Treatment of multiple sclerosis with T-cell receptor peptides: Results of a double-blind pilot trial

ARTHUR A. VANDENBARK[1–3], YUAN K. CHOU[2], RUTH WHITHAM[1,2], MICHELE MASS[2], ABIGAIL BUENAFE[2], DIANE LIEFELD[2], DANIEL KAVANAGH[1], SHELLEY COOPER[2], GEORGE A. HASHIM[4], HALINA OFFNER[1,2] & DENNIS N. BOURDETTE[1,2]

[1]*Neuroimmunology Research 151D, Veterans Affairs Medical Center, 3710 SW Veterans Hospital Road, Portland, Oregon 97201, USA*
[2]*Department of Neurology and* [3]*Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97201, USA*
[4]*Council for Tobacco Research, 900 Third Avenue, New York, New York 10022, USA*
Correspondence should be addressed to A.A.V.

A T-cell receptor (TCR) peptide vaccine from the Vβ5.2 sequence expressed in multiple sclerosis (MS) plaques and on myelin basic protein (MBP)-specific T cells boosted peptide-reactive T cells in patients with progressive MS. Vaccine responders had a reduced MBP response and remained clinically stable without side effects during one year of therapy, whereas nonresponders had an increased MBP response and progressed clinically. Peptide-specific T helper 2 cells directly inhibited MBP-specific T helper 1 cells *in vitro* through the release of interleukin-10, implicating a bystander suppression mechanism that holds promise for treatment of MS and other autoimmune diseases.

Network recognition[1] of antigen-specific T-cell receptors (TCRs) represents an important peripheral mechanism for controlling self-reactive T cells that escape the thymus. Regulatory T and B cells are naturally induced within the "immune homunculus"[2] to recognize unique determinants, or idiotopes, thought to be located preferentially within the hypervariable regions of the TCR sequence, including the complementarity-determining regions (CDRs) that interact with the antigen–MHC complex[3–5]. Whereas anti-idiotypic antibodies may recognize conformational determinants on the intact TCRs, anti-idiotypic T cells would necessarily be directed at naturally processed TCR peptides expressed on the T-cell surface in association with self MHC molecules[6]. Identification of such immunogenic TCR idiotopes might allow selective stimulation of the antireceptor network with synthetic peptides that correspond to defined segments of the TCR sequence, potentially unleashing this powerful immunoregulatory mechanism.

Experimental autoimmune encephalomyelitis (EAE) provided a prototypic model to test the hypothesis that TCR sequences could induce anti-idiotypic regulation. In EAE in Lewis rats and PL/J mice, encephalitogenic T cells specific for myelin basic protein (MBP) were highly restricted, expressing similar TCRs that consisted of Vα2 and Vβ8.2 (ref. 7) and that could be targeted for therapy by Vβ8.2-specific monoclonal antibodies. Moreover, vaccination with attenuated encephalitogenic T cells induced cell-mediated protection against EAE (ref. 8), indicating the presence of immunogenic structures on the pathogenic T cells. The common expression of TCR Vβ8.2 by these encephalitogenic effector cells allowed us to predict an immunogenic region within the CDR2, and vaccination of rats with the corresponding Vβ8.2 CDR2 peptide induced anti-idiotypic T cells and antibodies that inhibited the activation of pathogenic T cells and prevented and treated EAE (ref. 9, 10). Similarly, others have demonstrated that TCR peptides from CDR2 or other TCR regions can induce immunoregulation of pathogenic T cells specific for MBP, collagen, heat shock protein, and the P2 protein of peripheral myelin[11–18], implicating this regulatory network in experimental arthritis and neuritis, as well as in EAE.

T-cell receptor peptides might also induce network regulation in human diseases such as multiple sclerosis (MS) that may be mediated in part by oligoclonal T cells directed at tissue-specific autoantigens (for example, myelin basic protein). Indeed, based on the overexpression of TCRs Vβ5.2 and Vβ6.1 by MBP-specific T cells from the periphery and central nervous system plaques of HLA-DR2$^+$ (DRβ1*1501$^+$) multiple sclerosis patients[19–21], we found that intradermal injection of CDR2 peptides from Vβ5.2 and Vβ6.1 boosted the frequency of TCR peptide-specific T cells[22] *in vivo*. However, in this early study, there was no firm indication of whether the TCR peptide vaccination could induce immunoregulation or clinical benefit. The need to demonstrate an immunoregulatory effect in humans is especially important, as treatment might not be effective if V gene expression in response to autoantigens is oligoclonal[19,23,24], rather than essentially monoclonal, as in rodents with EAE. To evaluate immunoregulation *in vivo*, we designed a double-blind, placebo-controlled trial to evaluate clinical and immunological changes after vaccination with Vβ5.2 peptides in patients with progressive MS. Because there conceivably could be natural tolerance to germline self sequences, we compared the native Vβ5.2-38-58 sequence with a Vβ5.2-38-58 peptide in which the tyrosine at position 49 was replaced with threonine (Y49T-Vβ5.2-38-58), which was used in our previous open label study[22].

Study design

In this study, TCR peptide immunogenicity, effects on MBP response, and clinical outcome were evaluated under double-blind

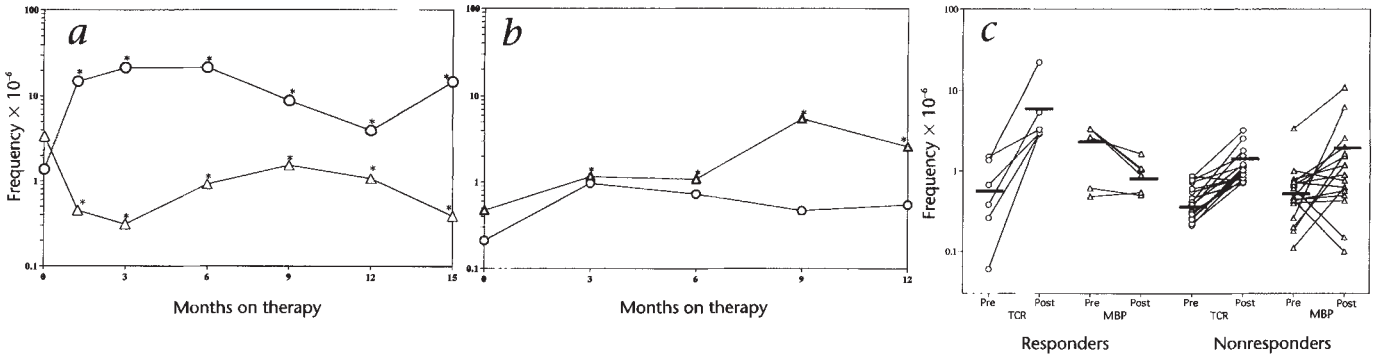

Fig. 1 Profiles of immunologically reactive MS patients. Changes in the frequencies of T cells responsive to Vβ5.2-38-58 peptide (native or Y49T-substituted sequences) and MBP are shown at the times indicated relative to the reference determinations that were obtained just before peptide injection. *a*, Peptide responder L.G.T. had significantly increased (*) T-cell frequencies to peptide injection (○) and concomitant significant decreases (*) in T-cell frequencies to MBP (△), with clinical improvement that continued beyond the 12 months exit evaluation. *b*, Peptide nonresponder K.B.K. had increased T-cell frequencies (*) to MBP (△), but not peptide (○), and clinical progression during the study. *c*, Changes in T-cell frequencies to Vβ5.2-38-58 peptides (TCR) (entry vs. peak responses) and to MBP (entry vs. exit responses) in peptide responders compared with nonresponders. T-cell frequencies were estimated using a limiting dilution assay[47]. Patients were classified as responders or nonresponders as described in Table 1. The arithmetic mean of each group is indicated by a solid horizontal bar.

conditions in 23 HLA-DRβ1*1501⁺ patients with chronic progressive MS (Table 1) who were treated for 12 months with native Vβ5.2-38-58 peptide, a 1:1 mixture of the native peptide and Y49T-substituted Vβ5.2-38-58 peptide, or placebo. Patients with chronic progressive MS were selected from the Oregon Health Sciences University MS clinic. All patients entered into the trial were prescreened and shown to express HLA-DRβ1*1501, as determined by molecular typing. Before entry, each patient was given a neurological examination and was assessed for T-cell frequencies specific for Vβ5.2-38-58 peptide and MBP. Each patient received 100 μg peptide or diluent in 0.1 ml weekly for 4 weeks, and then monthly for an additional 10 months, for a total of 14 injections. Patients were scored, by a neurologist blinded to treatment assignment, for clinical status at entry, 3, 6, 9 and 12 months and were assessed for blood T-cell responses to Vβ5.2 peptides and MBP, quantified by a limiting dilution analysis (LDA), on weeks –2, 0, 4, 5, 8, 12, 24, 36 and 52.

T-cell response to TCR peptide vaccination

Vaccination with Vβ5.2 peptides, but not placebo, induced strong T-cell recognition of the TCR Vβ5.2 sequences, as measured by the LDA. Significant boosting of T-cell responses to Vβ5.2-38-58 peptides occurred only in patients that received peptides (Table 1 and Fig. 1), with a trend toward a higher frequency of responses to the substituted peptide (5 out of 9) as compared with the native peptide (1 out of 8) (Table 2*a*). In responders, vaccination boosted peptide-specific T-cell frequencies from a baseline of <1 cell/million to peak responses of 3–6 cells/million, and in one patient (L.G.T.), to more than 20 cells/million. Responses to peptide were first detected 4–5 weeks after treatment was initiated in four patients who entered the trial with elevated responses to MBP, as is exemplified in Fig. 1*a*. In contrast, responses to peptide were not detected until 12–24 weeks of treatment in two patients (S.L.L. & T.H.R.) who never developed elevated T-cell frequencies to MBP. Taken together, these results suggested that boosting of Vβ5.2-38-58 peptide-specific T cells was more efficient with the substituted Vβ5.2-38-58 peptide and occurred more rapidly in patients with elevated MBP frequencies at entry. In spite of continued vaccination, T-cell frequencies to peptide generally decreased after the initial boosting effect, but still remained significantly elevated in four of the six responders at exit from the trial. It is noteworthy that none of the study patients had reduced cell-mediated immune responses to recall antigens, or abnormal blood cell counts, blood chemistries, or urinalyses, or any other side effects.

Responses to TCR peptide and MBP correlated inversely

Successful TCR peptide treatment of animals with EAE resulted in a decreased frequency of MBP-responsive T cells[25]. Similarly, in four of the six patients that were successfully vaccinated to substituted or native Vβ5.2-38-58 peptide, there was a coordinate and significant decrease in the frequency of MBP-specific T cells that occurred within 5–12 weeks of treatment and that remained significantly lower at exit (Fig. 1, *a* and *c*). In the other two TCR peptide responders, the low MBP responses at entry did not increase at any time during the study or at exit. In contrast, one placebo patient with an elevated entry response to MBP had an even higher MBP response at exit. Responses to MBP tended to increase among patients who did not develop a significant or sustained response to TCR peptide, or who were not vaccinated (Fig. 1, *b* and *c*), with four patients demonstrating significantly increased frequencies of MBP-reactive T cells at exit. Taken together, these data demonstrate a highly significant inverse relationship between response to TCR peptide and response to MBP ($P < 0.001$, Table 2*b*), a strong indication that responses to MBP are influenced by responses to Vβ5.2-38-58 peptide.

No clinical progression in TCR peptide responders

A clinical composite score was used to determine whether patients were improved, stable or worse at exit as compared with entry (Table 1). Owing to the small sample size, there were no statistically significant differences in clinical outcome of TCR peptide responders compared with placebo-treated patients. Conversely, patients who did not respond immunologically to TCR peptide vaccination did not differ clinically from patients who received placebo ($P = 0.16$), alleviating concern that exposure to peptide might produce clinical worsening in nonresponders[26]. However, there was a significant clinical benefit in patients that responded to TCR peptide vaccination (0 of 6 were clinically worse) versus nonresponders receiving either peptide or placebo (10 of 17 were clinically worse) ($P = 0.019$, Table 2*c*).

Table 1 Patient data

Pt	Age /Sex	Duration (yr) /Type[a]	Injection[b]	TCR peptide frequency[c]			MBP frequency[c]			Clinical baseline testing[d]		Clinical outcome[e]
				Entry	Max	Resp	Entry	Exit	Resp	EDSS	TW (s)	
Peptide responders												
M.L.M.	53/F	23/2°	Pep (nat)	0.3	2.9	Mod	3.4	0.9	↓	4	6.2	S
L.G.T.	44/M	12/2°	Pep (sub)	1.4	22.0	Str	3.4	1.1	↓	6.0	7.2	I (9HPT)
A.L.S.	52/F	10/1°	Pep (sub)	1.5	3.2	Mod	3.4	1.0	↓	4.0	6.5	S
K.E.E	52/F	21/2°	Pep (sub)	0.1	3.0	Mod	2.6	1.6	↓	6.5	11.0	S
S.L.L.	56/F	35/2°	Pep (sub)	0.4	5.4	Mod	0.5	0.5	–	5.0	6.3	S
T.H.R.	45/M	15/2°	Pep (sub)	0.7	3.0	Mod	0.6	0.5	–	6.0	7.5	S
Peptide nonresponders												
W.G.A.	58/F	11/1°	Pep (nat)	0.2	0.7	Non	0.2	0.5	↕	6.5	24.0	W (TW, EDSS, AI)
K.B.K.	42/M	17/2°	Pep (nat)	0.2	1.0	Non	0.5	2.6	↑	6.0	6.4	W (TW)
H.H.J.	45/M	13/1°	Pep (nat)	0.4	1.2	Non	0.2	1.7	↑	5.5	7.2	W (9HPT)
Z.L.D.	52/F	6/2°	Pep (nat)	0.4	2.6	Non	0.7	1.5	–	4.0	13.0	W (EDSS)
W.A.J.	57/F	14/1°	Pep (nat)	0.7	1.2	Non	0.4	0.6	–	5.5	8.0	W (TW, AI)
J.K.P.	61/F	18/1°	Pep (nat)	0.5	0.7	Non	0.1	0.6	–	6.0	14.8	S
H.R.C.	52/F	34/2°	Pep (nat)	0.9	0.8	Non	0.6	0.2	–	4.0	5.7	S
B.W.R.	56/M	15/1°	Pep (sub)	0.3	1.0	Non	0.7	2.0	↑	6.0	8.2	W (TW)
R.D.K.	43/F	19/2°	Pep (sub)	0.3	0.9	Non	0.8	1.2	–	5.5	10.2	W (TW)
B.R.S.	42/M	24/2°	Pep (sub)	0.8	1.6	Non	0.4	0.5	–	6.5	15.3	W (TW, 9HPT, AI)
P.A.R.	38/F	17/2°	Pep (sub)	0.6	0.8	Non	1.0	0.8	–	6.5	29.9	S
K.A.S.	50/F	10/2°	Placebo	0.3	1.0	Non	0.3	6.3	↑	6.5	14.0	W (TW)
G.A.B.	59/F	9/1°	Placebo	0.4	1.8	Non	3.4	11.0	↕	5.0	7.2	S
M.O.H.	55/M	4/1°	Placebo	0.8	3.3	Non	0.4	0.2	↕	3.5	6.8	S
F.K.V.	55/M	33/2°	Placebo	0.3	1.1	Non	0.4	0.4	–	6.5	71.2	S
S.J.D.	54/F	19/2°	Placebo	0.3	0.8	Non	0.7	0.6	–	4.5	8.4	W (EDSS)
M.A.N.	48/F	6/2°	Placebo	0.6	0.8	Non	0.7	0.9	–	4.5	6.5	I (EDSS)

[a]Type: 1°, primary progressive; 2°, secondary progressive.

[b]Patients were injected i.d. weekly for 4 weeks and monthly thereafter for 1 year with one of the following: Pep (nat), 100 μg native Vβ5.2-38-58 peptide; Pep (sub), 50 μg native + 50 μg Y49T-Vβ5.2-38-58 peptide; or placebo, lactated Ringer's solution.

[c]T-cell frequencies to TCR peptide or MBP were estimated using chi-square minimization[47] of proliferation carried out at limiting dilution. Pre- and postimmunization frequencies are given as estimated frequencies per million blood mononuclear cells. Patients were designated as responders if two or more frequency determinations to Vβ5.2-38-58 peptides were significantly elevated above the reference frequency, with at least one of these values in excess of 2 cells/million. Responses were termed moderate (Mod) if the highest postimmunization frequency was from 2 to 8 cells/million, and strong (Str), >8 cells/million. Responses to MBP were decreased (↓) if the exit frequency was significantly lower than baseline and the baseline frequency was >1 cell/million, increased (↑) if the exit frequency was >1 cell/million and was significantly greater than the baseline frequency, or (↕) if both entry and exit frequencies were elevated (patient G.A.B.) or at baseline (patients W.G.A. and M.O.H.) levels with significant intervening fluctuations, or negative (–) if no postimmunization frequencies were significantly different from baseline frequency.

[d]Patients underwent clinical evaluations at baseline and every 3 months until exit. The same neurologist, blinded to treatment category, determined the expanded disability status score (EDSS)[48] and the ambulation index (AI)[49] for all patients throughout the study. Mean baseline values for the nine-hole peg test (9HPT)[50], box and block test (BBT)[50], and 25-foot timed walk (TW) were obtained by measuring these on three separate days over 2 weeks. Criteria for determining whether an individual clinical test was improved or worse were as follows: EDSS, change of ≥1.0 for subjects with a baseline EDSS of ≤5.5 or change of ≥0.5 for subjects with baseline EDSS of 6.0 or 6.5; AI, change of ≥1; TW, change of >2 standard deviations from the mean baseline; 9HPT, change >20% from the mean baseline; BBT, change >20% from the mean baseline. At exit, subjects were classified as being improved (I), stable (S) or worse (W) using a composite clinical score[50]. To be classified as improved, a subject must have improved on at least one of the clinical tests and not worsened on any of the tests, and the improvement must have been sustained for 3 or more months. To be classified as worse, a subject must have worsened on at least one of the clinical tests and not improved on any of the tests, and the worsening must have been sustained for 3 or more months. All other subjects were classified as stable. Criteria for subject classification using the clinical composite score were established before study initiation, and all subjects were classified without knowledge of treatment status or frequency data.

Response to MBP correlated with clinical worsening

Significant changes in MBP-specific T-cell frequencies occurred in 11 of the 23 study patients. All four patients with an increase in MBP-specific T-cell frequency were clinically worse at exit, as was one peptide nonresponder (W.G.A.) in whom the MBP frequency increased transiently. Conversely, all six responders with a decrease or no net change in MBP T-cell frequency were improved or stable, as were two patients in whom entry and exit responses to MBP were similar with intervening fluctuations (Table 1). Taken together, these data demonstrate a highly significant correlation between response to MBP and clinical progression ($P = 0.024$, Table 2*d*). This correlation was even stronger when analysis included only the 11 patients with significant MBP fluctuations ($P = 0.015$, Table 2*d*).

T-cell cytokine profiles

To characterize the regulatory mechanism(s) of TCR peptide vaccination in humans, 32 TCR peptide-specific clones from three MS patients boosted successfully with either Vβ5.2-38-58 (21 clones) or Vβ9-38-58 (11 clones), and 15 MBP-specific T-cell clones from eight MS patients were evaluated for cytokine secretion profiles. As shown previously, both TCR peptide- and MBP-specific clones were CD4 positive and MHC II restricted[27]. However, on the basis of cytokine secretion, the TCR peptide-specific clones were predominantly like T helper 2 cells (Th2-like), with 24 of 32 clones (75%) secreting substantial levels of IL-4 (0.2–2.5 ng/ml, not shown) or IL-10 (0.2–2.9 ng/ml, Fig. 2*a*), but not interferon-γ (IFN-γ). The remaining eight TCR peptide-specific clones (25%) were characterized as Th0-like, producing both IL-4/IL-10 and IFN-γ (Fig. 2*a*). Only 1 of 32 TCR peptide-specific T-cell clones secreted a detectable level of transforming growth factor-β (TGF-β) (not shown). In contrast, 12 of 15 (80%) MBP-specific T-cell clones obtained from eight MS patients were Th1-like, producing IFN-γ but not IL-4/IL-10 (Fig. 2*b*), with the remaining three clones being Th0-like.

IL-10 is the predominant regulatory cytokine

Supernatants from activated Th2 TCR peptide-specific clones could

Table 2 Statistical analyses

a, Response to peptide in different treatment groups

	Placebo	Substituted peptide	Native peptide
Peptide responders	0	5	1
Peptide nonresponders	6	4	7

Responders were those with two or more frequency determinations significantly greater than the reference determination, with at least one frequency ≥2 cells/million. Fisher's exact test: Placebo vs. subst. peptide: $P = 0.044$; placebo vs. native peptide: $P = 1.00$; subst. vs. native peptide: $P = 0.131$.

b, Change in MBP frequency vs. response to peptide

	↓ MBP response	No change	↑ MBP response
Peptide responder	4	2	0
Peptide nonresponder	0	13	4

Chi-square test: $P < 0.001$. ↓ MBP response: ≥1 frequency significantly below a baseline ≥1 cell/million. ↑MBP response: ≥1 frequency significantly above a baseline of <1 cell/million. No change: No significant changes from baseline, or exit responses the same as entry responses with intervening fluctuations.

c, Response to peptide vs. clinical outcome

	Improved/Stable	Worse
Responders	6	0
Nonresponders	7	10

Fisher's exact test for response to peptide: $P = 0.019$. Responders had ≥2 significantly increased T-cell frequency determinations, at least one of which was ≥2 cells/million.

d, MBP response vs. clinical outcome

	Improved/Stable	Worse
↓ or – MBP	13 (6*)	6 (1*)
↑ MBP	0	4*

Fisher's exact test: $P = 0.024$. *Patients with significant changes in MBP frequency: $P = 0.015$.

inhibit both proliferation and release of IFN-γ by Th1 MBP-specific clones in a dose-dependent manner (Fig. 3, *a* and *b*). In contrast, addition of 90% Th2 supernatants had no inhibitory effects on responses of TCR peptide-specific Th2 cells (not shown). An example using a culture supernatant from a Vβ5.2-38-58-specific clone in combination with an autologous MBP-specific clone is presented in Fig. 3, and is representative of five such experiments showing that supernatants from TCR peptide-specific clones could inhibit either autologous or heterologous Th1 but not Th2 responses.

On the basis of its known properties and level of secretion by TCR peptide-specific clones, IL-10 was the most likely candidate for inhibiting Th1 cells. As shown in the inset in Fig. 3*a*, the 50% inhibition concentration (IC_{50}) for recombinant (rh)IL-10 inhibition of MBP-induced proliferation was 0.77 ng/ml (that is, the amount of rhIL-10 required to inhibit proliferation by 50%), and that for inhibition of IFN-γ release was 0.23 ng/ml (Fig. 3*b*, *inset*). In contrast, the same range of concentrations of rhIL-4 had no inhibitory effect on proliferation or IFN-γ release (not shown). The mean concentration of IL-10 in the 31 IL-10-positive TCR peptide-specific clonal supernatants was 1.1 ± 0.7 ng/ml, almost 1.5 times the IC_{50} value for inhibition of proliferation, and 5 times the IC_{50} value for inhibition of IFN-γ. The IC_{50} of supernatant from the Vβ5.2-38-58 peptide-specific clone shown in Fig. 3 was 47% for inhibition of proliferation and 14% for inhibition of IFN-γ release. If we assume that all of the inhibitory activity was due to IL-10, the supernatant would contain 1.64 ng/ml IL-10, as determined independently from each of the IC_{50} values. This value, based on the functional activities of IL-10, was indistinguishable from the 1.8 ± 0.2 ng/ml IL-10 quantified by enzyme-linked immunoabsorbent assay (ELISA). Moreover, inhibitory activity of the supernatant could be neutralized by anti-IL-10 antibody to a degree similar to that of rhIL-10 (Fig. 3). In the one clone that produced both IL-10 and TGF-β, approximately two-thirds of the inhibition was attributable to IL-10, and one-third to TGF-β (not shown). Taken together, these results demonstrate that IL-10 was the predominant regulatory cytokine produced by the TCR Vβ5.2-38-58-specific T cells.

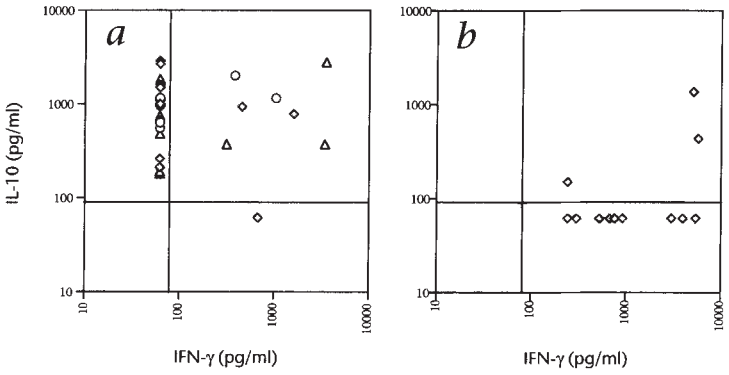

Fig. 2 Lymphokine secretion patterns of (*a*) TCR peptide-specific and (*b*) MBP-specific T-cell isolates. *a*, T cells were isolated from various donors' blood mononuclear cells (MNCs) by limiting dilution, activated in the presence of autologous antigen-presenting cells (irradiated blood MNCs) and Vβ5.2-38-58 peptide [patients M.R. (◇), who was vaccinated in the previous study[22], and L.G.T. (△) (see Table 1)] or Vβ9-39-59 peptide [patient J.C. (○)] or MBP. Supernatants were collected ($n = 32$ from the three patients) and quantified by ELISA for the concentration of IL-10, IL-4 (not shown), and IFN-γ. *b*, MBP-reactive clones were obtained from MS patients in the current and previous clinical studies ($n = 15$ from eight patients). Lines within the graphs indicate limits for detecting a significant elevation of lymphokine in the supernatant. Note that the majority of isolates responding to TCR peptides have a Th2-like pattern (elevated IL-10 but low IFN-γ levels), and the majority of isolates responding to MBP have a Th1-like pattern (elevated IFN-γ but low IL-10 levels).

Discussion

The data presented above demonstrate under double-blind conditions that successful vaccination with Vβ5.2 CDR2 peptides boosted the frequency of TCR peptide-specific T cells, reduced the frequency of MBP-specific T cells, and prevented clinical progression of MS without side effects. Conversely, lack of response to vaccination was associated with increased response to MBP and clinical progression. TCR peptide-reactive T cells were predominantly Th2-like, and directly inhibited MBP-specific Th1 responses *in vitro*, primarily through the release of IL-10. These data demonstrate the ability of synthetic peptides to activate an anti-idiotypic regulatory network directed at TCR determinants and provide evidence supporting the hypothesis that insufficient regulation of inflammatory T cells may contribute to the progression of MS. Moreover, the regulatory strategy described herein may be applicable to other tissue-specific human autoimmune diseases characterized by oligoclonal T-cell responses.

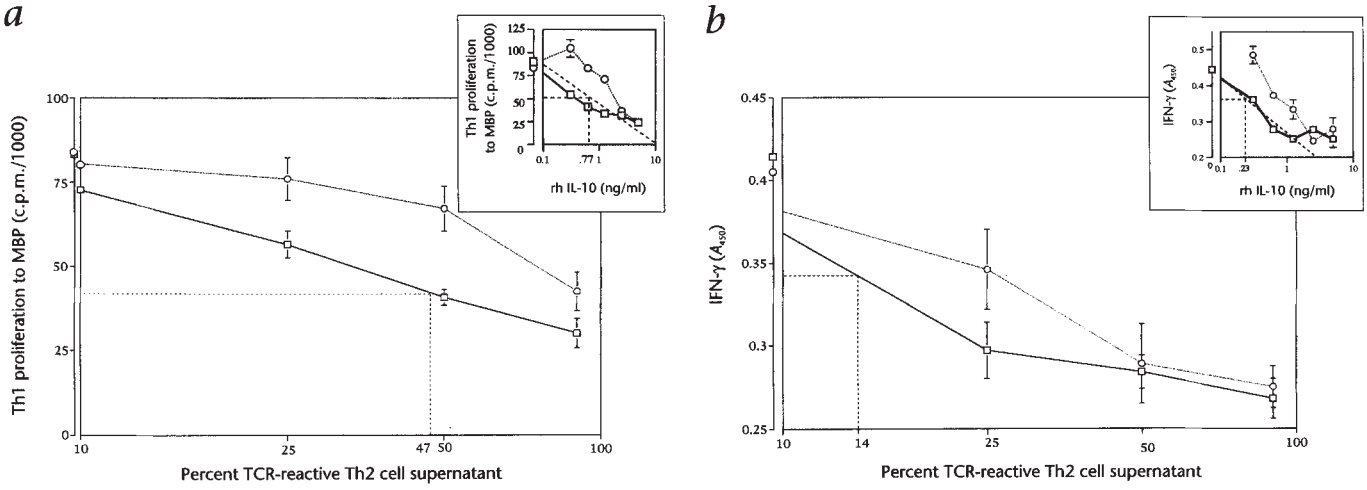

Fig. 3 Inhibition of Th1 response to MBP by IL-10 in the supernatant from a Th2 isolate responding to Vβ5.2-38-58 peptide. Supernatant from a Th2 cell isolate from patient L.G.T. (Table 1) or rhIL-10 was added at the indicated dilutions or concentration to cultures of an autologous MBP-specific isolate stimulated with antigen-presenting cells and MBP and assayed for (*a*) proliferation response, or (*b*) release of IFN-γ, with (○) or without (□) the addition of neutralizing anti-IL-10 antibody. Dotted vertical lines indicate the IC_{50}. No inhibition was observed with 90% Th1 supernatants from MBP-specific T-cell clones (not shown).

The rate of response after vaccination to the Y49T-Vβ5.2-38-58 peptide (56%) appeared to be higher than to the native Vβ5.2-38-58 sequence (13%), and was similar to that in the previous open label trial (64%). The apparent enhanced immunogenicity of the Y49T-Vβ5.2-38-58 peptide over the native germline sequence is of some theoretical interest. Although response to self TCR sequences was initially unexpected because of the pervasive presence of TCR chains in the thymus, where discrimination of self versus nonself takes place, there is extensive evidence to suggest that TCR idiotopes behave essentially like foreign antigens in their ability to induce both tolerance and responses in rodents[6]. Humans, like rodents, may have the intrinsic ability to respond to germline sequences, but in diseases such as MS with probable autoimmune abnormalities linked to TCR oligoclonality, peripheral tolerance mechanisms may have been triggered that mute responses to TCR sequences. In this situation, injection of cross-reactive or cryptic peptides might serve to break tolerance and to re-induce responses to germline sequences, as has been suggested[28]. Notably, the native Vβ5.2-38-58 and Y49T-Vβ5.2-38-58 sequences are strongly cross-reactive[29]. The 60% rate of response to vaccination with the Y49T-Vβ5.2-38-58 peptide may reflect the extent of involvement of Vβ5.2 in the disease process in DRβ1*1501 MS patients, thus defining the limitation of targeting only one of several contributing Vβ families. In this case, future therapies might require a cocktail of immunogenic peptides from several disease-associated Vβ families. Alternatively, favored selection of TCRs containing Vβ5.2 could be influenced by putative interactions[3-5] between CDR1 or CDR2 binding domains of Vβ5.2 with DRβ1*1501, or with peptide determinants[30] (for example, from MBP) restricted by DRβ1*1501, suggesting more widespread involvement of Vβ5.2. If this is the case, it might be possible to increase the rate of vaccination using more efficient immunization procedures such as the use of adjuvants.

Our findings suggest that TCR peptide vaccination may potentially have a therapeutic benefit by lowering circulating MBP-specific T cells that occur at an increased frequency within a subset of $DR2^+$ chronic progressive MS patients. Encephalitogenic Th1 cells specific for neuroantigens MBP or proteolipid protein are required to initiate EAE, and a similar Th1 cytokine profile has been reported previously[31,32] and is confirmed here for MBP-specific T cells from MS patients, demonstrating the pathogenic potential of these T cells. Conversely, our results showing clinical benefit and decreased MBP frequencies in MS patients with elevated Th2 cell frequencies after vaccination with Vβ5.2-38-58 peptide strongly suggest a pathogenic role for MBP-reactive T cells, as well as a regulatory role for TCR peptide-reactive cells in MS. These results concur with previous observations that implicate MBP as an encephalitogen in MS, including episodic increases in activated MBP-specific T cells in some patients[33-35] that are associated with clinical relapses (manuscript in preparation), and possible clinical benefit in patients in whom decreases in MBP responses were induced by oral MBP administration or vaccination with irradiated MBP-specific T cells[36,37].

The TCR peptide-reactive clones in our study produced elevated levels of both IL-4 and IL-10, but virtually no TGF-β, which has been shown by others to be the predominant regulatory cytokine secreted by mucosally derived Th2-like cells[38] and $CD4^+$ suppressor cells in EAE (ref. 39). IL-4 may be important in directing differentiation toward Th2 (ref. 40), but has no direct suppressive effect on Th1 responses[41]. In contrast, a protective role for IL-10 in EAE and MS has been inferred from studies that showed a reciprocal relationship between IL-10 mRNA or protein levels and disease activity[42], as well as from direct inhibition of EAE induction by rIL-10 administration[43].

The human TCR peptide-specific Th2 cells described herein may thus downregulate Th1 cell activation, proliferation and cytokine production, primarily through the inhibitory effects of IL-10 released locally upon activation. Additionally, release of IL-4 by these clones may contribute to a skewing effect on the maturation of neuroantigen-specific precursors toward a nonencephalitogenic Th2 or Th0 phenotype. Based on TCR peptide vaccination studies in EAE, we have proposed that the initial induction of TCR peptide-specific T cells occurs through a naturally processed form of Vβ5.2-38-58 peptide present on activated neuroantigen-specific Th1 effector cells[6]. Administration intradermally of soluble peptide at relatively low concentrations would serve to arm tissue-specific dendritic cells to present peptide and to expand the natural precursor population. Once primed, the TCR peptide-specific Th2 cells would recirculate throughout the periphery and tissues, with the capability of being reactivated by encountering neuroantigen-specific $V\beta5.2^+$ Th1 effec-

tor cells. It is noteworthy that TCR peptide-specific T cells have been isolated from the CSF of peptide-treated MS patients (data not shown) and from the CNS of peptide-treated rats[24]. In this sequence of events, presentation of naturally processed Vβ5.2 peptides to DR-restricted Th2 cells would occur more efficiently with increased expression of MHC II, a process known to occur in activated neuroantigen-specific Th1 cells in MS patients with disease activity. Activation of the Th2 cells would result in the local release of Th2 cytokines, including IL-4 and IL-10, that could inhibit activation of Th1 cells in the immediate vicinity, including cells bearing Vβ genes other than Vβ5.2. Thus, although activation of Th2 regulatory cells is peptide specific, their inhibitory effects would be expected to be neuroantigen nonspecific but restricted to Th1 cells. This mechanism of suppression might allow therapeutic effects under conditions involving limited but not homogeneous Vβ gene expression by encephalitogenic or other autopathogenic Th1 specificities.

Although the current study demonstrates that TCR peptide vaccination can affect responses to MBP and clinical course in MS patients, a more widespread application of this approach will likely require (1) more sensitive and reliable predictors of response (for example, V gene expression in response to MBP or other myelin antigens, levels of natural sensitization to TCR determinants); (2) more efficient means for inducing response (for example, adjuvants, different substituted peptides); and (3) more sensitive disease-associated parameters that are affected by treatment (for example, expanded clinical scales, magnetic resonance imaging correlations). With a similar knowledge base, the TCR peptide vaccination approach may also have application to other tissue-specific autoimmune diseases involving oligoclonal T cells.

Methods

Patients. Patients entered into the trial (shown in Table 1) included 15 females and 8 males with clinically or laboratory-supported definite MS diagnosis[44] with a mean age of 51 ± 6 years. The mean expanded disability status score (EDSS) (see Table 1) was 5.4 ± 1.0, and average duration of MS was 17 ± 9 years. Eight patients had primary (progressive course from onset) and 15 had secondary (initial relapsing-remitting phase followed by a progressive course) progressive MS, with the average duration of the progressive phase of disease being 9 ± 4 years. There were no significant differences in any of the demographic or clinical parameters among the three treatment groups at entry.

Antigens. TCR peptides, Vβ5.2-38-58 (ALGQGPQFIFQYYEEEERQRG) and Y49T-Vβ5.2-38-58, were synthesized, purified by HPLC, sequenced, redissolved in lactated Ringer's solution (pH 7–7.5), filter sterilized, pyrogen tested and stored frozen at –20 °C until use as described previously[22]. In this state, the peptides retained biological activity for >2 years. Patients received 100 μg of peptides intradermally (i.d.) in the forearm in 0.1 ml volumes at one site at a concentration of 1 mg/ml. MBP was extracted and purified using frozen brains supplied by the National Disease Research Interchange (NDRI, Philadelphia, Pennsylvania). Methods, including brain tissue homogenization, choice of organic solvents, protein extraction, and ion-exchange purification were described previously[45].

Identification of HLA-DRβ1*1501$^+$ MS patients. DNA was isolated from peripheral blood lymphocytes using standard techniques. Briefly, cells were boiled for 2 min (10^6 cells/50 μl water), then treated with Proteinase K, followed by extraction with phenol and chloroform. DNA (3 μl) was used in each amplification reaction. HLA-DRβ1*1501$^+$ patients were identified using a method described previously[46]. This method utilizes a system of primers and probes that specifically amplify and hybridize to alleles of the DR2 haplotype. DR2-specific alleles were amplified using specific primers in a two-step amplification profile. Amplified cDNA samples were transblotted to membrane filters, hybridized with horseradish peroxidase (HRP)-labeled probes, and then washed under the conditions specified. All probes were synthesized at Operon Technologies, Inc. (Alameda, California). ECL (enhanced chemiluminescent) detection reagents (Amersham, Arlington Heights, Illinois) were used to visualize positive bands.

Toxicity monitoring. To monitor for nonspecific immunosuppression, cell-mediated immunity skin testing to seven antigens (tetanus toxoid, diphtheria toxoid, *Streptococcus*, old tuberculin, *Candida*, *Trichophyton*, and *Proteus*) was carried out at entry, 6 months, and exit using the Multitest CMS (Merieux Institute). At entry and every 3 months thereafter, samples were taken from patients for a complete blood count, a 24-channel chemistry panel, and a urinalysis.

Antigen-specific T-cell frequencies. Limiting dilution assays (LDA) were used to estimate the circulating frequencies of T cells specific for Vβ5.2-38-58 and MBP. Peripheral blood mononuclear cells were separated by Ficoll gradient centrifugation and cultured in sets of 24 or 10 replicates with antigen in microtiter plates at 0.5, 0.25, 0.125 and 0.0625 × 10^6 cells/well, as described previously[22]. Proliferation after 5 days was measured by [^{3}H]thymidine uptake. Individual wells were scored as antigen responsive if the counts per minute exceeded 2 s.d. of the mean counts per minute from 12 wells cultured at the same cell concentration without antigen. By using the percentage of non-responding wells at each cell concentration, antigen-specific T-cell frequencies and their 95% confidence intervals were estimated by the chi-square minimization method[47], employing a program adapted for use with a personal computer containing a math coprocessor chip.

Antigen-specific T-cell isolates. T cells specific for Vβ5.2-38-58 or MBP were selected as described[27] from the blood of ten MS donors to characterize cytokine secretion profiles. Patients included L.G.T. from the current study, and nine additional donors with definite MS that were characterized previously. TCR peptide-specific isolates were obtained from patients L.G.T. (9 isolates) and M.R. (12 isolates), who developed strong responses after vaccination with Y49T-Vβ5.2-38-58, and patient J.C. (11 isolates), who responded to vaccination with Vβ9-39-59 (SKKFLKIMFSYNNKELIINET)[27]. Fifteen MBP-specific T cells were obtained from patients M.R. (five isolates), S.L. (four isolates), and L.G.T., A.F., T.R., S.D.S., E.S.N. and A.L. (one isolate each).

Cytokine quantification. Interferon-γ, IL-4 and IL-10 contained in T-cell supernatants were quantified by ELISA using combinations of capture and detecting antibodies. For IL-10, Maxisorp NUNC plates (Intermountain Scientific Corp., Bountiful, Utah) were coated with 2 μg/ml (50 μl/well) of purified rat anti-human IL-10 (PharMingen, San Diego, California) in 0.1 M $NaHCO_3$, pH 8.2, coating solution. After an overnight incubation, plates were washed with PBS/Tween, pH 7.4, then blocked with 4% BSA-PBS (Sigma), 150 μl/well at pH 7.4 for 2 h at room temperature. After further washing, 50 μl diluted supernatants or rhIL-10 was added in triplicate. Plates were again incubated overnight, washed with PBS/Tween, and secondary (detecting) biotinylated rat anti-human IL-10 monoclonal antibody (mAb) (PharMingen) was added at 2 μg/ml (100 μl/well) in 4% BSA-PBS, and incubated for 1 h at room temperature. After washing, plates were incubated for 30 min in 2.5 μg/ml avidin peroxidase (Sigma) in 4% BSA-PBS (100 μl/well). Plates were washed again and the color reagent (H_2O:0.1 M citrate:0.2 M Na_2HPO_4 as 49:24:27) containing

15 mg phenylenediamine dihydrochloride and 50 µl H_2O_2, pH 5.0, was added to each well. Color was allowed to develop for 30 min at room temperature before absorbance was measured at 450 nm using a Bio-Tek Instruments EL-309 ELISA reader. Absorbance readings of the standards were averaged from triplicate wells, and plotted using a linear curve fit ($r > 0.990$, Cricket Graph III). The minimal concentration that could be accurately assessed was 0.062 ± 0.014 ng/ml, and positive values were established as ≥0.09 ng/ml.

A similar procedure was used to quantify IL-4 and IFN-γ. For IL-4, the capture antibody was a mouse anti-human IL-4 mAb, and the detecting antibody was a biotinylated rat anti-human IL-4 mAb (both antibodies from PharMingen). For IFN-γ, the capture antibody (Endogen, Cambridge, Massachusetts) was diluted to 3 µg/ml, and the detecting antibody (biotinylated anti-human IFN-γ mAb, Endogen) was diluted to 0.25 µg/ml.

Acknowledgments

The authors wish to thank Eva Niehaus, Ian Robey, and Laura Unsicker for their assistance in preparing the manuscript. This work was supported by National Institutes of Health grants NS 23221 and NS 23444, and by the Department of Veterans Affairs.

RECEIVED 13 MAY; ACCEPTED 15 AUGUST 1996

1. Jerne, N.K. Idiotypic networks and other preconceived ideas. *Immunol. Rev.* **79**, 5–24 (1984).
2. Cohen, I.R. & Young, D.B. Autoimmunity, microbial immunity and the immunological homunculus. *Immunol. Today* **12**, 105–110 (1991).
3. Claverie, J.M., Prochnicka-Chalufour, A. & Bougueleret, A. Implications of a Fab-like structure for the T-cell receptor. *Immunol. Today* **10**, 10–14 (1989).
4. Davis, M.M. & Bjorkman, P.J. T-cell antigen receptor genes and T-cell recognition. *Nature* **334**, 395–402 (1988).
5. Williams, W.V., Weiner, D.B., Wadsworth, S. & Greene, M.I. The antigen-major histocompatibility complex–T-cell receptor interaction: A structural analysis. *Immunol. Rev.* **7**, 339–344 (1988).
6. Vandenbark, A.A., Hashim, G.A. & Offner, H. T cell receptor peptides in treatment of autoimmune disease: Rationale and potential. *J. Neurosci. Res.* **43**, 391–402 (1996).
7. Heber-Katz, E. & Acha-Orbea, H. The V-region disease hypothesis: Evidence from autoimmune encephalomyelitis. *Immunol. Today* **10**, 164–169 (1989).
8. Lider, O., Reshef, T., Beraud, E., Ben-Nun, A. & Cohen, I.R. Anti-idiotypic network induced by T cell vaccination against experimental autoimmune encephalomyelitis. *Science* **239**, 181–183 (1988).
9. Vandenbark, A.A., Hashim, G. & Offner, H. Immunization with a synthetic T-cell receptor V-region peptide protects against experimental autoimmune encephalomyelitis. *Nature* **341**, 541–544 (1989).
10. Offner, H., Hashim, G.A. & Vandenbark, A.A. T cell receptor peptide therapy triggers autoregulation of experimental encephalomyelitis. *Science* **251**, 430–432 (1991).
11. Howell, M.D. *et al.* Vaccination against experimental allergic encephalomyelitis with T cell receptor peptides. *Science* **246**, 668–670 (1989).
12. Stevens, D.B., Karpus, W.J., Gould, K.E. & Swanborg, R.H. Studies of Vβ8 T cell receptor peptide treatment in experimental autoimmune encephalomyelitis. *J. Neuroimmunol.* **37**, 123–129 (1991).
13. Kumar, V. & Sercarz, E.E. The involvement of T cell receptor peptide-specific regulatory CD4⁺ T cells in recovery from antigen-induced autoimmune disease. *J. Exp. Med.* **178**, 909–916 (1993).
14. Gregorian, S.K., Lee, W.P., Doudikian, N. & Amento, E.P. Two distinct T cell receptor peptides independently regulate experimental autoimmune neuritis. *Am. Assoc. Immunol.* **150**, 28A (1993).
15. Broeren, C.P.M. *et al.* CDR1 T-cell receptor β-chain peptide induces major histocompatibility complex class II-restricted T-T cell interactions. *Proc. Natl. Acad. Sci. USA* **91**, 5997–6001 (1994).
16. Matsumoto, Y., Tsuchida, M., Hanawa, H. & Abo, T. T cell receptor peptide therapy for autoimmune encephalomyelitis: Stronger immunization is necessary for effective vaccination. *Cell. Immunol.* **153**, 468–478 (1994).
17. Kuhrober, A., Schirmbeck, R. & Reimann, J. Vaccination with T cell receptor peptides primes anti-receptor cytotoxic T lymphocytes (CTL) and anergizes T cells specifically recognized by these CTL. *Eur. J. Immunol.* **24**, 1172–1180 (1994).
18. Haqqui, T.M., Qu, X.-M., Ma, J. & Sy, M.S. Immunization with TCR Vβ region peptides completely prevents the induction of collagen induced arthritis. *Ninth Int. Cong. Immunol. Abstr.* **5014**, 845 (1995).
19. Kotzin, B.L. *et al.* Preferential T cell receptor Vβ gene usage in myelin basic protein reactive T cell clones from patients with multiple sclerosis. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 9161–9165 (1991).
20. Oksenberg, J.R. *et al.* Selection of T-cell receptor Vβ-Dβ-Jβ gene rearrangements with specificity for a myelin basic protein peptide in brain lesions of multiple sclerosis. *Nature* **362**, 68–70 (1993).
21. Shimonkevitz, R., Murray, R. & Kotzin, B. Characterization of T-cell receptor Vβ usage in the brain of a subject with multiple sclerosis. *Ann. N. Y. Acad. Sci.* **756**, 305–306 (1995).
22. Bourdette, D.N. *et al.* Immunity to T cell receptor peptides in multiple sclerosis. I. Successful immunization of patients with synthetic Vβ5.2 and Vβ6.1 CDR2 peptides. *J. Immunol.* **152**, 2510–2519 (1994).
23. Wucherpfennig, K.W. *et al.* Shared human T cell receptor Vβ usage to immunodominant regions of myelin basic protein. *Science* **248**, 1016–1019 (1990).
24. Ben-Nun, A. *et al.* Restricted T-cell receptor Vβ gene usage by myelin basic protein- specific T-cell clones in multiple sclerosis: Predominant genes vary in individuals. *Proc. Natl. Acad. Sci. USA* **88**, 2466–2470 (1991).
25. Vandenbark, A.A., Vainiene, M., Celnik, B., Hashim, G. & Offner, H. TCR peptide therapy decreases the frequency of encephalitogenic T cells in the periphery and the central nervous system. *J. Neuroimmunol.* **39**, 251–260 (1992).
26 Desquenne-Clark, L., Esch, T.R., Otvos, L., Jr., & Heber-Katz, E. T cell receptor peptide immunization leads to enhanced and chronic experimental allergic encephalomyelitis. *Proc. Natl. Acad. Sci. USA* **88**, 7219–7223 (1991).
27. Chou, Y.K. *et al.* MHC-restriction, cytokine profile, and immunoregulatory effects of human T cells specific for TCR Vβ CDR2 peptides: Comparison with myelin basic protein-specific T cells. *J. Neurosci. Res.* (in the press).
28. Falcioni, F. *et al.* Self tolerance to T cell receptor Vβ sequences. *J. Exp. Med.* **182**, 249-254 (1995).
29. Vandenbark, A.A. [Letter to the editor]. *J. Immunol.* **153**, 910 (1994).
30. Sant'Angelo, D.B. *et al.* The specificity and orientation of a TCR to its peptide-MHC class II ligands. *Immunity* **4**, 367–376 (1996).
31. Voskuhl, R.R. *et al.* T helper 1 (Th1) functional phenotype of human myelin basic protein-specific T lymphocytes. *Autoimmunity* **15**, 137–143 (1993).
32. Chou, Y.K. *et al.* Immunity to T cell receptor peptides in multiple sclerosis. II. T cell recognition of Vβ5.2 and Vβ6.1 CDR2 peptides. *J. Immunol.* **152**, 2520–2529 (1994).
33. Allegretta, M., Nicklas, J.A., Sriram, S. & Albertini, R.J. T cells responsive to myelin basic protein in patients with multiple sclerosis. *Science* **247**, 718–721 (1990).
34. Vandenbark, A.A. *et al.* Episodic changes in T cell frequencies to myelin basic protein in patients with multiple sclerosis. *Neurology* **43**, 2416–2417 (1993).
35. Zhang, J. *et al.* Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J. Exp. Med.* **179**, 973–984 (1994).
36. Weiner, H.L. *et al.* Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. *Science* **259**, 1321–1324 (1993).
37. Zhang, J., Medaer, R., Stinissen, P., Hafler, D.A. & Raus, J.C.M. MHC-restricted depletion of human myelin basic protein-reactive T cells by T cell vaccination. *Science* **261**, 1451–1454 (1993).
38. Miller, A., Al-Sabagh, A., Santos, L.M.B., Prabhu-Das, M. & Weiner, H.L. Epitopes of myelin basic protein that trigger TGF-β release after oral tolerization are distinct from encephalitogenic epitopes and mediate epitope-driven bystander suppression. *J. Immuno.* **151**, 7307–7315 (1993).
39. Karpus, W.J. & Swanborg, R.H. CD4⁺ suppressor cells inhibit the function of effector cells of EAE through a mechanism involving transforming growth factor-beta. *J. Immunol.* **146**, 1163–1168 (1991).
40. Swain, S.L. Regulation of the development of distinct subsets of CD4⁺ T cells. *Res. Immunol.* **142**, 14–22 (1991).
41. Racke, M.K. *et al.* Cytokine-induced immune deviation as a therapy for inflammatory autoimmune disease. *J. Exp. Med.* **180**, 1961–1966 (1994).
42. Kennedy, M.K., Torrance, D.S., Picha, K.S. & Mohler, K.M. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J. Immunol.* **149**, 2496–2505 (1992).
43. Rott, O., Fleischer, B. & Cash, E. Interleukin-10 prevents experimental allergic encephalomyelitis in rats. *Eur. J. Immunol.* **24**, 1434–1440 (1994).
44. Poser, C.M. *et al.* New Diagnostic criteria for multiple sclerosis: Guidelines for research protocols. *Ann. Neurol.* **13**, 227–236 (1983).
45. Diebler, G.E., Boyd, L.F. & Kies, M.W. Enzymatic and nonenzymatic degradation of myelin basic protein. *Neurochemistry* **9**, 1371–1385 (1984).
46. Scharf, S.J., Griffith, R.L. & Erlich, H.A. Rapid typing of DNA sequence polymorphism at the HLA-DRB1 locus using the polymerase chain reaction and nonradioactive probes. *Hum. Immunol.* **30**, 190–201 (1991).
47. Taswell, C. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* **26**, 1614–1619 (1981).
48. Kurtzke, J.F. Further notes on disability evaluation in multiple sclerosis with scale modification. *Neurology* **15**, 654–662 (1965).
49. Hauser, S.L., Dawson, D.M. & Lehrich, J. C. Intensive immunosuppression in progressive multiple sclerosis: A randomized, three-arm study of high-dose intravenous cyclophosphamide, plasma exchange, and ACTH. *N. Engl. J. Med.* **308**, 173–180 (1983).
50. Goodkin, D.E. *et al.* Low-dose (7.5 mg) oral methotrexate reduces the rate of progression in chronic progressive multiple sclerosis. *Ann. Neurol.* **37**, 30–40 (1995).