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Multi-Peptide Coupled-Cell Tolerance Ameliorates Ongoing Relapsing EAE Associated with Multiple Pathogenic Autoreactivities[1]

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Abstract

The probability that epitope spreading occurs in multiple sclerosis (MS) and the fact that patients have been shown to respond to multiple myelin epitopes concurrently makes the use of peptide-specific tolerance therapies targeting single epitopes problematic. To attempt to overcome this limitation, we have employed cocktails of peptides in the ECDI coupled-APC tolerance system in mice to determine if T cell responses to multiple autoepitopes can be targeted simultaneously. *Preventative* tolerance induced with splenocytes coupled with a peptide cocktail of four distinct encephalitogenic epitopes ($PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$) inhibited initiation of active EAE induced with each individual peptide and by a mixture of the four peptides by preventing activation of autoreactive Th1 cells and subsequent infiltration of inflammatory cells into the CNS. Most relevant to treatment of clinical MS, *therapeutic* tolerance initiated by splenocytes coupled with the peptide cocktail administered at the peak of acute disease prevented clinical relapses due to epitope spreading and ameliorated a diverse disease induced with a mixture of the four peptides. Interestingly, therapeutic tolerance appeared to be mediated by a mechanism distinct from preventative tolerance, *i.e.* by significantly increasing the levels of production of the anti-inflammatory cytokines TGF-β and/or IL-10 in both the periphery and the CNS.

Keywords

Tolerance/Suppression/Anergy; Antigens/Peptides/Epitopes; Autoimmunity; T Cells; EAE/MS

Introduction

Multiple sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system (CNS) that afflicts approximately 1.1 million people worldwide[1]. The etiology of MS is unknown, but it is well-accepted that both genetic and environmental factors (likely infectious) play key roles in the susceptibility to and initiation of the disease [2–5]. While the precise pathogenic progression of MS is unknown, evidence from both MS patients [6–9] and

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from experimental autoimmune encephalomyelitis (EAE) [10–14], an animal model of MS, supports a major pathogenic role for autoreactive myelin-specific $CD4^+$ T cells. It is unknown at this time how these myelin-specific T cells are initially activated, but the activated T cells produce proinflammatory cytokines, which in turn chemoattract and activate CNS-resident (microglia) and peripheral (macrophages) mononuclear cells which mediate bystander myelin destruction. Progressive disease in relapsing EAE (R-EAE) [15–20] has been shown to be primarily mediated by *de novo* activation of naïve T cells specific for endogenously released myelin antigens, a process known as *epitope spreading*. For example, in $PLP_{139–151}$-induced R-EAE in the SJL mouse, there is a sequential and hierarchical order of epitope spreading in which the first relapse is associated with $CD4^+$ Th1 cell reactivity to $PLP_{178–191}$, and the second to $MBP_{84–104}$. In both the SJL and (SWR × SJL)F_1 R-EAE models, only tolerance to the primary spread epitope after recovery from acute clinical disease could prevent relapses and/or disease progression [17,18]. Relevant to MS therapy, a number of reports have also demonstrated epitope spreading in relapsing-remitting MS (RR-MS) patients [21–25].

Most of the currently approved therapies for the treatment of RR-MS use various antigen-nonspecific immunosuppressive and/or anti-inflammatory strategies and are only partially effective [26–28]. Currently, glatiramer acetate (GA) (copaxone®), a random polymer of L-alanine, L-glutamic acid, L-lysine, and L-tyrosine, is the only approved therapy for MS that is purported to act in a semi-antigen-specific manner [29]. GA is thought to induce T cells that produce Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13 which suppress inflammatory cells [30,31]. GA treatment requires daily subcutaneous (s.c.) injections, is beneficial to only a minority of relapsing-remitting MS patients [32], and 10% of patients experience significant side effects [33]. Antigen-specific therapeutic approaches, including oral tolerance and altered peptide ligands (APLs), have shown promise in EAE, but were not particularly efficacious in subsequent patient trials [34–36]. Thus, there is clearly still a need for new antigen-specific therapies for MS and other autoimmune diseases. Ideally these would be effective with a less severe treatment schedule and have reduced side effects.

There are multiple issues which must be addressed when devising an antigen-specific therapy for MS. First, because there is clear evidence of diversity in the activated T cell repertoire in MS patients [21,24,25], any antigen-specific therapy must be capable of simultaneously targeting T cells specific for multiple myelin epitopes. Second, as the etiology of MS is unknown, disease prevention is impractical at this time, and therefore any treatment must be capable of ameliorating ongoing disease. Thirdly, because changes in the repertoire of activated T cells over time have been demonstrated [21–24], any antigen-specific therapy must be able to affect previously activated autoreactive T cells as well as naïve autoreactive T cells. We have previously employed tolerance induced by the i.v. administration of ethylene carbodiimide (ECDI)-fixed syngeneic splenic APCs (Ag-SP) coupled to individual myelin peptides or to whole myelin proteins to safely and effectively prevent the induction of active R-EAE [37], to treat active R-EAE at peak disease [38], to inhibit the expression of adoptive R-EAE [39,40], and to treat clinical relapses in SJL/J mice by tolerizing to the appropriate spread epitope after the initial acute episode [15,17,41]. However, whether multiple myelin epitopes could be simultaneously coupled to syngeneic splenocytes and presented to naïve and activated autoreactive $CD4^+$ T cells efficiently enough to induce immune tolerance to treat established R-EAE has not been determined. The current experiments indicate that tolerance to a cocktail of peptides can be successfully induced using the ECDI-coupled cell tolerance protocol to simultaneously target T cells of multiple specificities that are actively engaged in CNS pathology. Moreover, the data indicate that tolerance in naïve vs. activated T cells is mediated by distinct mechanisms.

Materials and Methods

Mice

SJL mice, 6–7 weeks old, were purchased from Harlan Laboratories, Bethesda, MD. All mice were housed under specific pathogen-free conditions (SPF) in the Northwestern University Center for Comparative Medicine. Paralyzed animals were afforded easier access to food and water.

Reagents

Synthetic peptides $PLP_{139–151}$ (HSLGKWLGHPDKF), $PLP_{178–191}$ (NTWTTSQSIAFPSK), $MBP_{84–104}$ (VHFFKNIVTPRTPPPSQGKGR), $MOG_{92–106}$ (DEGGYTCFFRDHSYQ), $PLP_{56–70}$ (DYEYLINVIHAFQYV), $VP2_{70–86}$ (WTSQEAFSHIRIPLP), $VP1_{233–250}$ (SASVRIRYKKMKVFCPRP), and $OVA_{323–339}$ (ISQAVHAAHAEINEAGR) were purchased from Genemed, San Francisco, CA.

Induction and Clinical Evaluation of EAE

Female mice 8–10 weeks old were immunized s.c. at three spots on the flank with 100 μl of an emulsion of peptide in CFA containing 200 μg *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI) on day 0. In some experiments, mice also received another 100 μl peptide/CFA emulsion on day 7 and/or 200 ng *Bordetella pertussis* toxin (List Biological Laboratories, Campbell, CA) in 200 μl of PBS i.p. on days 0 and 2. Individual animals were observed every 1–3 days, and clinical scores were assessed on a scale of 0–4 as follows: 0 = no abnormality; 1 = limp tail or hind limb weakness; 2 = limp tail and hind limb weakness; 3 = partial hind limb paralysis; 4 = complete hind limb paralysis. Data are reported as the mean daily clinical score.

Antigen-coupled splenocyte tolerance

Peripheral tolerance induction using peptide-coupled splenocytes was performed as previously described [42]. Briefly, erythrocyte-free (Tris-NH_4Cl-treated) splenocyte suspensions were coupled with peptides using ethylene carbodiimide (ECDI). Cells were washed 2X in PBS, resuspended at 3.2×10^6/ml in PBS with 1 mg/ml of each peptide and 30.75 mg/ml ECDI (CalBiochem, La Jolla, CA), and incubated for 1 h at 4°C with periodic shaking. Peptide-coupled cells were then washed 3X in PBS, filtered through a 70 μm cell strainer, and resuspended at 250×10^6 cells/ml in PBS. Each mouse received 50×10^6 peptide-coupled cells in 200 μl PBS i.v. Coupling efficiency has previously been determined to be approximately 30% [42].

Delayed-Type Hypersensitivity (DTH) Responses

DTH responses were quantitated using a 24 hr ear swelling assay as previously described [43]. Pre-challenge ear thickness was determined using a Mitutoyo model 7326 engineer's micrometer (Schlesinger's Tools, Brooklyn, New York). Immediately thereafter, DTH responses were elicited by injecting 10 μg of peptide in 10 μl of PBS into the dorsal surface of the ear using a 100 μl Hamilton syringe fitted with a 30 gauge needle. The increase in ear thickness over pre-challenge measurements was determined 24 hr after ear challenge. Results are expressed in units of 10^{-4} inches ± SEM.

In Vitro Antigen-Specific Recall Responses

Spleens and draining lymph nodes (axillary, brachial, and inguinal) were harvested, and single-cell suspensions were obtained by mashing through sterile 60-mesh wire screens. In 96-well microtiter plates, 5×10^5 erythrocyte-free (Tris-NH_4Cl-treated) splenocytes or lymph node cells per well were incubated in supplemented culture medium with or without antigen or with

anti-CD3 antibody at 37°C in 7% CO_2 for 48 hr and then pulsed with 1 μCi/well [^{3}H]Thy for the final 24 h of culture. Proliferation was determined by uptake of [^{3}H]Thy detected using a Topcount Microplate Scintillation Counter (Packard Instruments, Meridan, CT). Results are expressed as the mean of triplicate cultures ± SEM. Alternatively, supernatants were collected at 48 hr for cytokine analysis. Cytokine measurements were performed using the Mouse Cytokine 10-Plex system and Luminex Liquidchip analyzer (Qiagen, Valencia, CA) or ELISA.

Immunohistochemistry

Mice were anesthetized and perfused with 30 ml PBS on the indicated days post-immunization. Spinal cords were removed by dissection, and 2- to 3-mm spinal cord blocks were immediately frozen in OCT (Miles Laboratories; Elkhart, IN) in liquid nitrogen. The blocks were stored at −80°C in plastic bags to prevent dehydration. Six micrometer thick cross-sections from the lumbar region (approximately L2-L3) were cut on a Reichert-Jung Cyocut CM1850 cryotome (Leica, Deerfield, IL), mounted on Superfrost Plus electrostatically charged slides (Fisher, Pittsburgh, PA), air dried, and stored at −80°C. Slides were stained using a Tyramide Signal Amplification (TSA) Direct kit (NEN, Boston, MA) according to manufacturer's instructions. Lumbar sections from each group were thawed, air-dried, fixed in 2% paraformaldehyde at room temperature, and rehydrated in 1x PBS. Nonspecific staining was blocked using anti-CD16/CD32, (FcIII/IIR, 2.4G2; BD PharMingen), and an avidin/biotin blocking kit (Vector Laboratories) in addition to the blocking reagent provided by the TSA kit. Tissues were stained with biotin-conjugated Abs anti-mouse CD4 (H129.19) (BD Biosciences, San Jose, CA) and anti-mouse F4/80 (BM8) (Caltag, Burlingame, CA). Sections were coverslipped with Vectashield mounting medium including DAPI (Vector Laboratories, Burlingame, CA). Slides were examined and images were acquired via epifluorescence using the SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI) and Metamorph imaging software (Universal Imaging, Downingtown, PA). Eight non-serial lumbar sections from each sample per group were analyzed at 100x and 200x magnification.

Statistical Analyses

Comparison of the percentage of animals showing clinical disease symptoms or of DTH responses between any two groups of mice was done by two-tailed unpaired student's t test. p values < 0.05 were considered significant.

Results

Preventative coupled-cell tolerance to multiple myelin peptides prevents clinical EAE induced either with individual encephalitogenic myelin peptides or with multiple myelin peptides

To determine whether multiple myelin peptides could be coupled to syngeneic splenocytes and presented to naïve $CD4^+$ T cells efficiently enough to protect against EAE induced with individual myelin peptides as well as multiple myelin peptides, SJL mice were given 50×10^6 syngeneic splenocytes coupled to four encephalitogenic myelin peptides ($PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$) (Tolerized) or four irrelevant peptides ($PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$) (Sham) seven days before priming with each peptide individually (Figure 1), or a combination of all four encephalitogenic myelin peptides (Figure 2). Not only did the tolerization protocol completely prevent or drastically reduce clinical symptoms in every situation, but DTH responses, as an *in vivo* measure of myelin peptide-specific Th1/17 activity, were also significantly decreased compared to sham-tolerized controls. In addition, peripheral peptide-specific T cell recall proliferative responses were significantly decreased in tolerized vs. sham-tolerized mice (Figure 3a). This was true for mice primed with any of the peptides individually (data not shown) and for mice primed with a combination of all four peptides (Figure 3). Analysis of culture supernatants revealed a decrease

in the levels of myelin peptide-induced proinflammatory cytokines IL-2 (Figure 3b) and IFN-γ (Figure 3c) produced *in vitro* by cells from tolerized vs. sham-tolerized mice and equivalent or slightly decreased levels of the regulatory cytokine TGF-□ (Figure 3d). IL-4, IL-5, and IL-10 responses were not seen upon peptide recall in either the tolerized or sham groups (data not shown). Together, these data indicate that preventative tolerance with Ag-SP coupled with multiple peptides leads to induction of anergy in naive myelin-specific T cells even when multiple specificities are targeted simultaneously. Furthermore, immunohistochemical evaluation of the spinal cord revealed a drastically decreased amount of infiltrating T cells and macrophages in tolerized mice compared to sham-tolerized mice (Figure 4). This is likely the result of peripheral anergy induction since only activated T cells are capable of crossing the blood-brain barrier.

Therapeutic coupled-cell tolerance to multiple myelin peptides in established R-EAE induced by individual myelin peptides ameliorates disease relapses

Since the etiology of MS is unknown at this time, prevention of this disease is not yet an option. That leaves treatment of ongoing disease as the only alternative for clinicians. Therefore, it is imperative to demonstrate that any experimental treatment regimen proposed as a possible therapy for MS can ameliorate ongoing disease. In addition, due to the phenomenon of epitope spreading in EAE [15–18] and its possible role in MS clinical relapses [21], it is important to demonstrate that multiple antigen coupled-cell tolerance can inhibit relapses to spread epitopes distinct from the initiating epitope. To test this, female SJL mice were primed with $PLP_{139–151}$ or $PLP_{178–191}$, which both result in relapsing-remitting disease in which the first relapse is due to intramolecular epitope spreading [15–17]. At the peak of acute disease, during which there is only $CD4^+$ T cell reactivity to the initiating epitope, 50×10^6 syngeneic splenocytes coupled to four encephalitogenic myelin peptides ($PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$) (Tolerized), including both the initiating and spread epitopes, or four irrelevant peptides ($PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$) (Sham) were given i.v., and mice were monitored for the development of clinical relapses. In both cases, the multi-peptide tolerization protocol significantly decreased the incidence and severity of clinical relapses to the spread epitope (Figure 5a). In addition, peripheral T cell responses were diminished to both the initiating and the spread epitopes as measured by DTH (Figure 5b). This demonstrates that both previously activated and naïve T cells can be tolerized simultaneously with splenocytes coupled to a myelin peptide cocktail.

Therapeutic coupled-cell tolerance to multiple myelin peptides in established R-EAE induced by immunization with a cocktail of encephalitogenic myelin peptides ameliorates clinical disease

There is clear evidence of diversity in the activated T cell repertoire in MS patients [21–25, 44]. Consequently, it is necessary to establish that a specific treatment regimen can affect ongoing disease in which T cells of multiple specificities are actively playing pathologic roles. In established SJL models of relapsing-remitting EAE, the acute phase is primarily due to autoreactivity specific for only one myelin epitope. In single peptide-induced models, the acute phase is mediated by $CD4^+$ T cells specific for the initiating epitope, and even in disease models that are initiated by priming with whole protein or mouse spinal cord homogenate (MSCH), acute disease is primarily mediated by T cells reactive to the immunodominant myelin epitope [12,15,37,43]. Therefore, in order to evaluate the ability of multiple antigen coupled-cell tolerance to ameliorate clinical symptoms caused by simultaneous reactivity to multiple self-antigens, mice were primed with a combination of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$/CFA. At the peak of acute disease, mice were tolerized with 50×10^6 syngeneic splenocytes coupled to a cocktail of all four encephalitogenic myelin peptides or a cocktail of four control peptides. Remarkably, clinical symptoms in tolerized mice were significantly ameliorated compared to sham-tolerized controls and remained suppressed until the

termination of the experiment (Figure 6a). Additionally, DTH responses were significantly decreased to each of the four myelin antigens after tolerance induction (Figure 6b).

To further examine T cell peripheral immune responses to the immunizing epitopes after therapeutic tolerization, spleens were harvested five days after coupled-cell administration and *in vitro* proliferation and cytokine recall responses were assayed. In response to incubation with the target antigens, the tolerized spleen cells had decreased proliferation compared to sham-tolerized spleen cells (Figure 7a). IL-2 production was decreased to $MBP_{84–104}$ and $PLP_{139–151}$, but not to $PLP_{178–191}$ (Figure 7b). $MOG_{92–106}$ did not induce recall proliferation of IL-2 production over background in cells from either tolerized or sham-tolerized mice. Interestingly, in contrast to the results after preventative tolerization (Figure 3c), IFN-γ production was either comparable, or even slightly increased in cells from tolerized mice (Figure 7c). However, there was a significant enhancement of production of transforming growth factor □ (TGF-□) by T cells from the tolerized mice in response to restimulation with all four of the myelin peptides (Figure 7d). TGF-β has been associated with the *in vivo* function of regulatory T cells (T_{regs}) [45,46]. IL-4 and IL-5 responses were not seen upon peptide recall in either the tolerized or sham groups (data not shown).

To complement the analyses of peripheral immune results, we also determined the effects of therapeutic coupled-cell tolerance on T cell responses in the CNS target organ. The lumbar regions of the spinal cords of representative mice in each group were harvested and evaluated for $CD4^+$ T cell and macrophage infiltrate via immunohistochemical staining. As opposed to preventative tolerance, mice tolerized with the cocktail of myelin peptides at the peak of acute R-EAE were found to have similar amounts of infiltrating cells as sham-tolerized controls (Figure 8). In addition, infiltrating leukocytes were isolated from whole spinal cords after tolerance induction and were restimulated *in vitro* with the priming peptides and supernatants collected for cytokine analysis. Surprisingly, compared to sham-tolerized controls, the levels of proinflammatory cytokines produced by cells from tolerized mice were remarkably varied, ranging from decreased levels in response to some peptides to comparable or even greater levels in response to other peptides (Figure 9). However, similar to the results from the spleen, CNS T cells isolated from therapeutically tolerized mice produced significantly greater amounts of TGF-β than those from sham-tolerized mice (Figure 9c). In addition, they also produced significantly increased levels of IL-10 (Figure 9d), which has also been associated with *in vivo* regulatory T cell function [47,48].

Discussion

In this study, multiple peptide coupled-cell tolerance was shown to be effective at preventing EAE initiated with either individual peptides or a combination of multiple peptides, at preventing relapses to spread epitopes by acting on both naïve and previously activated autoreactive T cells, and at ameliorating ongoing EAE in which T cells specific for multiple myelin antigens were simultaneously playing a pathogenic role. This provides the first demonstration that syngeneic ECDI-fixed cells coupled simultaneously with multiple peptides is an effective method of tolerance induction for both naïve and activated $CD4^+$ T cells. This is an important demonstration relevant to the therapy of MS for several reasons. Ideally, a tolerance-inducing regimen such as this could employ whole proteins coupled to syngeneic cells [37,49] which would operate via a cross-tolerance mechanism involving re-presentation of self epitopes expressed on apoptotic ECDI-treated splenic APCs by host splenic regulatory DCs (unpublished results). By using intact proteins, all of the epitopes contained within those proteins would theoretically be available for presentation to the specific autoreactive T cells in a tolerogenic context. Unfortunately, in the case of MS, whole myelin proteins are not currently available in quantities necessary for treatment purposes. The use of intact myelin proteins isolated from human nervous tissue or produced in bacteria have safety concerns which

preclude their use in the clinical setting. Therefore, it is necessary to employ synthetic myelin peptides produced under GMP conditions if coupled-cell tolerance is to be used in human patients.

The efficient use of peptide-specific tolerogenic therapies consequently requires prior knowledge of the autoepitopes involved in driving chronic disease pathogenesis. This is problematic in MS, a heterogeneous disease of unknown origin in which the specificities of both the inciting and spread epitope-specific autoreactive T cells will certainly vary from patient to patient, due to differences in HLA haplotypes. However, based on high precursor frequencies, high antigen avidities, and functional studies, Bielekova *et al.* have recently identified six immunodominant myelin epitopes most often discriminatory between MS patients and controls [44]. This provides a pool of candidate peptides that can be used for coupled-cell tolerance in future clinical trials in early RR-MS patients. In addition, a sensitive new technique for the functional characterization of antigen-specific T cells present in patient samples has recently been developed. This high-throughput method uses arrays of peptide-MHC complexes to capture antigen-specific T cells with antibodies against secreted cytokines, allowing for a broad-based analysis of the specificities and functional states of patient T cell repertoires [50]. This technology will no doubt facilitate the implementation of antigen-specific therapies in various autoimmune diseases.

There have been several previous studies demonstrating the ability to prevent or ameliorate EAE by targeting multiple encephalitogenic myelin antigens. These studies employed administration of either an engineered fusion protein of PLP and MBP (MP4) [51], an engineered “multiantigen/multiepitope” protein containing epitopes of PLP, MBP, and MOG (hmTAP) [52], or multiple myelin peptides [53]. However, in all of these examples of multi-epitope treatment, the soluble peptides or proteins were administered intravenously. It has recently been shown that both i.v. [38] and intraperitoneal (i.p.) [54] soluble peptide treatment as a therapy of ongoing EAE can result in fatal anaphylaxis. This puts the safety of any soluble peptide/protein treatment for human MS patients into serious question. Indeed, a recent phase II clinical trial employing subcutaneous injections of a soluble MBP APL in MS patients which was terminated due to systemic hypersensitivity reactions that developed in 13 of 142 patients [36]. We have previously demonstrated that peptide-coupled cells, as used in this study, are an effective alternative which is not complicated by the deleterious side effects of anaphylactic shock [38]. This is likely due to the fact that the cell-bound peptide fails to access IgE-coated mast cells in peripheral tissues.

Therefore, we set out to determine if multiple myelin peptides can be efficiently coupled to syngeneic cells and used to induce immune tolerance to multiple myelin antigens. Figures 1 and 2 show that multi-peptide coupled-cell tolerance is effective at preventing EAE initiated both by individual peptides and by a combination of four potent encephalitogenic peptides. In addition, because the etiology of MS is unknown, making prevention of the disease unrealistic at this time, it was essential to demonstrate that the multi-peptide coupled-cell tolerance treatment regimen can ameliorate ongoing disease. Furthermore, as there is evidence that the repertoire of encephalitogenic T cells evolves over time in MS patients, likely due to epitope spreading [21–24], it was important to verify that multi-peptide coupled-cell tolerance can specifically inhibit responses of both previously activated as well as naïve T cells. The data show that multi-peptide coupled-cell tolerance is capable of preventing relapses due to T cell reactivity to spread epitopes, tolerizing not only previously activated T cells (those specific for the priming epitopes), but also naïve T cells (those specific for the spread epitopes) (Figure 5). Finally, because there appears to be considerable diversity in the autoreactive T cell repertoire in MS patients [21,24,44], it was necessary to establish that multi-myelin peptide coupled-cell tolerance can ameliorate ongoing disease associated with multiple concurrent pathogenic reactivities. Figure 6 shows that administration of multi-peptide coupled-cells at the peak of

acute EAE, in which T cells specific for four distinct encephalitogenic myelin epitopes are actively participating, can ameliorate clinical disease progression and decrease peripheral DTH responses to all four peptides. Collectively, these results are very encouraging with regard to the clinical potential of multi-peptide coupled-cell tolerance in the treatment of MS.

With regard to the mechanism(s) by which multi-peptide coupled-cell tolerance might operate, the location and activation/functional capacity of myelin-specific $CD4^+$ T cells were evaluated after tolerance induction. Anergy induction appears to play a major functional role in protective tolerization of naïve T cells. Myelin peptide-specific recall proliferative and pro-inflammatory cytokine responses are significantly suppressed (Figure 3) in the absence of any induction of IL-4. IL-5, or IL-10 (data not shown) in T cells from peripheral immune organs of mice tolerized with multi-peptide coupled splenic APCs seven days prior to priming. In addition, there is a marked decrease in the number of inflammatory leukocytes in the spinal cords of myelin peptide-tolerized mice vs. sham-tolerized mice. This is likely a reflection of the induction of anergy in the peripheral autoreactive T cells prior to immunization, but could also be partially the result of an independent effect of the tolerization protocol on the natural pattern of T cell migration in response to pro-inflammatory stimulation. These results are similar to findings from previous studies in which either whole myelin protein or individual peptides were coupled to the syngeneic donor cells and used to induce tolerance before EAE induction [37,55–57].

In contrast, when previously activated myelin-specific T cells are tolerized, anergy appears to play a lesser role in concert with other mechanisms. This is illustrated by the finding that while myelin-specific T cells from mice tolerized during ongoing disease, in which disease progression is ameliorated (Figure 6), have a decreased ability to proliferate upon restimulation with myelin peptides, $CD4^+$ T cells from both the spleen and spinal cord produce equivalent quantities of IFN-□ as T cells from sham-tolerized mice (Figures 7 and 9). There was also no apparent decrease of infiltrating $CD4^+$ T cells or macrophages in the spinal cords of mice tolerized during ongoing disease. However, therapeutic tolerance led to significantly increased peptide-induced production of both TGF-β and IL-10 by spinal cord-infiltrating T cells compared to sham-tolerance (Figure 9). While IL-10 production from splenic cells of tolerized mice was inconsistent from experiment to experiment (data not shown), TGF-β production was consistently significantly upregulated in splenic T cells from tolerized mice compared to sham-tolerized mice upon myelin peptide restimulation. This pattern of regulatory cytokine production is consistent with a role for T_{reg} cell function in therapeutic tolerance. The fact that IL-10 and TGF-β are upregulated in sham-tolerized mice compared to naive mice is likely an indication that there is a natural increase in T_{reg} cell function during ongoing EAE in an effort to keep the inflammation resulting from the autoimmunity in check.

Although there have been several reports that *in vitro* $CD4^+CD25^+$ T_{reg} cell function is contact-, but not cytokine-dependent [58–60], others have demonstrated roles for secreted IL-10 and TGF-β in *in vivo* T_{reg} survival and function. For example, TGF-β1 has been shown to be necessary for $CD4^+CD25^+$ T_{reg} homeostasis, Foxp3 expression, and regulatory function [45, 46]. Furthermore, $CD4^+CD25^+$ T_{reg} cells from wildtype, but not $IL10^{-/-}$, B6 mice protect against EAE [47], and IL-10-producing $CD4^+CD25^+$ T_{reg} cells in the CNS mediate recovery from EAE [48]. In addition, naturally occurring $CD4^+CD25^+$ T_{reg} cells have been shown to be capable of inducing conventional T cells to become suppressive, and the suppression by the induced regulatory T cells is IL-10 and TGF-β-dependent [61–63]. Thus, it is plausible that the induction/activation/expansion of T_{reg} cells, either in the periphery or locally at the site of inflammation, is a critical action of regulation by multi-myelin peptide coupled-cells administered at the peak of acute disease. However, it is also possible that TGF-β and IL-10 are being produced unrelated to the presence or action of T_{reg} cells, *e.g.* via immune deviation. Both IL-10 and TGF-β are produced by multiple cell types and have immunoregulatory

function. TGF-β suppresses the production of nitric oxide and reactive oxygen intermediates by macrophages [64–66] and has also been shown to inhibit the differentiation of T cells into Th1 and Th2 cells by blocking expression of T-bet and GATA-3 [67–69]. IL-10 has been shown to increase monocytic phagocytosis [70,71] while reducing antigen presentation and CD80/86 expression [72,73]. We are currently evaluating the requirement, or lack thereof, for natural T_{reg} and induced Tr1 cells and/or TGF-β and IL-10 in multi-peptide coupled-cell tolerance utilizing GFP-Foxp3 knock-in mice [74].

In conclusion, multi-peptide coupled-cell tolerance is a novel approach to multiple sclerosis therapy with great clinical potential. While the mechanisms of action must be further examined, this study demonstrates the efficiency of this treatment in simultaneously tolerizing both naïve and previously activated T cells of multiple targeted specificities and to ameliorate ongoing disease associated with multiple pathogenic autoreactivities. This makes it an ideal candidate for treatment of heterogeneous autoimmune disorders such as multiple sclerosis.

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Abbreviations

Ag-SP
: antigen-coupled splenocytes

APL
: altered peptide ligand

EAE
: experimental autoimmune encephalomyelitis

ECDI
: ethylene carbodiimide

MBP
: myelin basic protein

MOG
: myelin oligodendrocyte protein

MS
: multiple sclerosis

PLP
: myelin proteolipid protein

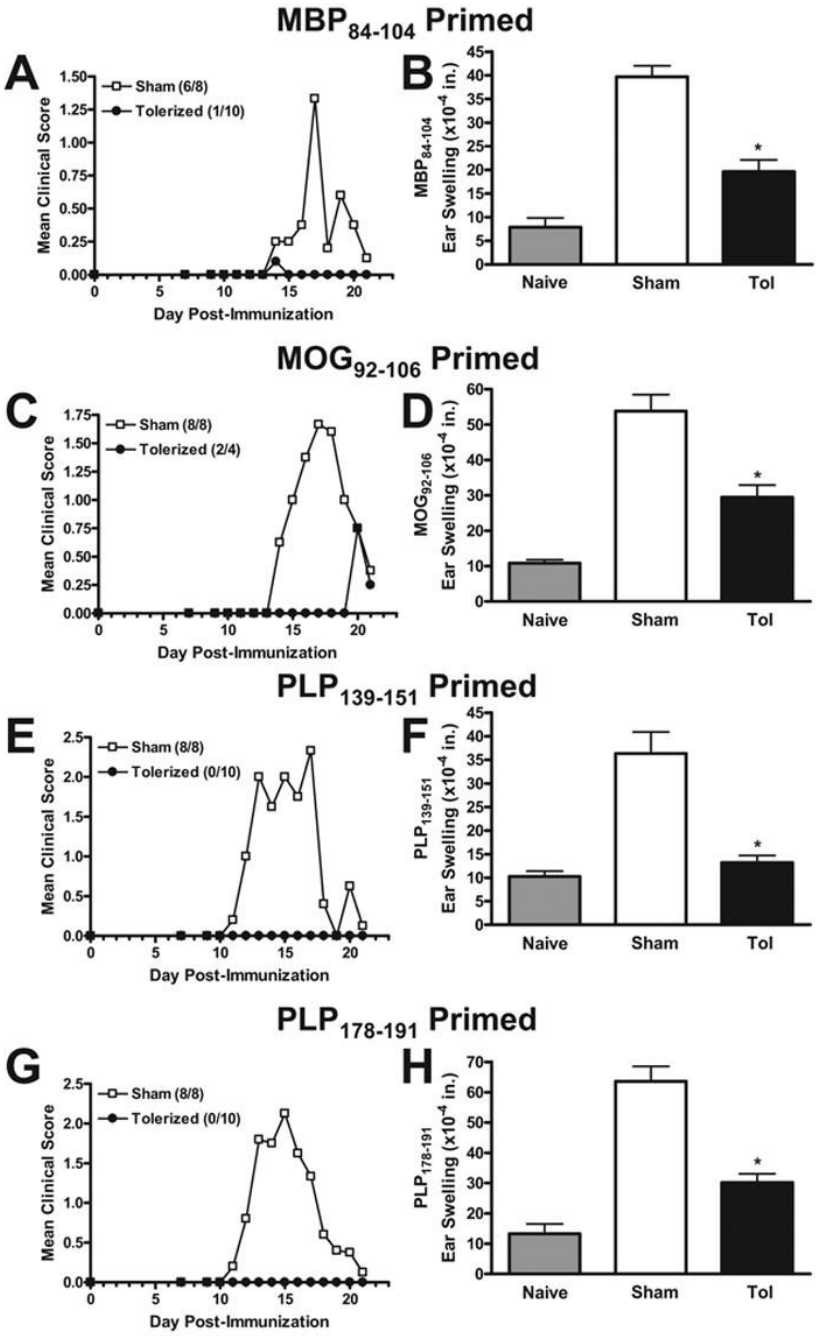

Figure 1. Preventative coupled-cell tolerance to multiple myelin peptides prevents clinical EAE induced with individual encephalitogenic myelin peptides

Female SJL mice were treated i.v. with 50×10^6 syngeneic splenocytes coupled to a myelin peptide cocktail consisting of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$ (Tolerized) or to a control peptide cocktail consisting of $PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$ (Sham) seven days before priming. Four to ten mice per group were immunized s.c. with 200 μg $MBP_{84–104}$/CFA on days 0 and 7 (**A & B**), 200 μg $MOG_{92–106}$/CFA on days 0 and 7 (**C & D**), 50 μg $PLP_{139–151}$/CFA on day 0 (**E & F**), or 100 μg $PLP_{178–191}$/CFA on day 0 (**G & H**). Mice receiving $MBP_{84–104}$ or $MOG_{92–106}$ were also given 200 ng *Bordatella pertussis* toxin i.p. on days 0 and 2. For DTH, mice were ear challenged with 10 μg of the

indicated peptides on day 20, and swelling was measured 24 h later. Data represents two separate experiments. *DTH response significantly less than sham-tolerized controls, $p < 0.01$.

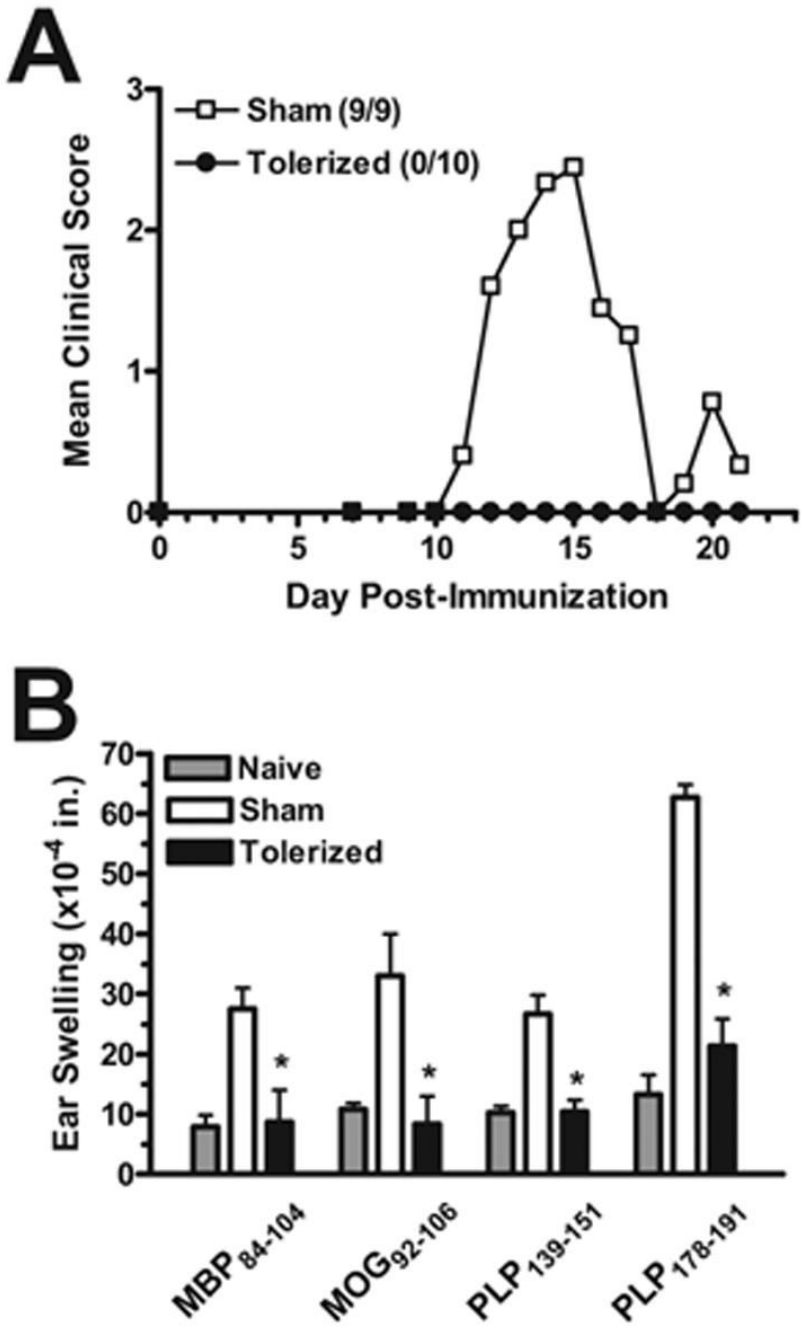

Figure 2. Preventative coupled-cell tolerance to multiple myelin peptides prevents clinical EAE induced with a cocktail of encephalitogenic myelin peptides

Female SJL mice were treated i.v. with 50×10^6 syngeneic splenocytes coupled to a myelin peptide cocktail consisting of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$ (Tolerized) or to a control peptide cocktail consisting of $PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$ (Sham) seven days before priming. Nine to ten mice per group were immunized s.c. with a cocktail of 100 μg $MBP_{84–104}$, 100 μg $MOG_{92–106}$, 25 μg $PLP_{139–151}$, and 50 μg $PLP_{178–191}$/CFA on day 0. For DTH, mice were ear challenged with 10 μg of the indicated peptides on day 20, and swelling was measured 24 h later. *DTH response significantly less than sham-tolerized controls, $p < 0.01$. Data represents two separate experiments.

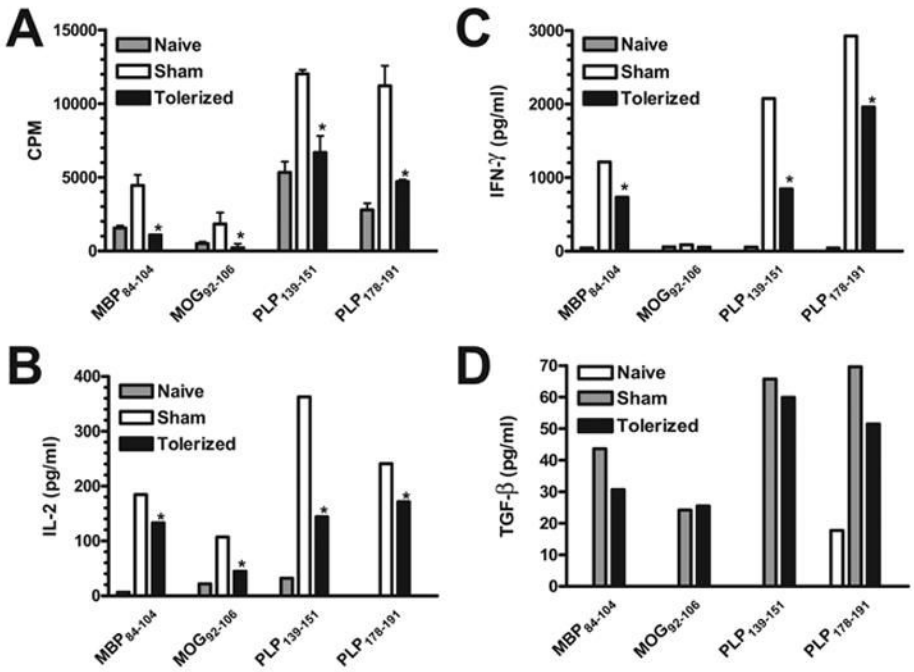

Figure 3. Preventative coupled-cell tolerance to multiple myelin peptides inhibits peripheral peptide-specific T cell recall responses

Female SJL mice were treated i.v. with 50×10^6 syngeneic splenocytes coupled to a myelin peptide cocktail consisting of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$ (Tolerized) or to a control peptide cocktail consisting of $PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$ (Sham) seven days before priming. Three to five mice per group were immunized s.c. with a cocktail of 100 μg $MBP_{84–104}$, 100 μg $MOG_{92–106}$, 25 μg $PLP_{139–151}$, and 50 μg $PLP_{178–191}$/CFA on day 0. Draining lymph nodes were harvested from 3 representative mice per group on day 21. Single cell suspensions were incubated for 3 days with the indicated peptides and proliferative responses assessed by uptake of [^{3}H] thymidine (**A**). Data is presented as mean CPM ± SEM. Single cell suspensions were incubated for 48 hr with the indicated antigens prior to measurement of IL-2 (**B**) and IFN-γ (**C**) levels via cytokine array analysis and TGF-β **(D)** levels via ELISA. Cytokine data is presented as mean pg/ml. *Responses significantly less than sham-tolerized controls, $p < 0.05$.

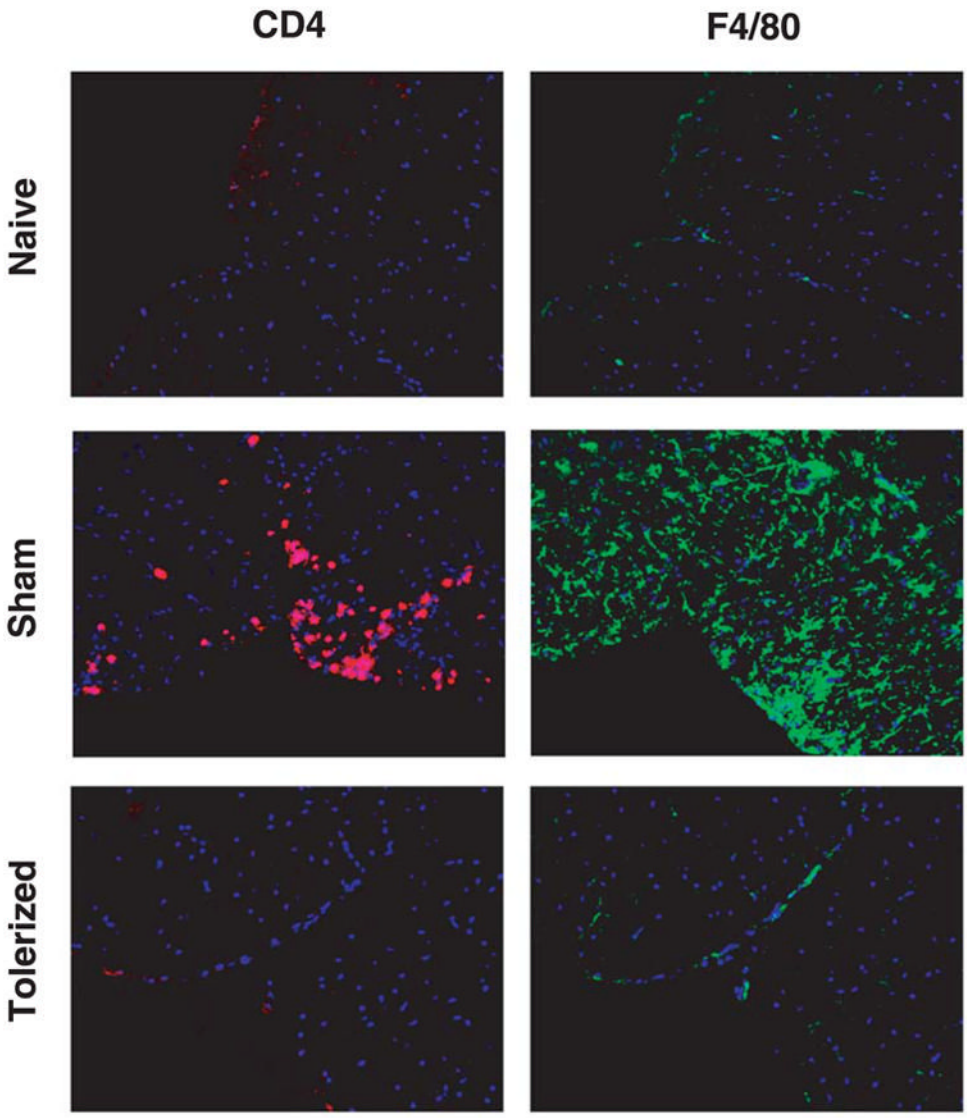

Figure 4. Preventative coupled-cell tolerance to multiple myelin peptides inhibits inflammatory spinal cord infiltrate

Female SJL mice were treated i.v. with 50×10^6 syngeneic splenocytes coupled to a myelin peptide cocktail consisting of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$ (Tolerized) or to a control peptide cocktail consisting of $PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$ (Sham) seven days before priming. Three to five mice per group were immunized s.c. with a cocktail of 100 μg $MBP_{84–104}$, 100 μg $MOG_{92–106}$, 25 μg $PLP_{139–151}$, and 50 μg $PLP_{178–191}$/CFA on day 0. Representative mice from each group were perfused on day 21. Spinal cords were harvested and frozen in OCT. Lumbar regions were sectioned into 6 μm slices and stained with anti-CD4 (red) or anti-F4/80 (green) and counterstained with DAPI (blue). Sections shown at 200x and are representative of 3 individual mice per group.

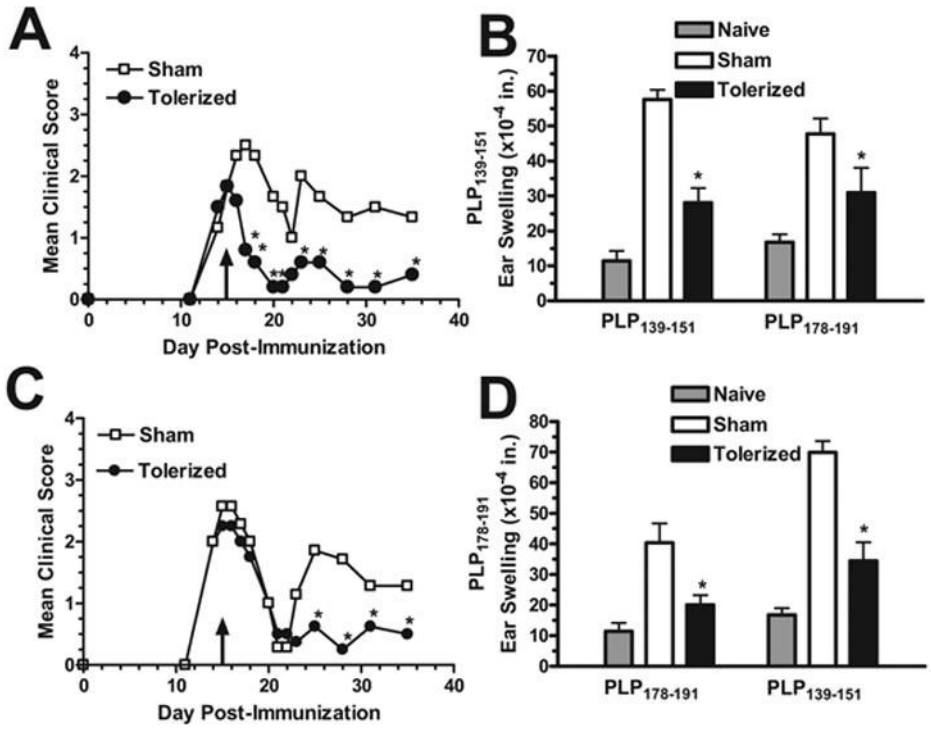

Figure 5. Therapeutic coupled-cell tolerance to multiple myelin peptides in established R-EAE induced by individual myelin peptides ameliorates disease relapses

Twenty-eight female SJL mice were primed s.c. with either 50 μg $PLP_{139–151}$ (**A & B**) or 100 μg $PLP_{178–191}$ (**C & D**) in CFA on day 0 and monitored for clinical disease. At day 15 (indicated by arrows), during the acute phase of the disease, mice were given $50×10^6$ syngeneic splenocytes coupled to a myelin peptide cocktail consisting of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$ (Tolerized) or to a control peptide cocktail consisting of $PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$ (Sham) i.v. For DTH, mice were ear challenged with 10 μg peptide on day 30, and swelling was measured 24 h later. *Response significantly less than sham-tolerized controls, $p < 0.01$. Data is representative of two separate experiments.

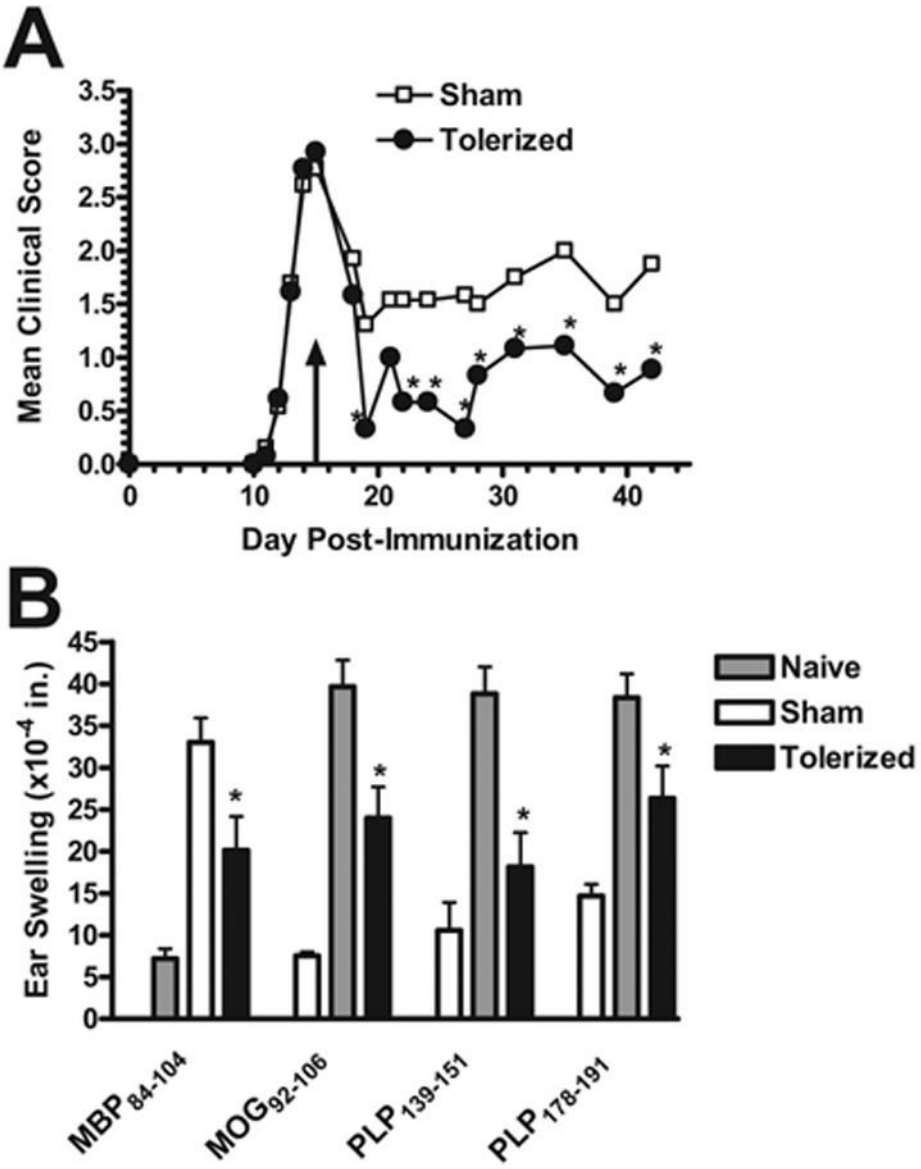

Figure 6. Therapeutic coupled-cell tolerance to multiple myelin peptides in established R-EAE induced by immunization with a cocktail of encephalitogenic myelin peptides ameliorates disease relapses

Twenty-six female SJL mice were primed s.c. with a cocktail of myelin peptides consisting of 25 μg $PLP_{139–151}$, 50 μg $PLP_{178–191}$, 100 μg $MBP_{84–104}$, and 100 μg $MOG_{92–106}$ and CFA on day 0. On day 15 (indicated by arrow), at the peak of acute disease, mice were given $50×10^6$ syngeneic splenocytes coupled to a myelin peptide cocktail consisting of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$ (Tolerized) or to a control peptide cocktail consisting of $PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$ (Sham) i.v. and monitored for clinical disease (**A**) and peptide-specific DTH responses (**B**). For DTH, mice were ear challenged with 10 μg peptide on day 27, and swelling was measured 24 hours later. Data is representative of three separate experiments. *Responses significantly less than sham-tolerized controls, $p < 0.05$.

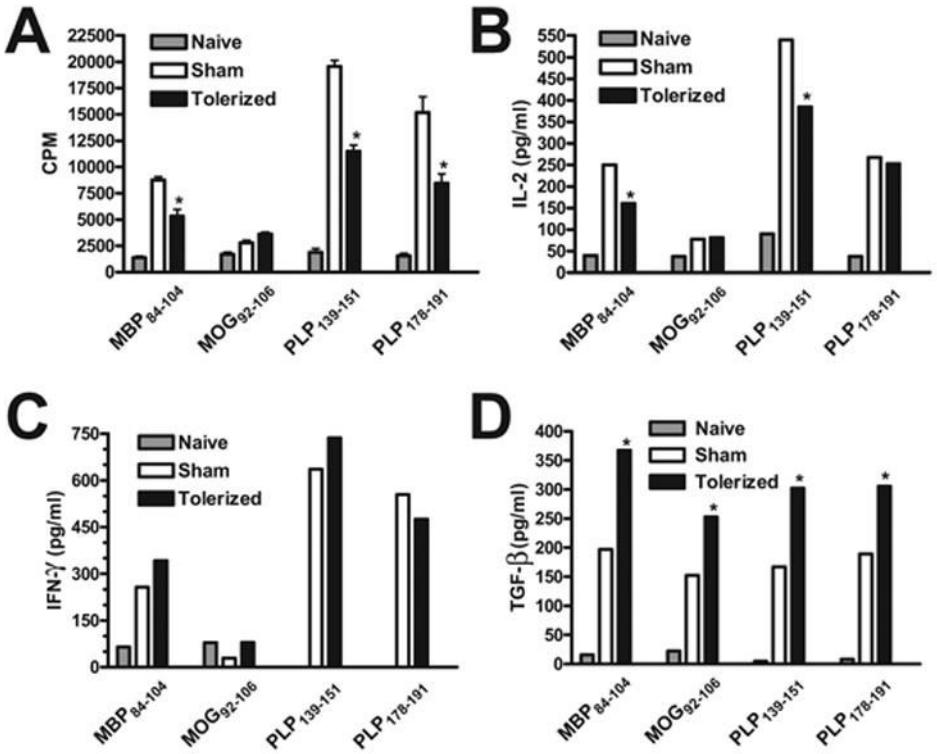

Figure 7. Effect of therapeutic coupled-cell tolerance to multiple myelin peptides on peripheral peptide-specific T cell recall responses

Twenty-six female SJL mice were primed s.c. with a cocktail of myelin peptides consisting of 25 μg $PLP_{139–151}$, 50 μg $PLP_{178–191}$, 100 μg $MBP_{84–104}$, and 100 μg $MOG_{92–106}$ and CFA on day 0. On day 15, at the peak of acute disease, mice were given 50×10^6 syngeneic splenocytes coupled to a myelin peptide cocktail consisting of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$ (Tolerized) or to a control peptide cocktail consisting of $PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$ (Sham) i.v. Spleens were harvested from 3 representative mice from each group on day 20. Single cell suspensions were incubated for 3 days with the indicated antigens and assessed for proliferative responses by uptake of [^{3}H] thymidine (**A**). Data is presented as mean CPM ± SEM. Single cell suspensions were incubated for 48 hr with the indicated antigens prior to measurement of IL-2 (**B**) and IFN-γ (**C**) levels via cytokine array analysis and TGF-β (**D**) levels via ELISA. Cytokine data is presented as mean pg/ml. *Responses significantly different than sham-tolerized controls, $p < 0.05$. Data is representative of two experiments.

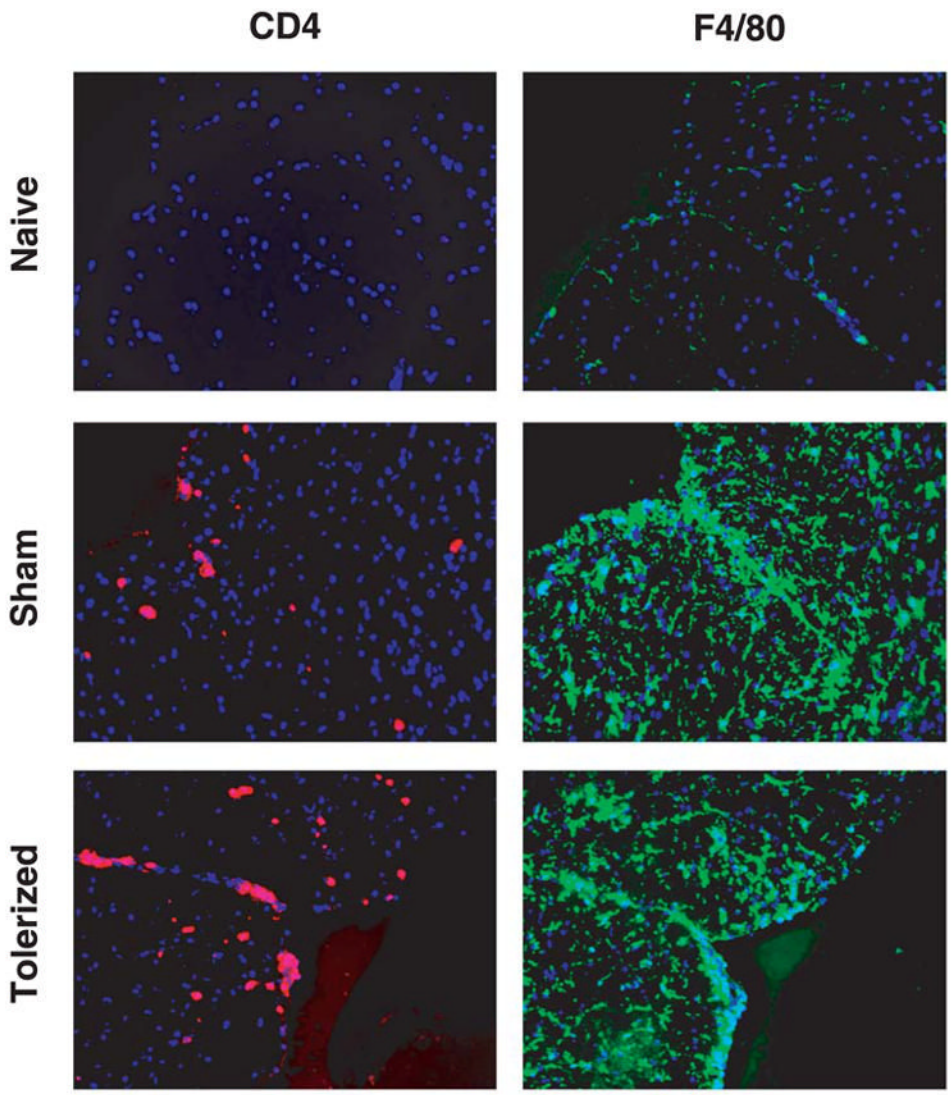

Figure 8. Effect of therapeutic coupled-cell tolerance to multiple myelin peptides on CNS inflammatory cell infiltrates in established EAE

Twenty female SJL mice were primed s.c. with a cocktail of myelin peptides consisting of 25 μg $PLP_{139–151}$, 50 μg $PLP_{178–191}$, 100 μg $MBP_{84–104}$, and 100 μg $MOG_{92–106}$ and CFA on day 0. On day 15, at the peak of acute disease, mice were given 50×10^6 syngeneic splenocytes coupled to a myelin peptide cocktail consisting of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$ (Tolerized) or to a control peptide cocktail consisting of $PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$ (Sham) i.v. Three mice from each group were perfused on day 31. Spinal cords were harvested, snap-frozen, sectioned into 6μm slices, stained with anti-CD4 (red) or anti-F4/80 (green) mAbs, and counterstained with DAPI (blue). Lumbar regions are shown. Sections shown at 200x and are representative of two experiments.

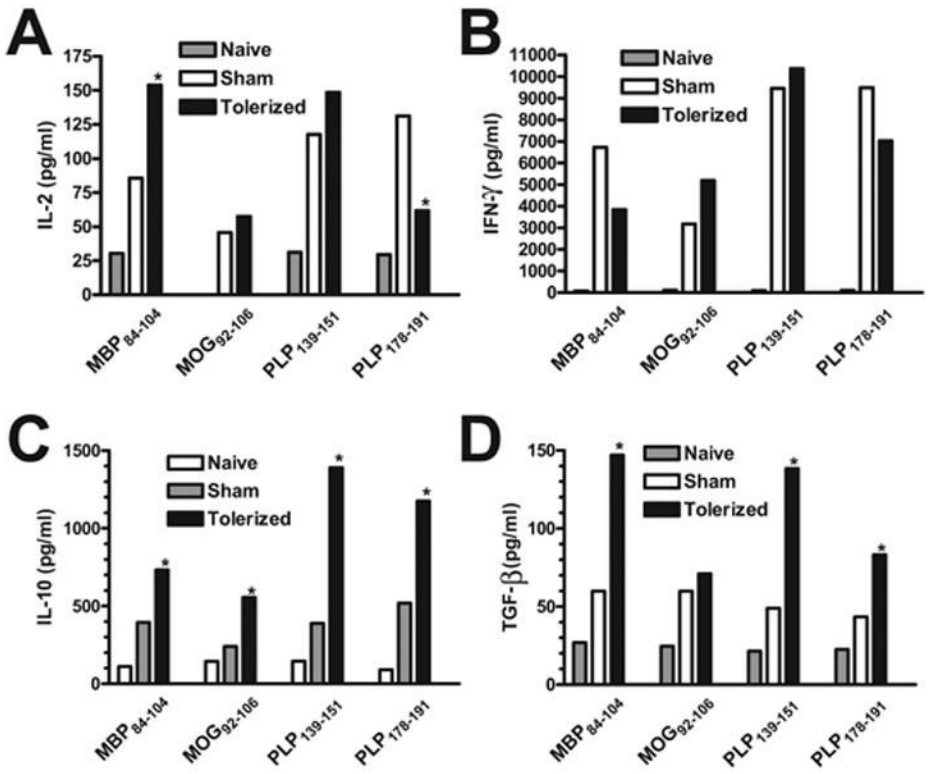

Figure 9. Effect of therapeutic coupled-cell tolerance to multiple myelin peptides on CNS peptide-specific T cell recall responses in established EAE

Twenty-six female SJL mice were primed s.c. with a cocktail of myelin peptides consisting of 25 μg $PLP_{139–151}$, 50 μg $PLP_{178–191}$, 100 μg $MBP_{84–104}$, and 100 μg $MOG_{92–106}$ and CFA on day 0. On day 15, at the peak of acute disease, mice were given $50×10^6$ syngeneic splenocytes coupled to a myelin peptide cocktail consisting of $PLP_{139–151}$, $PLP_{178–191}$, $MBP_{84–104}$, and $MOG_{92–106}$ (Tolerized) or to a control peptide cocktail consisting of $PLP_{56–70}$, $OVA_{323–339}$, $VP1_{233–250}$, and $VP2_{70–86}$ (Sham) i.v. On day 20, ten mice from each group were perfused, and spinal cords were harvested. Infiltrating leukocytes were isolated and single cell suspensions were incubated for 48 hr with the indicated antigens prior to measurement of IL-2 (**A**), IFN-γ (**B**) and IL-10 (**C**) levels via cytokine array analysis and TGF-β (**D**) levels via ELISA. Cytokine levels are reported as mean pg/ml and are representative of two separate experiments. *Responses significantly different than sham-tolerized controls, $p < 0.05$.