lassmann. 1996. Distinct patterns of multiple sclerosis pathology indicate heterogeneity on pathogenesis. *Brian Pathol* 6: 259-274

292. Reingold, S. C. 1990. Fatigue in multiple sclerosis. *MS News Br Mult Scler Soc* 142: 30-31

293. Confavreux, C., S. Vukusic, T. Moreau, and P. Adeleine. 2000. Relapsed and progression of disability in multiple sclerosis. *N Engl J Med* 343: 1430-1438

294. Hohol, M. J., M. J. Olek, E. J. Orav, L. Stazzone, D. A. Hafler, S. J. Khoury, D. M. Dawson, and H. L. Weiner. 1999. Treatment of progressive multiple sclerosis with pulse cyclophosphamide/methylprednisolone: response to therapy is linked to the duration of progressive disease. *Mult Scler* 5(6): 403-409

295. Montalban, X. 2005. Primary progressive multiple sclerosis. *Curr Opin Neurol* 18: 261-266

296. Markovic-Plese, S., C. Pinilla, and R. Martin. 2004. The initiation of the autoimmune response in multiple sclerosis. *Clin Neurol Neurosurg* 106(3): 218-222

297. Ebers, G. C., K. Kukay, D. E. Bulman, and A. D. Sadovnick. 1996. A full genome search in multiple sclerosis. *Nature Genet* 13: 472-476

298. Sawcer, S., H. B. Jones, R. Feakes, J. Gray, and N. Smaldon. 1996. A genome screen in multiple sclerosis reveals susceptibility loci on chromosome 6p21 and 17q22. *Nature Genet* 13: 464-468

299. Haines, J. L., M. Ter-Minassian, A. Bazyk, J. F. Gusella, and D. J. Kim. 1996. A complete genomic screen for multiple sclerosis underscores a role for the major histocompatibility complex. The Multiple Sclerosis Genetics Group. *Nature Genet* 13: 469-471

300. Svejgaard, A. 2008. The immunogenetics of multiple sclerosis. *Immunogenetics* 60: 275-286

301. Oksenberg, J. R., and S. L. Hauser. 2005. Genetics of multiple sclerosis. *Neurol Clin* 23: 61-75

302. Williams, A., R. Eldridge, H. McFarland, S. Houff, H. Krebs, and D. McFarlin. 1980. Multiple sclerosis in twins. *Neurol* 30: 1139-1147

303. Ebers, G. C., D. E. Bulman, A. D. Sadovnick, D. W. Paty, S. Warren, W. Hader, T. J. Murray, T. P. Seland, P. Duquette, and T. Grey. 1986. A population-based study of multiple sclerosis in twins. *N Engl J Med* 315: 1638-1642

304. Ramagopanlan, S. V. 2008. Genes for multiple sclerosis. *Lancet* 371(9609): 283-285

305. Fogdell-Hahn, A., A. Ligers, M. Gronning, J. Hillert, and O. Olerup. 2000. Multiple sclerosis: a modifying influences of HLA class I genes in an HLA II associated autoimmune disease. *Tissue Antigens* 55L 140-148

306. Harbo, H. F., B. A. Lie, S. Sawcer, E. G. Celius, K. Z. Dai, A. Oturai et al. 2004. Genes in the HLA class I region may contribute to the HLA class II-associated genetic susceptibility to multiple sclerosis. *Tissue Antigens* 63: 237-247

307. Waksman, B. H., and W. E Reynolds. 1984. Minireview: Multiple sclerosis as a disease of immune regulation. *Proc Soc Exp Biol Med 175*: 282-294

308. Ebers. G. C. 2008. Environmental factors and multiple sclerosis. *Lancet Neurol* 7: 268-277

309. Hayes, C. E., M. T. Cantorna, and H. F. DeLuca. 1997. Vitamin D and multiple sclerosis. *Proc Soc Exp Biol Med* 216(1): 21-27

310. Raghuwanshi, A., S. S. Joshi, and S. Christakos. 2008. Vitamin D and multiple sclerosis. *J Cell Biochem* Jul 24, Epublication ahead of print

311. Fleming, J., Z. Fabry. 2007. The hygiene hypothesis and multiple sclerosis. *Ann Neurol* 61(2): 85-91

312. Martin, R., B. Gran, Y. Zhao, S. Markovic-Plese, B. Bielakova, A. Marques, M. Sung, B. Hammer, R. Simon, H. F. McFarland, and C. Pinilla. 2000. Molecular mimicry and antigen-specific T cell responses in multiple sclerosis and chronic CNS Lyme disease. *JAI* 16: 187-192

313. Sibley, W. A., C. K. Bramford, and K. Clark. 1985. Clinical viral infections and multiple sclerosis. *Lancet* 1: 1313-1315

314. Warren, K. G., I. Catz, and L. Steinman. 1995. Fine specificity of the antibody response to myelin basic protein in the central nervous system in multiple sclerosis: the minimal B cell epitope and a model of its unique features. *PNAS* 92: 11061-11065

315. Wucherpfennig, K. W. and J. L Strominger. 1995. Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. *Cell* 80: 695-705

316. Goverman, J., A. Woods, L. Larson, L. Weiner, L. Hood, and D. Zaller. 1993. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell* 72: 551-560

317. Schmidt, D., J. Goronzy, and C. Weyand. 1996. CD4+CD7-CD28- T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest* 97: 2027-2037

318. Hafler D. A., J. M. Slavik, D. E. Anderson, K. C. O'Connor, P. De Jager, and C. Baecher-Allan. 2005. Multiple Sclerosis. *Immunol Rev* 204: 208-231

319. Link, H. 1998. The cytokine storm in multiple sclerosis. *Mult Scler* 4: 12-15

320. Cannella, B., and C. S. Raine. 1995. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol* 37: 424-435

321. Deloire, M. S., T. Touil, B. Brochet, V. Dousset, J. M. Caillé, and K. G. Petry. 2004. Macrophage brain infiltration in experimental autoimmune encephalomyelitis is not completely compromised by suppressed T-cell invasion: *in vivo* magnetic resonance imaging illustration in effective anti-VLA-4 antibody treatment. *Mult Scer* 10(5): 540-648

322. Burger, D. R., D. Ford, R. M. Veto, A. Hamblin, A. Goldstein, M, Hubbard et al. 1981. Endothelial cell presentation of antigen to human T cells. *Hum Immunol* 3: 209-230

323. McCarron, R. M., M, Spatz, O. Kempski, R. N. Hogan, L. Muehl, and D. E. McFarlin. 1986. Interaction between myelin basic protein-sensitized T lymphocytes and murine cerebral vascular endothelial cells. *J Immunol* 137: 3428-3435

324. Gijbels, K., R. Geraldy, and L. Steinman. 1994. Reversal of EAE with hydroxymate inhibitor of matrix metalloproteases. *J Clin Invest* 94: 2177-2182

325. Brosnan, C. F., and C. S. Raine. 1996. Mechanisms of immune injury in multiple sclerosis. *Brain Pathol* 6: 243-257

326. Markovic-Plese, S., and H. McFarland. 2001. Immunopathogenesis of the multiple sclerosis lesions. *Curr Neurol Neurosci Rep* 1: 257-262

327. Cornet, A., E. Bettelli, M. Oukka, C. Cambouris, V. Avellana-Adalid, K. Kosmatopoulus et al. 2000. Role of astrocytes in antigen presentation and naïve T cell activation. *J Neuroimmunol* 106: 69-7

328. Bornstein, M. B., and S. H. Appel. 1961. The application of tissue culture to the study of experimental "allergic" encephalomyelitis. I. Pattern of demyelination. *J Neuropathol Exp Neurol* 1(20): 141-147

329. Genian, C. P., B. Cannella, S. L. Hauser, and C. S. Raine. 1999. Identification of the autoantibodies associated with myelin damage in multiple sclerosis. *Nature Med* 5: 170-175

330. Lalive, P. H., T. Menge, C. Delarasse, B. D. Garpera, D. Pham-Dinh, P. Villoslada, H.-C. von Büdingen, and C. P. Genian. 2006. Antibodies to native myelin oligodendrocyte glycoprotein are serologic markers of early inflammation in multiple sclerosis. *PNAS* 103(7): 2280-2285

331. Archelos, J. J., M. K. Stroch, and H. P. Hartung. 2000. The role of B cells ands autoantibodies in multiple sclerosis. *Ann Neurol* 47: 694-706

332. Warrington, A. E., K. Asakura, A. J. Bieber, B. Ciric, V. Van Keulen, S. V. Kaveri et al. 2000. Human monoclonal antibodies reactive to oligodendrocytes promote remyelination in a model of multiple sclerosis. *PNAS* 97: 6820-6825

333. Zhang, J., H. L. Weiner, and D. A Hafler. 1992. Autoreactive T cells in multiple sclerosis. *Int Rev Immunol* 9(3): 183-201

334. Raine, C. S. 1994. Multiple sclerosis: immune system molecule expression in the central nervous system. *J Neuropathol Exp Neurol* 53(4): 328-37

335. Chen, Z., M. S. Freedman. 2008. CD16 (+) gammadelta T cells mediate antibody dependent cellular cytotoxicity: Potential mechanism in the pathogenesis of multiple sclerosis. *Clin Immunol* 128(2): 219-227

336. Swanborg, R. H. 1995. Experimental autoimmune encephalomyelitis in rodent as a model for human demyelinating disease. *Clin Immunol Immunopathol* 77: 4-13

337. Iwakura, Y., and H. Ishigame. 2006. The IL-23/IL-17 axis in inflammation. *J Clin Invest* 116(5): 1218-1222

338. Steinman, L., 2001. Immunotherapy of multiple sclerosis: the end of the beginning. *Curr Opin Immunol* 13: 597-600

339. Hedegaard, C. J., M. Krakauer, K. Bendtzen, H. Lund, F. Sellebjerg, and C. H. Nielsen. 2008. T helper cell type 1 (Th1), Th2 and Th17 responses to myelin basic protein and disease activity in multiple sclerosis. *Immunol* April 4 (Epub ahead of print)

340. Van Boxel-Dezaire, A. H., S. C. Hoff, B. W. van Osten, C. L. Verweij, A. M. Drager et al. 1999. Decreased interleukin-10 and increased interleukin-12p40mRNA are associated with disease activity and characterize different disease stages in multiple sclerosis. *Ann Neurol* 45: 695-703

341. Trapp, B. D., R. Ransohoff, and R. Rudick. 1999. Axonal pathology in multiple sclerosis: relationship to neurologic disability. *Curr Opin Neurol* 12: 295-302

342. Kapoor, R., M. Davis, P. A. Blaker, S. M. Hall, and K. J. Smith. 2003. Blockers of sodium and calcium entry protect axons from nitric oxide-mediated degeneration. *Ann Neurol* 53: 174-180

343. Werner, P., D. Pitt, and C. S. Raine. 2001. Multiple sclerosis altered glutamate homeostasis in lesions correlates with oligodendrocyte and axonal damage. *Ann Neurol* 50: 169-180

344. Bielakova, B., N. Richert, T. Howard, G. Blevis, S. Markovic-Plese, J. McCartin, J. Würfel, J. Ohayon, T. A. Waldmann, H. F. McFarland, and R. Martin. 2004. Humanized anti-CD25 (daclizuman) inhibits disease activity in multiple sclerosis patients failing to respond to interferon *PNAS* 101(23): 8705-8708

345. Rose, J. W., J. B. Burns, J. Bjorklund, J. Klein, H. E. Watt, and N. G. Carlson. 2007. Daclizumab phase II trial in relapsing and remitting multiple sclerosis: MRI and clinical results. *Neurol* 69(8): 785-789

346. Vollmer, T., L. Key, V. Durkalski., W. Tayor, J. Corboy, S. Markovic-Plese, J. Preiningerova, M. Rizzo, and I. Singh. 2004. Oral simvastatin treatment in relapsing-remitting multiple sclerosis. *Lancet* 363: 1607-1608

347. Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nature Rev Immunol* 3: 331-341

348. Kraus, T. A., J. Brimnes, C. Muong, J. H. Liu, T. M. Moran, K. A. Tappenden, P. Boros, and L. Mayer. 2005. Induction of mucosal tolerance in Peyer's patch-deficient, ligated small bowel loops. *J Clin Invest* 115: 2234-2243

349. Reiter, R., and K. Pfeffer. 2002. Impaired germinal center formation and humoral immune response in the absence of CD28 and interleukin-4. *Immunol* 106: 222-228

350. Rennert, P. D., J. L. Browining, and P. S. Hochman. 1997. Selective disruption of lymphotoxin ligands reveals a novel set of mucosal lymph nodes and unique effects on lymph node cellular organization. *Int Immunol* 9: 1627-1639

351. Kiyono, H., and S. Fukuyama. 2004. NALT- versus Peyer's-patch-mediated mucosal immunity. *Nature Rev Immunol* 4: 699-710

352. Wolf, J. L., R. S. Kauffman, R. Finberg, R. Dambrauskas, B. N. Fields, and J. S. Trier. 1983. Determinants of reovirus interaction with the intestinal M cells and absorptive cells of murine intestine. *Gastroenterol* 85: 291-300

353. Mah, D. C. W., G. Leone, J. M. Jankowski, and P. W. K. Lee. 1990. The N-terminal quarter of reovirus cell attachment protein σ1 possesses intrinsic virion-anchoring function. *Virol* 179: 95-103

354. Turner, D. L., R. Duncan, and P. W. Lee. 1992. Site-directed mutagenesis of the C-terminal portion of reovirus protein σ1: evidence for a conformation-dependent receptor binding domain. *Virol* 186: 219-227

355. Wang, X., D. M. Hone, A. Haddad, M. T. Shata, and D. W. Pascual. 2003. M cell DNA vaccination for CTL immunity to HIV. *J Immunol* 171: 4717-4725

356. Han, S., X. Zhang, E. Marinova, Z. Ozen, R. Bheekha-Escura, L. Guo, D. Wansley, G. Booth, Y. X. Fu, and B. Zheng. 2005. Blockade of lymphotoxin pathway exacerbates autoimmune arthritis by enhancing the Th1 response. *Arthritis Rheum* 52(10): 3202-3209

357. Csencsits, K. L., N. Walters, and D. W. Pascual. 2001. Cutting Edge: Dichotomy of homing receptor dependence by mucosal effector B cells: αE versus L-selectin. *J Immunol* 167: 2441-2445

358. Alignani, D., B. Maletti, M. Liskovsky, A. Rópolo, G. Morón, and M. C. Pistoresi-Palencia. 2005. Orally administered OVA/CpG-ODN induces specific mucosal and systemic immune response in young and aged mice. *J Leukoc Biol* 77: 898-905

359. Gonnella, P. A., H. P. Waldner, D. Kodali, and H. L. Weiner. 2004. Induction of low-dose oral tolerance in IL-10 deficient mice with experimental autoimmune encephalomyelitis. *J Autoimmun* 3: 193-200

360. Ghoreishi, M., and J. P. Dutz. 2006. Tolerance induction by transcutaneous immunization through ultraviolet-irradiated skin is transferable through CD4+CD25+ T regulatory cells and is dependent on host - derived IL-10. *J Immunol* 176: 2635-2644

361. Seewaldt, S., J. Alferink, and I. Förster. 2002. Interleukin-10 is crucial for maintenance but not for developmental induction of peripheral T cell tolerance. *Eur J Immunol* 32: 3607-3616

362. Veldhoen, M., and B. Stockinger. 2006. TGFβ1, a "Jack of all trades": the link with pro-inflammatory IL-17-producing T cells. *Trends Immunol* 27: 358-361

363. Matsumoto, M., Y.-X., Fu, H. Molina, and D. D. Chaplin. 1997. Lymphotoxin-a-deficient and TNF receptor-I-deficient mice define developmental and functional characteristics of germinal centers. *Immunol Rev* 156(1): 137-144

364. Van der Heijden, P. J., A. T. J. Bianchi, M. Dol, J. W. Pals, W. Stok, and B. A. Bokhout. 1991. Manipulation of intestinal immune response against ovalbumin by cholera toxin and its B subunit in mice. *Immunol* 72: 89-93

365. Yokohama, Y., and Y. Harabuchi. 2002. Intranasal immunization with lipoteichoic acid and cholera toxin evokes specific pharyngeal IgA, and systemic IgG responses and inhibits streptococcal adherence to pharyngeal epithelial cells in mice. *Int J Pediatr Otohinolaryngol* 63: 235-241

366. Lian, T., T. Bui, and R. J. Ho. 1999. Formulation of HIV-envelope protein with lipid vesicles expressing ganglioside GM1 associated to cholera toxin B enhances mucosal immune responses. *Vaccine* 18: 604-611

367. Grundstrom, S., L. Cederbom, A. Sundstedt, P. Scheipers, and F. Ivars. 2003. Superantigen-induced regulatory T cells display different suppressive functions in the presence or absence of natural CD4+CD25+ regulatory T cells *in vivo*. *J Immunol* 170: 5008-5017

368. Lee, J. H., L. C. Wang, Y. T. Lin, Y. H. Yang, D. T. Lin, and B. L. Chiang. 2006. Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in pediatric patients with systemic lupus erythematosus. *Immunol* 117: 280-286

369. Allan, S. E., S. O. Crome, N. K. Crellin, L. Passerini, T. S. Steiner, R. Bacchetta, M. G. Roncarolo, and M. K. Levings. 2007. Activation-induced FoxP3 in human T

effector cells does not suppress proliferation or cytokine production. *Int Immunol* 19: 345-354

370. Cong, Y., C. Liu, C. T. Weaver, and C. O. Elson. 2004. Early upregulation of T cell IL-10 production plays an important role in oral tolerance induction. *Ann NY Acad Sci* 1029: 319-320

371. Maurer, M., W. Seidel-Guyenot, M. Metz, J. Knop, and K. Steinbrink. 2003. Critical role of IL-10 in the induction of low zone tolerance to contact allergens. *J Clin Invest* 112: 432-439

372. Silva, S. R., J. F. Jacysyn, M. S. Macedo, and E. L. Faquim-Mauro. 2006. Immunosuppressive components of *Ascaris suum* down-regulate expression of co-stimulatory molecules and function of antigen-presenting cells via an IL-10-mediated mechanism. *Eur J Immunol* 36: 3227-3237

373. Joetham A., K. Takeda, C. Taube, N. Miyahara, S. Matsubara, T. Koya, Y. H. Rha, A. Dakahama, and E. W. Gelfand. 2007. Naturally occurring lung CD4+CD25+ T cell regulation of airway allergic response depends on IL-10 induction of TGF-β. *J Immunol* 178: 1433-1442

374. Mercier, G. T., J. A. Campbell, J. D. Chappell, T. Stehle, T. S. Dermody, and M. A. Barry. 2004. A chimeric adenovirus vector encoding reovirus attachment protein σ1 targets cells expressing junctional adhesion molecule 1. *PNAS* 101: 6188-6193

375. Connolly, J. V., E. S. Barton, and T. S. Dermody. 2001. Reovirus binding to cell surface sialic acid potentiates virus-induced apoptosis. *J Virol* 75: 4029-4031

376. Tyler, K. L., M. K. Squier, S. E. Rodgers, B. E. Schneider, S. M. Oberhaus, T. A. Grdina, J. J. Cohen, and T. S. Dermody. 1995. Differences in the capacity of reovirus strains to induce apoptosis are determined by the viral attachment protein σ1. *J Virol* 69: 6972-6979

377. Johnson, A. J., G. L. Suidan, J. McDole, and I. Pirko. 2007. The CD8 T cells in multiple sclerosis: suppressor cells or mediator of neuropathology? *Int Rev Neurobiol* 79: 73-97

378. Weiss, H. A, J. M. Millward, and T. Owens. 2007. CD8+ T cells in inflammatory demyelinating disease. *J Neuroimmunol* 191: 79-85

379. Gold, R., C Linington, and H. Lassmann. 2006. Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis. *Brain* 129: 1953-1971

380. Miller, S. D., B. L. McRae, C. L. Vanderlugt, K. M. Nicevich, L. G. Popel, and W. J. Karpus. 1995. Evolution of T cell repertoire during the course of experimental autoimmune mediated demyelinating disease. *Immunol Rev* 144: 225-244

381. Sun, D., J. N. Whitaker, Z. Huang, D. Liu, C. Coleclought , H. Werkle, and C. S. Raine. 2001. Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57 BL/6 mice. *J Immunol* 166(12): 7579-7587

382. Nicholson, L. B., J. M. Greer, R. A. Sobel, M. B. Less, V. K. Kuchroo. 1995. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity* 3(4): 397-405

383. Asano, M, M. Toda, N., Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 184: 387-396

384. McGeachy, M. J., L. A. Stephens, and S. M. Anderton. 2005. Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *J Immunol* 175: 3025-3032

385. Reddy, J., Z. Illes, X. Zhang, J. Encinas, J. Pyrdol, L. Nicholson, R. A. Sobel, K. W. Wucherpfenning, and V. J. Kuchroo. 2004. Myelin proteolipid protein-specific CD4+CD25+ regulatory cells mediate genetic resistance to experimental autoimmune encephalomyelitis. *PNAS* 101: 15434-15439

386. Morgan, M. E., R. Flierman, L. M. van Duivenvoorde, H. J. Witteveen, W. van Ewijk, J. M. van Laar, R. R. De Vries, and R. E. Toes. 2005. Effective treatment of collagen-induced arthritis by adoptive transfer of CD25+ regulatory T cells. *Arthritis Reum* 52: 2212-2221

387. Gonnella, P.A., D. Kodali, and H. L. Weiner. 2003. Induction of low-dose oral tolerance in monocyte chemoattractant protein-1- and CCR2-deficient mice. *J Immunol* 170: 2316-2322

388. Uchimura, K., M. Itoh, K. Yamamoto, S. Imamura, M. Makino, T. Kato, K. Fujiwara, and Y. Sawai. 2002. The effects of CD40-and interleukin (IL-4)-activated CD23+ cells on the production of IL-10 by mononuclear cells in Grave's disease: the role of CD8+ T cell. *J Exp Med* 128(2): 308-312

389. Chung, Y., S.-H. Lee, D.-H. Kim, and C-Y. Kang. 2005. Complementary role of CD4+CD25+ regulatory T cells and TGF-beta in oral tolerance. *J Leukoc Biol* 77: 906-913

390. Jiang, H., S. Curran, E. Ruiz-Vazguez, B. Liang, R. Winchester, and L. Chess. 2003. Regulatory CD8+ T cells fine-tune the myelin basic protein-reactive T cell receptor Vβ repertoire during experimental autoimmune encephalomyelitis. *PNAS* 100(14): 8378-8383

391. Martin, F., and A. C. Chan. 2004. Pathogenic roles of B cells in human autoimmunity: insights from the clinic. *Immunity* 20: 517-527

392. Rutella, S., S. Danese, and G. Leone. 2006. Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood* 108(5): 1435-1440

393. Li, H., G. X. Zhang, and A. Rostani. 2008. CD11c+CD11b+ dendritic cells play an important role in intravenous tolerance and suppress ongoing experimental autoimmune encephalomyelitis. *FASEB Journal* 22: 669.6

394. Valaperti, A., R. R. Marty, G. Kania, D. Germano, N. Mauermann, S. Dirnhofer, C. Leimenstoll, P. Blyszczuk, C. Dong, C. Mueller, L. Hunziker, and U. Eriksson. 2008. CD11b+ monocytes abrogate Th17 CD4+ T cell-mediated experimental autoimmune myocarditis. *J Immunol* 180(4): 2682-2695

395. Zhu, B., Y. Bando, S. Xiao, K. Yang, A. C. Anderson, V. K. Kuchroo and S. J. Khoury. 2007. CD11b+Ly-6C(hi) suppressive monocytes in experimental autoimmune encephalomyelitis. *J Immunol* 179(8): 5228-5237

396. Bullard, D. C., X. Hu, T. R. Schoeb, R. C. Axtell, C. Raman, and S. R. Barnum. 2005. Critical role of CD11b (Mac-1) on T cells and accessory cells for development of experimental autoimmune encephalomyelitis. *J Immunol* 175: 6327-6333f

397. Suzuki, H., S. Sekine, K. Kataoka, D. W. Pascual, M. Maddaloni, R. Kobayashi, K. Fujihashi, H. Kozono, J. R. McGhee, and K. Fujihashi. 2008. Ovalbumin-protein sigma1 M-cell targeting facilitates oral tolerance with reduction of antigen-specific CD4 (+) T cells. *Gastroenretol* (in press)

398. Robertson, S. J., R. J. Messer, A. B. Carmody, and K. J. Hasenkrug. 2006. *In vitro* suppression of CD8+ T cell function by friend virus-induced regulatory T cells. *J Immunol* 176: 3342-3349

399. Clements, C. S., H. H. Reid, T. Beddoe, F. E. Tynan, M. A. Perugini, T. G. Johns, C. C. A. Bernard, and J. Rossjohn. 2003. The crystal structure of myelin oligodendrocyte glycoprotein, a key autoantigen in multiple sclerosis. *PNAS* 100(19): 11059-11064

400. Issazadeh, S., K. Abdallah, T. Chitnis, A. Chandraker, A. D. Wells, L. A. Turka, M. H. Sayegh, and S. J. Khoury. 2000. Role of passive T-cell death in chronic experimental autoimmune encephalomyelitis. *J Clin Invest* 105(8): 1109-1115

401. Harris, D. P., S. Goodrich, K. Mohrs, M. Mohrs, and F. E. Lund. 2005. Cutting edge: The development of IL-4-producing B cells (B effector 2 cells) is controlled by IL-4, IL-4 receptor alpha, and Th2 cells. *J Immunol* 175: 7103-7107

402. Thompson, L. F., J. M. Rudei, A. Glass, M. G. Low, and A. H. Lucas. 1989. Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidyloinositol-anchored protein, cause human peripheral cells to proliferate. *J Immunol* 143:1815-1821

403. Mills, J. H., L. F. Thompson, C. Mueller, A. T. Waickman, and M. S. Bynoe. 2008. Caffeine and multiple sclerosis: Is protection in your coffee cup? *FASEB J* 22: 1074.9

404. Bulanova, E., V. Budagian, Z. Orinska, M. Hein, F. Petersen, L. Thon, D. Adam, and S. Bulfone-Paus. 2005. Extracellullar ATP induces cytokine expression and apoptosis through P2X7 receptor in murine mast cells. *J Immunol* 174: 3880-3890

405. Lomo, J., H. K. Blomhoff, K. Beiske, T. Stokke, and E. B. Smeland. 1995. TGF-beta 1 and cyclic AMP promote apoptosis in resting human B lymphocytes. *J Immunol* 154(4): 1634-1643

406. Hashikawa, T., M. Takedachi, M. Terakura, T. Saho, S. Yamada, L. F. Thompson, Y. Shimabukuro, and S. Murakami. 2003. Involvement of CD73 (ecto-5'-nucleotidase) in adenosine generation by human gingival fibroblasts. *J Dent Res* 82(11): 888-892

407. Kobie, J. J., P. R. Shah, L. Yang, J. A. Rebhahn, D. J. Fowell, and T. R. Mosmann. 2006. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J Immunol* 177: 6780-6786

408. Alam, M. S., C. C. Kurtz, B. K. Reuter, S. E. Crowe, and P. B. Ernst. 2008. Gastric Th cells express CD39 and CD73 leading to adenosine accumulation that suppresses Helicobacter-induced gastritis by modulating Th cell function. *FASEB J* 22: 852.13

409. Dwyer, K. M., S. Deaglio, W. Gao, D. Friedman, T. B. Strom, and S. C. Robson. 2007. CD39 and control of cellular immune response. *Purinergic Signal* 3: 171-180

410. Maliszewski, C. R., G. J. Delespesse, M. A. Schoenborn, R. J. Armitage, W. C. Fanslow, T. Nakajima, E. Baker, G. R. Sutherland, K. Poindexter, and C. Briks. 1994.

The CD39 lymphoid cell activation antigen, molecular cloning and structural characterization. *J Immunol* 153: 3574-3583

411. Koziak, E., J. Sevigny, S. C. Robson, J. B. Siegel, and K. Kaczmarek. 1999. Analysis of CD39/ATP diphosphohydrolase (ATPDase) expression in endothelial cells, platelets and leukocytes. *Thromb Haemost* 82: 1538-1544

412. Nowicki Montgomery, I., and H. C. Rauch. 1982. Experimental allergic encephalomyelitis (EAE) in mice: primary control of EAE susceptibility is outside the H-2 complex. *J Immunol* 128(1): 421-425

413. Tuohy, V. K., M. Yu, L. Yin, J. A. Kawczyk, J. M. Johnson, P. M. Mathisen, B. Weinstock-Guttman, and R. P. Kinkel. 1998. The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol Rev* 164: 93-100

414. Kroenke, M. A., T. J. Carlson, A. V. Andjelkovic, and B. M. Segal. 2008. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J Exp Med* 205(7): 1535-154