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

Felix S. Lichtenegger,*† Stefanie Kuerten,* Susan Faas, † Bernhard O. Boehm, †
Magdalena Tary-Lehmann,* and Paul V. Lehmann2*

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.

Immune deviations, such as the induction of type 2 immune responses, can occur when the immune system is exposed to autoantigens. These responses are associated with Th2-type cytokines and IgG1 Abs, which can protect against autoimmune diseases. However, they can also lead to allergic reactions. We studied the therapeutic benefit and risk of immune deviation in experimental allergic encephalomyelitis (EAE) induced in SJL mice using MP4, a fusion protein of myelin basic protein and proteolipid protein. MP4 was administered in Freund’s complete adjuvant (CFA) or incomplete adjuvant (IFA), leading to type 2 T cell immunity, IgG1 Abs, and EAE protection. Repeated injections enhanced both, but the risk of anaphylaxis was limited to IFA because it contains thioglycollate, which retains Ags. In contrast, s.c. injection of soluble PLP:139–151 peptide led to lethal anaphylaxis. This suggests that anaphylaxis is more likely when large Ags are retained in an adjuvant long enough to elicit an allergic response. Therefore, the risk of anaphylaxis can be mitigated by using smaller Ags that are more readily disseminated, to design therapeutic protocols that boost protective type 2 T cell responses while minimizing allergic side effects.
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 FcγRII 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 were performed in accordance with the institutional guidelines. MP4 (Apopgen) 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 (EMSScience). 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 a specified vehicle. 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. RA-66A2 (DB Pharmeding) 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 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.

Statistics

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, 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 dLN 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, dLN 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.
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 1-IV in Fig. 2B). 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 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. 2A (groups I and V-VII). Spleen cells from 14 mice per group were tested for the MP4-induced proliferative response by [3H]thymidine incorporation (Fig. 2B) 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.
entirely type 2 polarized with a prevalence of IL-5 over IL-4 pro-
zations; after the fourth injection, the T cell response became
tertiary injection). Overall, the frequency measurements of cyto-
p
to undetectable levels after the fourth injection (group VII). 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 representa-
tive of two individual experiments per group. The primary response refers
to treatment group I of Fig. 2A, the secondary to group V, the tertiary to
group VI, and the quaternary to group VII.

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 spec-
ified in Fig. 2A for primary (1°), secondary (2°), tertiary (3°), and quarter-
ary (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 titer was 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 representa-
tive of two individual experiments per group. The primary response refers
to treatment group I of Fig. 2A, the secondary to group V, the tertiary to
group VI, and the quaternary to group VII.

A similar trend was noted for MP4-induced IL-2 production (Fig. 2C); 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 sec-
ondary 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 de-
cline of IL-2-producing MP4-specific cells was seen in mice in-
jected four times (group VII, 92/million; p < 0.001 vs tertiary injection).
MP4-specific IL-4-producing cells showed a similar pattern (Fig. 3D); 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 tertiar-
ily 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. 2E), their frequencies reaching intermediate numbers between IL-2 and IL-4 producers (the maximal frequency of MP4-induced IL-5-produ-
cing cells was 129/million as shown for group V). The increase
from primary to secondary injection was highly significant (p < 0.001), the additional increase from secondary to tertiary in-
jected mice was still significant (p = 0.039). In contrast to the oth-
er 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. 2F, 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 cyto-
kine-producing MP4-specific T cells showed that a type 0/2 re-
sponse was induced whose magnitude peaked after three immu-
nizations; after the fourth injection, the T cell response became
entirely type 2 polarized with a prevalence of IL-5 over IL-4 pro-
ducers. 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. 2A). As described before, these mice were inoculated with 75
μg of MP4 per dose. Another experimental group (six mice) re-
ceived 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 pre-
treatments did not have a detectable effect on the time point and
severity of disease onset. However, all of these treatments ame-
liorated 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 interme-
diate 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
A type 2 immunity, but also increase the therapeutic efficacy of im-
mune 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. Al-
though 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

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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.

### 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 inactive 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 knockout 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. 2C). 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-induced T 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 reactivation 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 naïve 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, pre-existing 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. Rejection 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
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