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Dissociation of Experimental Allergic Encephalomyelitis Protective Effect and Allergic Side Reactions in Tolerization with Neuroantigen[1]

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Administration of autoantigens under conditions that induce type 2 immunity frequently leads to protection from T cell-mediated autoimmune diseases. Such treatments, however, are inherently linked to the induction of IgG1 Abs and to the risk of triggering anaphylactic reactions. We studied the therapeutic benefit vs risk of immune deviation in experimental allergic encephalomyelitis of SJL mice induced by MP4, a myelin basic protein-proteolipid protein (PLP) fusion protein. MP4 administration in IFA induced type 2 T cell immunity, IgG1 Abs, and experimental allergic encephalomyelitis protection, and all three were enhanced by repeat injections. Despite high Ab titers, anaphylactic side reactions were not observed when MP4 was repeatedly injected in IFA or as soluble Ag s.c. In contrast, lethal anaphylaxis was seen after s.c. injection of soluble PLP:139–151 peptide, but not when the peptide was reinjected in IFA. Therefore, the Ab response accompanying the immune therapy constituted an anaphylactic risk factor only when the autoantigen was not retained in an adjuvant and when it was small enough to be readily disseminated within the body. Taken together, our data show that treatment regimens can be designed to boost the protective type 2 T cell response while avoiding the risk of Ab-mediated allergic side effects. *The Journal of Immunology,* **2007, 178: 4749–4756.**

Immunizations with neuroantigens such as myelin basic protein (MBP),[3] proteolipid protein (PLP), and myelin oligodendrocyte protein result in experimental allergic encephalitis (EAE), a T cell-mediated autoimmune disease of the CNS that resembles multiple sclerosis (MS). In mice, the induction of EAE requires the neuroantigens to be injected together with adjuvants that trigger a type 1 T cell response (1). In addition, most murine EAE models depend on the use of pertussis toxin (PTX) that has type 1 adjuvant effects (2, 3), disrupts the blood brain barrier (4), and licenses the APC in the brain for recognition by the effector T cells (5, 6). The administration of the neuroantigens in essentially any other way does not lead to EAE, but rather protects mice from the subsequent induction of the autoimmune disease. Classical protocols for EAE prevention include the injections with IFA (7–9), oral (10, 11) or nasal administration of neuroantigen (12, 13), or i.v. injection of soluble Ag (14, 15). These treatments result in "immune deviation" that is associated with a T cell response of type 2/3 cytokine signature (16, 17) and an Ab response. Common to all of these regimens is the administration of Ag in the absence of TLR stimulation.

IFA is a plain mineral oil (without the mycobacteria that CFA contains in addition). Its "adjuvant" effects are due to the creation of a depot from which the Ag is slowly released: even 90 days postinjection, half of the original Ag dose is still contained in the inoculum (18). In contrast, soluble Ag administered via any route has a very short half-life, within the range of mere minutes. When injected in IFA, most Ags studied to date, including protein autoantigens as well as peptides, were found to induce type 2 T cell and strong Ab responses (19–22).

Autoantigen injections in IFA have long been classical protocols for disease prevention (reviewed in Ref. 22). The protection is specific: for example, injection of PLP:IFA prevents EAE, whereas renal tubular Ag:IFA does not. Similarly, the latter treatment, but not the former, abrogates the development of the autoimmune antitubular basement membrane disease in the kidney. This protection can be adoptively transferred with CD4 cells that display an Ag-specific type 2 cytokine signature (22). Type 2 T cells seem to be viable candidates for mediating this type of "infectious tolerance." That is because the cytokines they secrete can be suppressive to type 1 T cells, and they can create a type 2 T cell differentiation environment in the target organ that shifts the determinant-spreading reaction toward a type 2 cytokine profile (23).

However, recent evidence suggests that type 2 immunity can be a double-edged sword in autoimmune disease. It has been described that autoreactive type 2 T cells are able to induce autoimmune pathology on their own (24–27). Moreover, mast cells are essential for early onset and severe EAE, suggesting an amplificatory role for these cells in the T cell-mediated immune pathology (28–30). Eosinophils also play a critical role in the pathogenesis of EAE (31). Multiple other elements of the

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[3] Abbreviations used in this paper: MBP, myelin basic protein; PLP, proteolipid protein; MS, multiple sclerosis; PTX, pertussis toxin; HEL, hen egg lysozyme; drLN, draining lymph node; Thpp, T helper-primed precursor.

allergic arm of the immune response have been shown to be involved in EAE; for example, histamine receptors 1 and 2 are present on inflammatory cells in brain lesions, and EAE severity is decreased in mice genetically deficient in FcγRIII and FcεRI (32). As an intrinsic part of type 2 immunity, Abs are typically induced by the autoantigen or peptide administered for accomplishing immune deviation (21). Such Abs can cause severe anaphylactic reactions in mice and humans and have brought clinical trials to a halt (33–36).

Clinical protocols frequently involve repeat injections of Ag with the intent to boost the protective class of T cell response. Although, so far, it has not been formally established that such repeat injections augment type 2 T cell immunity and are indeed of therapeutic benefit, they do inevitably boost the levels of anaphylatoxins and therefore increase the risk of allergic side effects. Using MBP-ΔPLP4 fusion protein (MP4) and PLP:139–151 as neuroantigens in SJL mice, we studied the impact of repeated Ag injections on the T cell response and scrutinized whether the therapeutic benefit can be dissociated from anaphylactic side reactions.

Materials and Methods

Mice, Ags, and treatments

Female SJL/J mice were obtained from The Jackson Laboratory and maintained in specific pathogen-free animal facilities of Case Western Reserve University. At the time of first injection, all mice were 6–8 wk old. All treatments complied with the institutional guidelines. MP4 (Apogen) was obtained from Alexion Pharmaceuticals. PLP:139–151 peptide was purchased from Princeton Biomolecules, and hen egg lysozyme (HEL) was obtained from Sigma-Aldrich. IFA was prepared as a mixture of mannide monooleate (Sigma-Aldrich) and paraffin oil (EMScience). CFA was prepared by mixing *Mycobacterium tuberculosis H37 RA* (Difco Laboratories) at 5 mg/ml into IFA. For immunization purposes, MP4 was used at doses ranging from 37.5 to 300 μg per mouse using CFA or IFA as specified. HEL was administered at a dose of 200 μg/mouse, and the PLP peptide at a dose of 100 μg/mouse. Three hundred microliters of Ag-adjuvant emulsion for the administration of 300 μg of MP4 and 200 μl for all other immunizations was injected per mouse s.c., at two different sites on the flank. For EAE induction, PTX (200 ng; List Biological Laboratories) was injected i.p. in 500 μl of saline directly after the immunization with MP4 in CFA, and then a second time 48 h later. Starting from day 5 after injection of the neuroantigen, the mice were assessed daily for the development of paralytic symptoms, and the severity of disease was recorded according to the standard scale: grade 1, floppy tail; grade 2, hind leg weakness; grade 3, full hind leg paralysis; grade 4, quadriplegia; grade 5, death. Mice demonstrating symptoms in between the clear-cut gradations were scored intermediately in increments of 0.5.

ELISPOT assays and ELISPOT image analysis

ImmunoSpot M200 plates (Cellular Technology) were coated overnight with the capture Abs in sterile PBS. R4-6A2 (BD Pharmingen) was used at 4 μg/ml for capturing IFN-γ; JES6-1A12 (eBioscience) at 4 μg/ml for IL-2; 11B11 (purified from hybridoma in our laboratory) at 8 μg/ml for IL-4; TRFK5 (eBioscience) at 1 μg/ml for IL-5. The plates were blocked for 1 h with sterile PBS containing 1% BSA (Sigma-Aldrich) and washed three times with sterile PBS. Mice were sacrificed at specific time points and draining lymph nodes (drLN) or spleen were harvested. Single-cell suspensions were prepared and cells were plated in HL-1 (BioWhittaker) supplemented with L-glutamine at 1 mM without (medium) or with Ag (20 μg/ml MP4), which we have shown to induce the maximal recall response. For splenocytes, 10^6 cells were plated per well, whereas for drLN cells, 5×10^5 cells were used per well. The plates were cultured at 37°C with 7% CO_2 (24 h for IFN-γ and IL-2 assays; 48 h for IL-4 and IL-5 assays). After thorough washing, the detection Abs were added for overnight incubation. Biotinylated XMG1.2 (BD Pharmingen) was used at 2 μg/ml for detecting IFN-γ, JES6-5H4-biotin (BD Pharmingen) at 2 μg/ml for IL-2, BVD6-24G2-biotin (BD Pharmingen) at 1 μg/ml for IL-4, and TRFK4-biotin (BD Pharmingen) at 2 μg/ml for IL-5. The plates were then washed four times in PBS/BSA containing 0.025% Tween 20 (Fisher Scientific International). As a tertiary reagent, streptavidin-AP (DakoCytomation) was added at a 1/1000 dilution in PBS/BSA/Tween 20 and incubated for 2 h, followed by three washes with PBS containing 0.025% Tween 20 (PBST) and three washes with PBS. The plates were then developed for 10–30 min using NBT/5-bromo-4-chloro-3-indolyl phosphatase substrate (Kirkegaard & Perry Laboratories). The resulting spots were counted on an ImmunoSpot Series 3B Analyzer (Cellular Technology) specifically designed for morphometric ELISPOT analysis. All results were normalized to 10^6 cells, and the difference between stimulated and nonstimulated cells was calculated.

Proliferation assays

Spleen cell proliferation assays were performed as described previously (37). Briefly, single-cell suspensions were prepared, and 10^6 spleen cells per well were plated in triplicates in flat-bottom, 96-well microtiter plates in serum-free HL-1 medium supplemented with L-glutamine at 1 mM. MP4 was added at a final concentration of 20 μg/ml, which we have established to induce the maximal recall response. During the last 18 h of a 4-day culture, [^{3}H]thymidine was added (1 μCi/well); incorporation of label was measured by liquid scintillation counting, and the difference with respect to non-Ag-stimulated values was calculated.

ELISA for detecting MP4-specific IgG1 serum Abs

ELISA plates (Nunc Immunoplate MaxiSorp; Fisher Scientific) were coated with 3 μg/ml MP4 in bicarbonate buffer overnight at 4°C. The plates were blocked for 2 h with PBST enriched with 1% BSA at room temperature, and serial dilutions of serum, starting at 1/200 in PBST/BSA, were added in triplicates to the plate. After overnight incubation at 4°C, plate-bound serum Abs were detected by alkaline phosphatase-coupled goat anti-mouse IgG1 Abs (Southern Biotechnology Associates). A total of 100 μl/well of freshly prepared *p*-nitrophenylphosphate solution (Research Organics) was used for the development of the colorimetric reaction. The OD was read in an ELISA reader at 405 nm, and values of different plates were adjusted for 1,000 in positive control wells.

Statistics

Results are presented as mean ± SEM. Differences between groups were assessed using the Student's *t* test or, in case the Normality Test or the Equal Variance Test failed, the Mann-Whitney *U* rank sum test was used (calculated by SigmaStat, version 7.0; SPSS). Statistical significance was set at $p < 0.05$.

Results

Unlike MP4:IFA, injections of MP4:CFA induce EAE

MBP and PLP are major constituents of the myelin sheath, and both have been implicated in the pathogenesis of MS. Comprising both the MBP and the PLP protein, the MP4 fusion protein has been generated with the purpose of inducing tolerance (or autoimmunity) to both neuroantigens simultaneously (15, 38). We injected SJL mice with either MP4 in CFA and PTX or with MP4 in IFA. The CFA-injected mice developed severe EAE (mean score 3.0) after injection of 300, 150, or 75 μg of MP4, with similar disease courses and severities at all three doses (data not shown; EAE induced by immunization with 75 μg shown for the controls). Because the mice injected with only 37.5 μg of MP4 recovered faster and showed no signs of relapses, we elected the 75 μg dose to be used for induction of disease and tolerance in subsequent studies. In contrast, SJL mice tolerated an injection of 300 μg (as well as 75 μg) MP4 in IFA without displaying any clinical symptoms (data not shown, also see the data below).

Single MP4:CFA injection induces type 1 immunity; single MP4:IFA injection induces a weak IL-2-positive, IL-4/5-negative T cell response

The type 1/type 2 T cell response profile associated with injection of MP4 in CFA or IFA has not yet been established. Studies of cytokine signatures showed MP4-induced IFN-γ and IL-2, but no IL-4 or IL-5 production in drLN cells of MP4:CFA-injected mice tested on day 9 (Fig. 1), consistent with the induction of a polarized type 1 immunity. On day 9 after the injection, drLN cells of once MP4:IFA-injected mice did not generate a clear IL-2, IL-4, IL-5, or IFN-γ recall response (Fig. 1). However, when the spleen cells of once MP4:IFA-injected mice were studied at later time points, a clear IL-2 recall response was detected on wk 4, 6, and 8

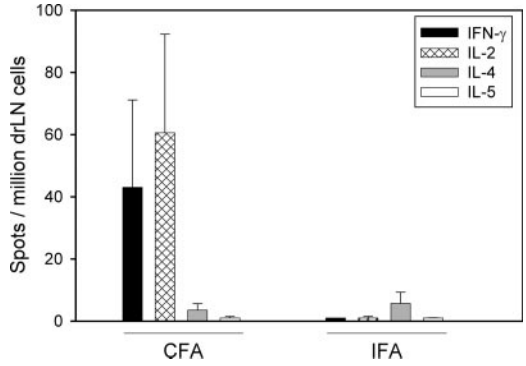

FIGURE 1. Single s.c. immunization with MP4:CFA induces type 1 T cell response, and single immunization with MP4:IFA results in a weak type 2 T cell response in drLNs. SJL mice were immunized s.c. with MP4 (75 μg) in either CFA or IFA, and drLN cells were tested in the specified cytokine ELISPOT assays for MP4-induced cytokine production on day 9. DrLN cells from eight mice per group were tested individually, each in triplicate wells. The mean values for each group and the SEM among the individual mice of the group are shown (the SEM between the triplicates of individual mice was <20%; data not shown). The number of cytokine-producing cells per million drLN cells is specified on the *y*-axis. The results shown are from one experiment that is representative of two performed on drLN cells.

(Fig. 2*C*), in the absence of significant IL-4, IL-5, or IFN-γ (Fig. 2, *D*, *E*, and *F*, respectively). MP4-specific IgG1 Abs could not be detected in once MP4:IFA-injected mice (Fig. 3). Therefore, a single injection of MP4:IFA induced a non-Th1/Th2-polarized T cell response in which IL-2 producers prevailed (T helper-primed precursor (Thpp) cell), in the absence of a detectable Ab response. With a frequency of ~100/million, the numbers of IL-2 producers after the single MP4:IFA injection were in the same order of magnitude as IL-2-producing T cells induced by single MP4:CFA injection (see Fig. 1). Proliferative recall responses were not detected in singly MP4:IFA-injected mice (groups I-IV in Fig. 2*B*). This likely results from a lower sensitivity of proliferation assays for the detection of Ag-specific T cells compared with the single-cell resolution ELISPOT assays (when the frequencies of IL-2 producers were higher, e.g., in the repeatedly injected group VI, also the proliferation assay picked up a weak signal).

Repeat immunizations with MP4:IFA boost type 2 T cell and IgG1 Ab response

SJL mice were immunized with MP4:IFA up to four times at intervals of 2 wk. Mice were tested 2 wk after the primary, secondary, tertiary, and quartiary injection, as specified in Fig. 2*A* (groups I and V-VII). Spleen cells from 14 mice per group were tested for the MP4-induced proliferative response by [^{3}H]thymidine incorporation (Fig. 2*B*) and for MP4-induced cytokine production by IL-2, IL-4, IL-5, and IFN-γ ELISPOT assays (Fig. 2, *C–F*). Both after primary and secondary MP4:IFA injection, the proliferative recall response was undetectable. The tertiary injection induced a strong response ($p = 0.006$ vs secondary injection). In mice injected four times, the MP4-induced proliferative response declined

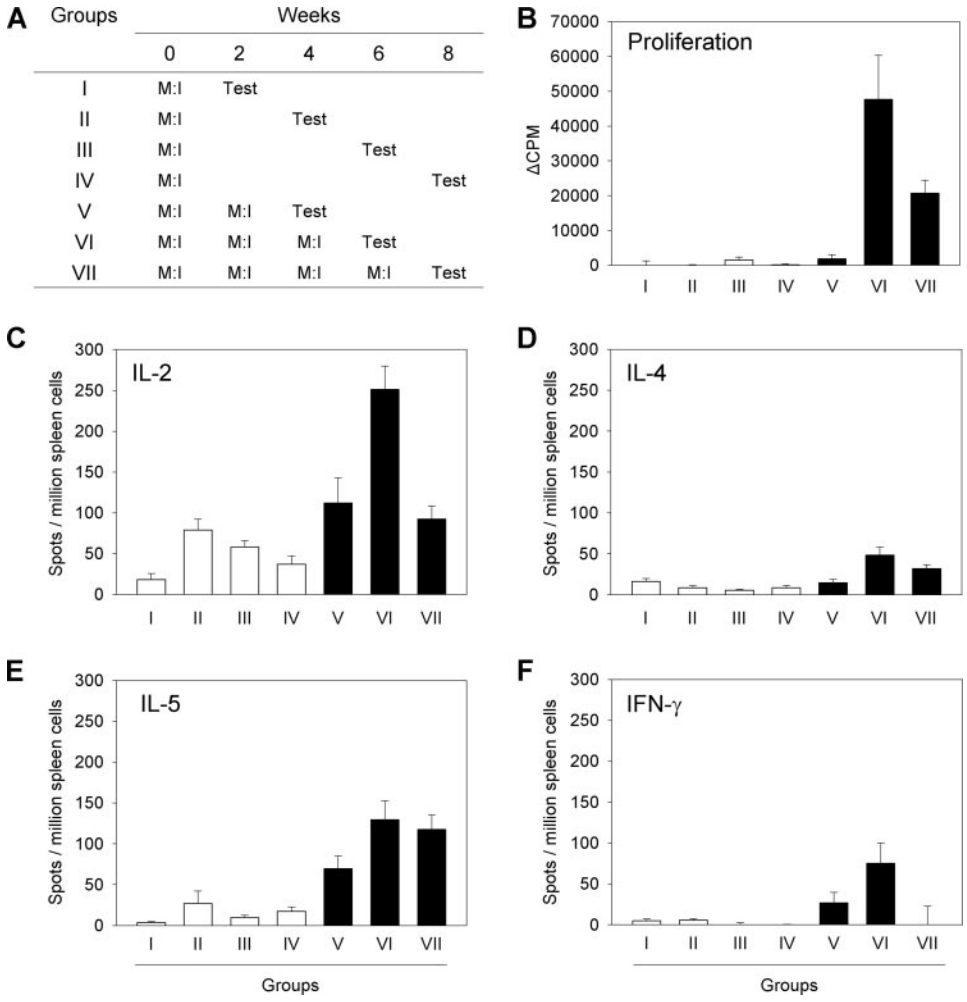

FIGURE 2. Repeat injections with MP4:IFA boost type 2 T cell response. SJL mice were repeatedly injected s.c. with MP4 (75 μg) in IFA (M:I), as specified in *A*. Groups of mice that had been injected once and tested at different time points thereafter are represented with □, groups that had been injected repeatedly, and tested 2 wks after the last injection, are represented with ■. At the defined time points (Test), the splenocytes were tested for MP4-induced proliferation by [^{3}H]thymidine incorporation (*B*) and cytokine production by ELISPOT assays (*C–F*). Spleen cells from 6 to 18 mice per group were tested individually ($n = 6$ for groups II-IV, $n = 14$ for groups I, V, and VI, and $n = 18$ for group VII). The data are represented as specified in the Fig. 1 legend.

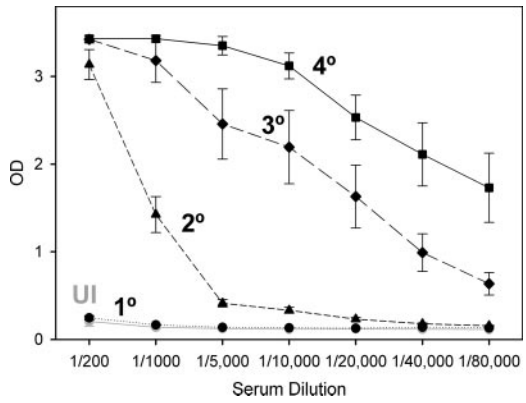

FIGURE 3. Repeat injections with MP4:IFA boost IgG1 Ab response. SJL mice were repeatedly injected s.c. with MP4 (75 μg) in IFA as specified in Fig. 2*A* for primary (1°), secondary (2°), tertiary (3°), and quarternary (4°) injections (groups I, V, VI, and VII, respectively). Two weeks after the last injection, the mice were bled, serum was obtained, and the MP4-specific IgG1 Ab titers were measured by ELISA. Sera of eight mice were tested in serial dilutions individually in triplicates; the mean and SEM of the OD for each group is shown for each dilution, compared with sera of two unimmunized control mice (UI). The results shown are representative of two individual experiments per group. The primary response refers to treatment group I of Fig. 2*A*, the secondary to group V, the tertiary to group VI, and the quaternary to group VII.

(statistically not significant). A similar trend was noted for MP4-induced IL-2 production (Fig. 2*C*); however, this assay proved to be more sensitive, detecting increased frequencies of MP4-specific cells already in mice immunized just once (group I, 22/million 2 wk after the primary injection). The frequencies rose after the secondary injection (group V, 112/million; $p = 0.002$ vs primary injection) and were further increased after the tertiary injection (group VI, 251/million; $p = 0.002$ vs secondary injection). A decline of IL-2-producing MP4-specific cells was seen in mice injected four times (group VII, 92/million; $p < 0.001$ vs tertiary injection). MP4-specific IL-4-producing cells showed a similar pattern (Fig. 3*D*); however, their frequencies were considerably lower, with 16/million after the primary injection (group I), 15/million for the secondary injection (group V), 48/million for the tertiarily injected mice (group VI), and 32/million for the group injected four times (group VII). Although the overall changes seen were moderate, the difference between the secondary and tertiary injection reached statistical significance ($p = 0.002$). The IL-5-producing cells also reproduced this overall pattern (Fig. 2*E*), their frequencies reaching intermediate numbers between IL-2 and IL-4 producers (the maximal frequency of MP4-induced IL-5-producing cells was 129/million as shown for group VI). The increase from primary to secondary injection was highly significant ($p < 0.001$), the additional increase from secondarily to tertiarily injected mice was still significant ($p = 0.039$). In contrast to the other cytokines, however, the numbers of IL-5 producers did not significantly decrease after the fourth injection (group VII, 117/million). Low frequency IFN-γ recall responses became detectable in secondarily injected mice (group V in Fig. 2*F*, 27/million; $p = 0.015$ vs primary injection); these increased to 75/million after the tertiary injection ($p = 0.041$ vs secondary injection), and declined to undetectable levels after the fourth injection ($p < 0.001$ vs tertiary injection). Overall, the frequency measurements of cytokine-producing MP4-specific T cells showed that a type 0/2 response was induced whose magnitude peaked after three immunizations; after the fourth injection, the T cell response became entirely type 2 polarized with a prevalence of IL-5 over IL-4 producers. In addition, the serum levels of MP4-specific IgG1 Abs increased with each booster injection as measured by ELISA (Fig. 3). Although MP4-specific IgG1 was detected from the second injection on, MP-4-induced IL-4 could be measured only after the third injection. Apparently, the frequency of the IL-4-producing MP4-specific T cells was still below the detection limit of the ELISPOT assay as performed with a sample size of 1 million spleen cells per well, and only after the third booster injection did the numbers of the IL-4 producers rise to a detectable level.

Repeat MP4:IFA injections result in increased EAE protection

Although repeat MP4:IFA injections boost type 2 T cell and Ab responses to MP4, it is unclear whether or not such repeatedly injected mice are better protected from MP4-induced EAE than mice receiving a single MP4:IFA injection. If indeed the protection is better, the question is raised whether the increased effect results from the increased net dose of Ag deposited, or from renewed immune exposures. Because the half-life of Ag in IFA is ~90 days (18), Ag persistence alone could not explain any differences observed.

Groups of 10 mice were injected with MP4:IFA one to four times at intervals of 2 wk (corresponding to groups I and V-VII in Fig. 2*A*). As described before, these mice were inoculated with 75 μg of MP4 per dose. Another experimental group (six mice) received a single injection of the 4-fold dose, that is, 300 μg of MP4 in IFA. Control mice (also 10 per group) were injected once or four times with HEL:IFA. Two weeks after the last injection of the four times pretreated group, all mice received the disease-inducing MP4:CFA injection with the additional PTX challenge. The results of this experiment are shown in Fig. 4: The four times HEL:IFA-injected mice developed severe EAE (the disease course for mice injected once with HEL:IFA was similar; data not shown). Single or double injections of 75 μg of MP4:IFA or the single inoculation of 300 μg of MP4 in IFA led to comparable results: these pretreatments did not have a detectable effect on the time point and severity of disease onset. However, all of these treatments ameliorated the subsequent disease to a similar extent. In contrast, the four times MP4:IFA-preinjected mice showed a marked reduction of disease severity at onset (maximum score of 0.8 vs 3.0), and by day 22, 8 of the 10 mice had completely recovered, the ninth had a score of 1, and the tenth a score of 0.5. The disease level of the group that received three MP4:IFA injections resulted in intermediate scores between the twice and four times injected groups. The enhanced protection seen in four times vs once MP4:IFA-injected mice was reproduced in an independent experiment using 10 mice per group (the data for this repeat experiment are not shown). Statistical analysis of mice in both experiments showed highly significant differences between once and four times injected mice on day 14 ($p = 0.001$), and significant differences on day 21 ($p = 0.048$). These results show that repeat injections not only boost type 2 immunity, but also increase the therapeutic efficacy of immune deviation by MP4:IFA treatment.

Anaphylactic side reactions do not occur after reinjection in IFA or of soluble MP4, but after reinjection of soluble PLP peptide

For the above experiments, 38 mice were injected four times with MP4:IFA, an additional 24 mice received three injections of MP4:IFA, and another 24 mice received two of these injections. Although such mice developed high titers of anti-MP4 Abs of the IgG1 class (Fig. 3) and these Abs excel in mediating anaphylactic reactions (39), none of the mice showed even mild symptoms of immediate hypersensitivity (piloerection, prostration, erythema, dyspnea, shallow breathing, or any other symptoms suggestive of

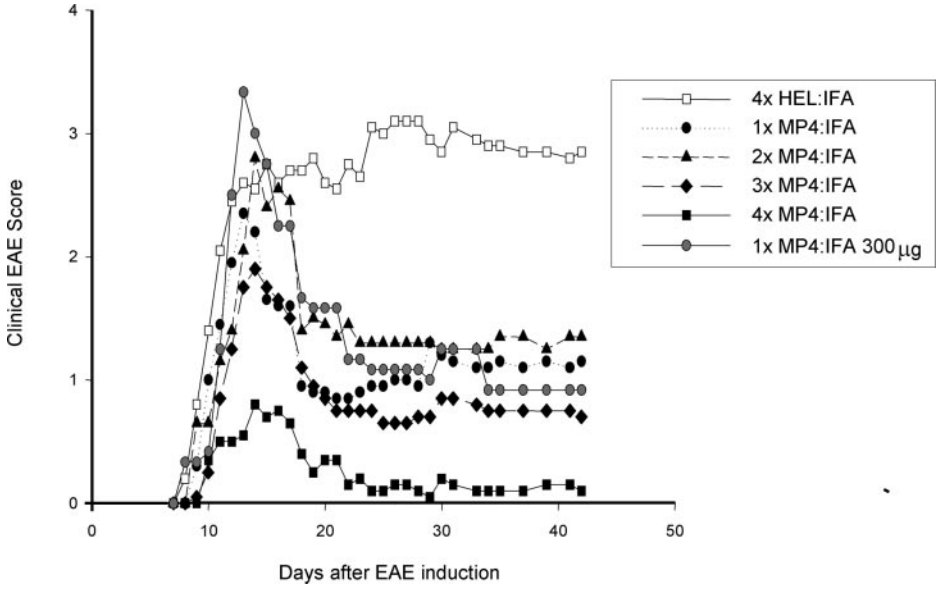

FIGURE 4. Increase of EAE protection in multiple MP4:IFA-preinjected mice. SJL mice were injected with 75 μg of MP4 in IFA, s.c., one to four times in 2-wk intervals, or a single time with a dose of 300 μg of MP4:IFA s.c., as specified. The control group received four s.c. injections with HEL: IFA. Two weeks after the last injection of the four times injected group, the mice were immunized for EAE induction with 75 μg of MP4 in CFA, with PTX as specified in *Materials and Methods*. The mice were monitored daily for clinical signs of EAE and assigned a score according to the standard scale. The mean disease score for each group is shown ($n = 10$ mice for all groups, except $n = 6$ for the 300 μg of MP4-injected group). The data shown are from one experiment representative of two performed.

side reactions). Presumably, the retention and slow release of the Ag from the adjuvant helps to prevent allergic reactions in this regimen. Because allergic side reactions have been reported during immune deviation therapies, we set out to systematically establish the conditions that favor their occurrence (Table I).

We induced type 2 immunity by injecting MP4:IFA up to three times and challenged such mice with MP4 in IFA or soluble MP4 s.c. (The number of MP4:IFA injections therefore added up to 4×, the same numbers as for the disease test.) No allergic reactions were observed in any of the mice, neither during the first 90 min after injection, nor when rescored after 3 and after 24 h; under these conditions, MP4 is first drained through the lymphatics before it can reach the blood circulation, and due to its high m.w., it may be trapped in the lymphoid tissues. Because allergic side reactions were observed after injection of soluble low m.w. peptides, we repeated these experiments with PLP:139–151. SJL mice were sensitized by a single injection of this peptide in IFA, followed by a s.c. challenge with 100 μg of this peptide in PBS. All mice (10 of 10) developed symptoms of immediate hypersensitivity, and 5 of the 10 mice died of anaphylactic shock within 62 min. In contrast, up to four injections of 100 μg of the peptide in IFA injected every second week were tolerated without any detectable allergic side reactions. The retention of the peptide in the adjuvant seemed to limit its diffusion into the blood circulation, avoiding systemic Ag concentrations high enough to cause generalized mast cell degranulation and the development of an anaphylactic shock.

Table I. *Allergic side reactions*[a]

Pretreatment[b]	Challenge[c]	Anaphylactic Side Reactions[d]	
		Total	Lethal
MP4:IFA s.c.			
1×	MP4:IFA s.c.	0/34	0/34
2×	MP4:IFA s.c.	0/34	0/34
3×	MP4:IFA s.c.	0/48	0/48
1×	MP4 sol s.c.	0/10	0/10
2×	MP4 sol s.c.	0/10	0/10
3×	MP4 sol s.c.	0/10	0/10
PLPp:IFA s.c.			
1×	PLPp:IFA s.c.	0/10	0/10
2×	PLPp:IFA s.c.	0/10	0/10
3×	PLPp:IFA s.c.	0/10	0/10
1×	PLPp sol s.c.[e]	10/10	5/10

[a] Anaphylactic reactions do not occur when MP4 or PLP:139–151 is reinjected in IFA, but occur after reinjection of PLP:139–151 in PBS.

[b] SJL mice were injected with 75 μg of MP4 in IFA, or PLP:139–151 in IFA, s.c., one to three times in 2-wk intervals, as specified.

[c] Four weeks after the last injection, the mice were challenged with the Ag (75 μg of MP4, or 100 μg of PLP:139–151, in IFA or in PBS, s.c., as specified).

[d] The development of anaphylactic symptoms was closely monitored during the first 90 min. The numbers of mice developing lethal and nonlethal reactions are shown under Total; mice developing a lethal anaphylactic shock are specified separately.

[e] PLPp sol, soluble PLP:139–151 peptide.

Discussion

To avoid a generalized immune compromised state, ideal immune therapies for autoimmune diseases specifically target autoantigen-specific T cell populations. Treatments either with the autoantigens themselves, or with modified T cell ligands, are prime candidates for accomplishing this goal. Such treatments can inactivate the autoreactive T cells, inducing Ag-specific "true tolerance." Alternatively, treatments with autoantigens can deviate the autoreactive T cells to a nonpathogenic class. Treatments with autoantigens or with their altered peptides frequently result in such immune deviation that entails a type 2 component. The therapeutic benefit of such immune deviation is that, in addition to the functional depletion of the effector cells, the "deviated" T cells frequently secrete cytokines in the target organ that inhibit Th1-like effector T cells of other Ag specificities in a site (organ)-specific manner. Therefore, benefits of immune deviation are that the autoantigen targeted by the endogenously primed effector cells does not have to be known, and that this strategy is promising in cases where the autoimmune response has undergone determinant spreading, now targeting a wide array of second wave autoantigens (37, 40).

Because the Ag-specific T cells induced by autoantigen treatment typically occur at very low frequencies, it has been challenging to delineate mechanisms by which these treatments lead to protection. Injections of autoantigens in IFA, typically i.p. in neonatal mice, have been a classical means of preventing autoimmune disease. Intraperitoneal injections with IFA at adult age are also protective—both operating by the engagement of type 2 immunity (19, 22, 41). In EAE models, i.p. injections are more protective than s.c. injections, and administrations with IFA are more effective than injections of soluble Ag (9), most likely because the Ag

is retained in the oil emulsion for hundreds of days while it is cleared from the circulation within minutes when injected in a soluble form (18). Although highly effective in mouse models, i.p. Ag injections with IFA are not a conceivable treatment for humans; however, s.c. injections with mineral oil (or with related lipids) are approved for clinical studies. Our previous studies have suggested that the protective effect of autoantigen:IFA injections in autoimmune models is Ag specific and T cell mediated: this treatment also protects B cell knock out mice, and the protection can be adoptively transferred with T cells (22). Unlike the immune-modulating effects of i.p. IFA injections that have been closely studied in mice (19, 21, 22, 42), the effects of s.c. IFA injections—and in particular of repeat injections—are not well characterized. Our first set of data aimed at filling this gap of knowledge.

A single s.c. injection of MP4 in IFA induced IL-2-producing T cells occurring at a relatively low frequency (<100 Ag-specific cells in 1 million spleen cells; Fig. 2*C*). Most of these IL-2-producing T cells did not produce IL-4 or IFN-γ; these T cells therefore qualify as uncommitted Thpp cells (43, 44). IL-5 producers, which are prevalent after a single i.p. Ag:IFA injection (21, 22) and which also become prominent after repeated s.c. injections (Fig. 2), were present in low but clearly detectable frequencies, at 27 per million spleen cells in primarily injected mice. The response induced by s.c. injection had delayed kinetics compared with i.p. immunization: whereas the latter triggers peak numbers of Ag-specific memory cells by day 10 in the spleen (21), it took 4 wk for the primary response to reach peak levels after s.c. immunization. Unlike primary i.p. injections with IFA that trigger high titers of IgG1 and IgE (but no IgG2a) Abs (21), the primary s.c. injections of MP4 did not induce detectable IgG1 levels.

Repeat s.c. MP4 injections with IFA resulted in increasing titers of specific IgG1 Abs. Interestingly, the frequency of the Ag-specific T cells was also raised. This outcome has not yet been documented and might seem surprising because the half-life of Ag in IFA is 90 days, thus, the reinjection of Ag every 14 days does not add to the continuity of Ag presence. Although repeat injections increase the net amount of Ag deposited, the booster effect cannot be explained by the Ag dose alone as a single s.c. injection of 300 μg of MP4 in IFA induced similar frequencies of MP4-specific T cells to the single injection of 75 μg of MP4 in IFA (data not shown). Rather than mere Ag presence or the increased Ag dose, we speculate that the booster effect of repeat injections results from the induction of new waves of dendritic cells migrating from the new sites of Ag deposition (45, 46). Secondary and tertiary injections increased the frequencies of IL-2 and to a lesser extent of IL-4, IL-5, and IFN-γ-producing cells, that is to say, the T cell response maintained an unpolarized cytokine expression profile. By the fourth injection, however, an almost complete type 2 polarization was accomplished: the numbers of uncommitted, IL-2-producing Thpp cells decreased and IFN-γ-producing T cells were no longer detectable, whereas IL-5 producers prevailed. The IL-5 producers outnumbered the IL-4 producers, consistent with the fact that these cytokines are frequently not coexpressed by T cells (47, 48). This dissociation of "Th2" cytokines is not surprising because IL-4 and IL-5 expression by T cells underlies independent instructed differentiation and different gene regulation pathways (48, 49).

Repeated s.c. MP4:IFA injections therefore induced and boosted a Th2-like MP4-specific immune response. Despite the high numbers of autoreactive type 2 T cells present and the high titers of autoantibodies induced, these repeatedly MP4:IFA-injected mice did not develop any symptoms of neurological or other disease. This result is not self-evident, because MP4 contains extracellular domains of PLP that these Abs can access, resulting in massive deposits on the surface of Schwann cells, but not causing detectable immune pathology (22). To the contrary, such mice were profoundly protected from MP4-induced EAE (Fig. 4). Importantly, mice injected repeatedly with MP4:IFA showed a more profound protection. Once again, the number of injections rather than the Ag dose alone defined the extent of protection; mice that were injected once with 300 or 75 μg of MP4 were similarly protected, whereas the injection of 300 μg of MP4 in four doses of 75 μg each resulted in a much more distinct effect.

Studying the impact of repeated Ag injections in IFA on the Ag-specific T and B cell response, as well as on disease protection, provides insights into the protective mechanism. Our data show that for the first three injections, boosting of type 2 immunity and of EAE protection go in parallel. After the fourth injection, when the protective effect was the most pronounced, a marked decrease of IL-2 producers was observed, whereas the number of type 2 T cells was largely unaffected (in fact, slightly decreased). These IL-2 producers are thought to be memory cells that are yet uncommitted to type 1/type 2 differentiation (43, 44). It appears that these uncommitted cells are increasingly converted into committed type 2 cells by the repeat injections. Because type 2 cells are not capable of autocrine proliferation, the proliferative recall response (Fig. 2) and the overall clonal sizes of the Ag-specific T cells seem to decline with the subsequent Ag:IFA injection. By the fourth MP4:IFA treatment, the Ag-specific T cell pool was highly type 2 polarized, with IFN-γ producers no longer detectable. Therefore, we can conclude that the extent of EAE protection paralleled the extent of type 2 polarization, that is to say, immune deviation was induced as opposed to clonal anergy or deletion.

The exact mechanism by which immune deviation leads to protection remains unknown. Several possibilities can be envisioned: First, the conversion of naive T cells and of Thpp cells (that are uncommitted with respect to type 1/2 differentiation) into committed type 2 cells exhausts the pool of precursor cells from which type 1 effector cells can be generated. Second, because type 2 differentiation is under positive cytokine feedback regulation, preexisting autoantigen-specific type 2 T cells will cause a type 2 bias in subsequent T cell responses; that is, they will cause type 2 determinant spreading (23). Third and last, cytokines secreted by such type 2 cells can create a microenvironment in the target organ that is suppressive to proinflammatory type 1 cells. IL-10 and TGF-β are among the prime cytokine candidates mediating such an effect. It is unclear whether "regulatory" T cells producing such cytokines coexpress IL-4 or IL-5 (that is, are the same cells that we have measured) or whether they are independent lineages engaged in parallel. We did not conduct single-cell resolution measurements of MP4-specific IL-10 and TGF-β-producing T cells because both cytokines are not readily amenable to ELISPOT T cell analysis: the former are masked by activation of cells of the innate immune system (50), whereas the latter are obscured by the present inability to distinguish between the active and passive form of the molecule (51). The frequencies of the cytokine-producing Ag-specific cells in most measurements was <100/million (<0.01%), that is, below the detection limit of intracytoplasmic cytokine staining by flow cytometry, and hence the detection of such rare cells was dependent on the high sensitivity of ELISPOT measurements.

Repeat injections with the Ag were therefore critical to augment the therapeutic effect, and these were inherently linked to the boosting of cellular and humoral type 2 immunity. Reinjection of Ag in a sensitized host entails the risk of allergic side reactions, and in the case of type 2 immunity, of hypersensitivity of the immediate type. Such anaphylactic reactions were seen in mice and humans after immune therapy with peptides (33–36). These reactions are mediated by IgE and IgG1 Abs that cause systemic

mast cell degranulation either by the binding of Ag to the sensitized mast cells, or indirectly, by systemic complement activation, whereby activated complement components (C3a, C4a, C5a) cause the mast cell degranulation. In both cases, for systemic anaphylaxis to occur, the Ag that elicits the reaction needs to disseminate in the body via the blood stream; failure to do so results in only localized mast cell degranulation. When MP4 or PLP:139–151 was injected repeatedly with IFA, even mild anaphylactic reactions did not occur (Table I). This result suggests that systemic Ag concentrations did not reach sufficient levels to elicit generalized mast cell degranulation, most likely due to the retention and gradual release of the Ag from the IFA depot. This Ag retention in the adjuvant favors immunogenicity, thereby enhancing the therapeutic effect while avoiding the life-threatening allergic side reactions. In contrast, half of the mice died of anaphylaxis, and the other half displayed severe symptoms of immediate hypersensitivity when soluble PLP:139–151 peptide was injected in mice that were sensitized with only one PLP:139–151 injection with IFA (Table I). Interestingly, s.c. injection of the soluble MP4 in even four times MP4:IFA-injected mice did not result in anaphylaxis despite the high titers of MP4-specific IgG1 Abs present in their blood. Unlike the 13-aa-long PLP peptide, MP4 is almost 400 aas long and compromises a multitude of B cell determinants. Subsequently, when MP4 is injected in a sensitized host, the immune complexes generated are of considerably greater size than PLP peptide complexes. The larger the immune complexes are, the more efficiently the reticuloendothelial system clears them. Therefore, it is conceivable that after s.c. injection, MP4 is filtered from the lymph as it passes the drLNs (resulting in the stimulation of T and B cell responses) and is prevented from entering into the blood stream (and thereby causing anaphylactic side reactions).

In summary, our data show that s.c. injections with the neuroantigen MP4 in IFA result in a type 2 T cell response that is boosted and polarized by repeat injections. The protective effect of the treatment paralleled the T cell boost. As a treatment-associated side effect, the repeat injections also induced high titers of Abs, but anaphylactic reactions did not occur with reinjection of the Ag when it was retained in adjuvant or when the Ag was a larger protein. Therefore, the therapeutic benefit of inducing at type 2 T cell response could be dissociated from the Ab-mediated complications of the treatment. These findings established in a murine model may have important implications for immune therapy of humans. Although immune therapy in mice is well established and initial clinical trials were promising in humans, Ab-mediated complications have impeded progress in their translation to the bedside. The ability to dissociate therapeutic effects from side effects might revive efforts around using autoantigens or altered peptide ligands for the therapy of human autoimmune diseases.

Disclosures

S. Faas is employed by Alexion Pharmaceuticals, which manufactured and supplied MP4.

References

1. Liblau, 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.
2. Shive, C., H. H. Hofstetter, L. Arredondo, C. Shaw, and T. G. Forsthuber. 2000. The enhanced antigen-specific production of cytokines induced by pertussis toxin is due to clonal expansion of T cells and not to altered effector functions of long-term memory cells. *Eur. J. Immunol.* 30: 2422–2431.
3. Agarwal, R. K., S. H. Sun, S. B. Su, C. C. Chan, and R. R. Caspi. 2002. Pertussis toxin alters the innate and the adaptive immune responses in a pertussis-dependent model of autoimmunity. *J. Neuroimmunol.* 129: 133–140.
4. Linthicum, D. S. 1982. Development of acute autoimmune encephalomyelitis in mice: factors regulating the effector phase of the disease. *Immunobiology* 162: 211–220.
5. Darabi, K., A. Y. Karulin, B. O. Boehm, H. H. Hofstetter, Z. Fabry, J. C. LaManna, J. C. Chavez, M. Tary-Lehmann, and P. V. Lehmann. 2004. The third signal in T cell-mediated autoimmune disease? *J. Immunol.* 173: 92–99.
6. Wakatsuki, A., P. Borrow, K. Rigley, and P. C. Beverley. 2003. Cell-surface bound pertussis toxin induces polyclonal T cell responses with high levels of interferon-γ in the absence of interleukin-12. *Eur. J. Immunol.* 33: 1859–1868.
7. Rauch, H. C., E. R. Einstein, J. Csejtey, and W. J. Davis. 1968. Protective action of the encephalitogen and other basic proteins in experimental allergic encephalomyelitis. *Immunochemistry* 5: 567–575.
8. Alvord, E., C. S. Hruby, K. Hughes, C. M. Shaw, and M. W. Kies. 1969. Comparisons of organ-specific antigens of the central nervous system. *Int. Arch. Allergy Appl. Immunol.* 36(Suppl.): 276–292.
9. Swierkosz, J. E., and R. H. Swanborg. 1977. Immunoregulation of experimental allergic encephalomyelitis: conditions for induction of suppressor cells and analysis of mechanism. *J. Immunol.* 119: 1501–1506.
10. Higgins, P. J., and H. L. Weiner. 1988. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments. *J. Immunol.* 140: 440–445.
11. Bitar, D. M., and C. C. Whitacre. 1988. Suppression of experimental autoimmune encephalomyelitis by the oral administration of myelin basic protein. *Cell. Immunol.* 112: 364–370.
12. Metzler, B., and D. C. Wraith. 1993. 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.
13. Bai, X. F., F. D. Shi, B. G. Xiao, H. L. Li, P. H. van der Meide, and H. Link. 1997. Nasal administration of myelin basic protein prevents relapsing experimental autoimmune encephalomyelitis in DA rats by activating regulatory cells expressing IL-4 and TGF-β mRNA. *J. Neuroimmunol.* 80: 65–75.
14. Miller, A., Z. J. Zhang, R. A. Sobel, A. al Sabbagh, and H. L. Weiner. 1993. Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein, VI: suppression of adoptively transferred disease and differential effects of oral vs. intravenous tolerization. *J. Neuroimmunol.* 46: 73–82.
15. Elliott, E. A., H. I. McFarland, S. H. Nye, R. Cofiell, T. M. Wilson, J. A. Wilkins, S. P. Squinto, L. A. Matis, and J. P. Mueller. 1996. Treatment of experimental encephalomyelitis with a novel chimeric fusion protein of myelin basic protein and proteolipid protein. *J. Clin. Invest.* 98: 1602–1612.
16. Rocken, M., and E. M. Shevach. 1996. Immune deviation: the third dimension of nondeletional T cell tolerance. *Immunol. Rev.* 149: 175–194.
17. Cobbold, S., and H. Waldmann. 1998. Infectious tolerance. *Curr. Opin. Immunol.* 10: 518–524.
18. Herbert, W. J. 1968. The mode of action of mineral-oil emulsion adjuvants on antibody production in mice. *Immunology* 14: 301–318.
19. Forsthuber, T., H. C. Yip, and P. V. Lehmann. 1996. Induction of TH1 and TH2 immunity in neonatal mice. *Science* 271: 1728–1730.
20. Lehmann, P. V., D. Matesic, G. Benichou, and P. S. Heeger. 1997. Induction of T helper 2 immunity to an immunodominant allopeptide. *Transplantation* 64: 292–296.
21. Yip, H. C., A. Y. Karulin, M. Tary-Lehmann, M. D. Hesse, H. Radeke, P. S. Heeger, R. P. Trezza, F. P. Heinzel, T. Forsthuber, and P. V. Lehmann. 1999. Adjuvant-guided type-1 and type-2 immunity: infectious/noninfectious dichotomy defines the class of response. *J. Immunol.* 162: 3942–3949.
22. Heeger, P. S., T. Forsthuber, C. Shive, E. Biekert, C. Genain, H. H. Hofstetter, A. Karulin, and P. V. Lehmann. 2000. Revisiting tolerance induced by autoantigen in incomplete Freund's adjuvant. *J. Immunol.* 164: 5771–5781.
23. Tian, J., P. V. Lehmann, and D. L. Kaufman. 1997. Determinant spreading of T helper cell 2 (Th2) responses to pancreatic islet autoantigens. *J. Exp. Med.* 186: 2039–2043.
24. Khoruts, A., S. D. Miller, and M. K. Jenkins. 1995. Neuroantigen-specific Th2 cells are inefficient suppressors of experimental autoimmune encephalomyelitis induced by effector Th1 cells. *J. Immunol.* 155: 5011–5017.
25. Genain, C. P., K. Abel, N. Belmar, F. Villinger, D. P. Rosenberg, C. Linington, C. S. Raine, and S. L. Hauser. 1996. Late complications of immune deviation therapy in a nonhuman primate. *Science* 274: 2054–2057.
26. Lafaille, J. J., F. V. Keere, A. L. Hsu, J. L. Baron, W. Haas, C. S. Raine, and S. Tonegawa. 1997. Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J. Exp. Med.* 186: 307–312.
27. Pakala, S. V., M. O. Kurrer, and J. D. Katz. 1997. T helper 2 (Th2) T cells induce acute pancreatitis and diabetes in immune-compromised nonobese diabetic (NOD) mice. *J. Exp. Med.* 186: 299–306.
28. Dietsch, G. N., and D. J. Hinrichs. 1989. The role of mast cells in the elicitation of experimental allergic encephalomyelitis. *J. Immunol.* 142: 1476–1481.
29. Brenner, T., D. Soffer, M. Shalit, and F. Levi-Schaffer. 1994. Mast cells in experimental allergic encephalomyelitis: characterization, distribution in the CNS and in vitro activation by myelin basic protein and neuropeptides. *J. Neurol. Sci.* 122: 210–213.
30. Secor, V. H., W. E. Secor, C. A. Gutekunst, and M. A. Brown. 2000. Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis. *J. Exp. Med.* 191: 813–822.
31. Gladue, R. P., L. A. Carroll, A. J. Milici, D. N. Scampoli, H. A. Stukenbrok, E. R. Pettipher, E. D. Salter, L. Contillo, and H. J. Showell. 1996. Inhibition of leukotriene B4-receptor interaction suppresses eosinophil infiltration and disease pathology in a murine model of experimental allergic encephalomyelitis. *J. Exp. Med.* 183: 1893–1898.
32. Pedotti, R., J. J. DeVoss, S. Youssef, D. Mitchell, J. Wedemeyer, R. Madanat, H. Garren, P. Fontoura, M. Tsai, S. J. Galli, et al. 2003. Multiple elements of the

allergic arm of the immune response modulate autoimmune demyelination. *Proc. Natl. Acad. Sci. USA* 100: 1867–1872.
33. Kappos, L., G. Comi, H. Panitch, J. Oger, J. Antel, P. Conlon, and L. Steinman. 2000. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial: The Altered Peptide Ligand in Relapsing MS Study Group. *Nat. Med.* 6: 1176–1182.
34. Pedotti, R., D. Mitchell, J. Wedemeyer, M. Karpuj, D. Chabas, E. M. Hattab, M. Tsai, S. J. Galli, and L. Steinman. 2001. An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat. Immunol.* 2: 216–222.
35. Liu, E., H. Moriyama, N. Abiru, D. Miao, L. Yu, R. M. Taylor, F. D. Finkelman, and G. S. Eisenbarth. 2002. Anti-peptide autoantibodies and fatal anaphylaxis in NOD mice in response to insulin self-peptides B:9–23 and B:13–23. *J. Clin. Invest.* 110: 1021–1027.
36. Pedotti, R., M. Sanna, M. Tsai, J. DeVoss, L. Steinman, H. McDevitt, and S. J. Galli. 2003. Severe anaphylactic reactions to glutamic acid decarboxylase (GAD) self peptides in NOD mice that spontaneously develop autoimmune type 1 diabetes mellitus. *BMC Immunol.* 4: 2.
37. 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.
38. Jordan, E. K., H. I. McFarland, B. K. Lewis, N. Tresser, M. A. Gates, M. Johnson, M. Lenardo, L. A. Matis, H. F. McFarland, and J. A. Frank. 1999. Serial MR imaging of experimental autoimmune encephalomyelitis induced by human white matter or by chimeric myelin-basic and proteolipid protein in the common marmoset. *AJNR* 20: 965–976.
39. Miyajima, I., D. Dombrowicz, T. R. Martin, J. V. Ravetch, J. P. Kinet, and S. J. Galli. 1997. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and FcγRIII: assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *J. Clin. Invest.* 99: 901–914.
40. Lehmann, P. V., E. E. Sercarz, T. Forsthuber, C. M. Dayan, and G. Gammon. 1993. Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol. Today* 14: 203–208.
41. Tian, J., M. Clare-Salzler, A. Herschenfeld, B. Middleton, D. Newman, R. Mueller, S. Arita, C. Evans, M. A. Atkinson, Y. Mullen, et al. 1996. Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat. Med.* 2: 1348–1353.
42. Marusic, S., and S. Tonegawa. 1997. Tolerance induction and autoimmune encephalomyelitis amelioration after administration of myelin basic protein-derived peptide. *J. Exp. Med.* 186: 507–515.
43. Yang, L., and T. Mosmann. 2004. Synthesis of several chemokines but few cytokines by primed uncommitted precursor CD4 T cells suggests that these cells recruit other immune cells without exerting direct effector functions. *Eur. J. Immunol.* 34: 1617–1626.
44. Yang, L., J. J. Kobie, and T. R. Mosmann. 2005. CD73 and Ly-6A/E distinguish in vivo primed but uncommitted mouse CD4 T cells from type 1 or type 2 effector cells. *J. Immunol.* 175: 6458–6464.
45. Figdor, C. G., I. J. de Vries, W. J. Lesterhuis, and C. J. Melief. 2004. Dendritic cell immunotherapy: mapping the way. *Nat. Med.* 10: 475–480.
46. Randolph, G. J., V. Angeli, and M. A. Swartz. 2005. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat. Rev. Immunol.* 5: 617–628.
47. Bucy, R. P., L. Karr, G. Q. Huang, J. Li, D. Carter, K. Honjo, J. A. Lemons, K. M. Murphy, and C. T. Weaver. 1995. Single cell analysis of cytokine gene coexpression during $CD4^+$ T-cell phenotype development. *Proc. Natl. Acad. Sci. USA* 92: 7565–7569.
48. Karulin, A. Y., M. D. Hesse, M. Tary-Lehmann, and P. V. Lehmann. 2000. Single-cytokine-producing CD4 memory cells predominate in type 1 and type 2 immunity. *J. Immunol.* 164: 1862–1872.
49. Okano, M., A. R. Satoskar, M. Abe, D. A. Harn, Jr., M. Okano, K. Nishizaki, Y. Takeda, T. Yoshino, F. Brombacher, and A. A. Satoskar. 2000. Interleukin-4-independent production of Th2 cytokines by nasal lymphocytes and nasal eosinophilia in murine allergic rhinitis. *Allergy* 55: 723–731.
50. Guerkov, R. E., O. S. Targoni, C. R. Kreher, B. O. Boehm, M. T. Herrera, M. Tary-Lehmann, P. V. Lehmann, and S. K. Schwander. 2003. Detection of low-frequency antigen-specific IL-10-producing $CD4^+$ T cells via ELISPOT in PBMC: cognate vs. nonspecific production of the cytokine. *J. Immunol. Methods* 279: 111–121.
51. Aung, H., J. Sherman, M. Tary-Lehman, and Z. Toossi. 2002. Analysis of transforming growth factor-β1 (TGF-β1) expression in human monocytes infected with *Mycobacterium avium* at a single cell level by ELISPOT assay. *J. Immunol. Methods* 259: 25–32.