Encephalitogenic potential of the myelin basic protein peptide (amino acids 83–99) in multiple sclerosis: Results of a phase II clinical trial with an altered peptide ligand

Bibiana Bielekova[1], Bonnie Goodwin[1], Nancy Richert[2], Irene Cortese[1], Takayuki Kondo[1], Ghazaleh Afshar[1], Bruno Gran[1], Joan Eaton[1], Jack Antel[3], Joseph A. Frank[2], Henry F. McFarland[1] & Roland Martin[1]

[1]Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke,
[2]Laboratory of Diagnostic Radiology Research, Clinical Center, National Institutes of Health, Bethesda, Maryland 20892-1400, USA, [3]Department of Neurology, Montreal Neurological Institute, 3801 University Avenue, Montreal, Quebec H3A 2B4, Canada
Correspondence should be addressed to R.M.

Myelin-specific T lymphocytes are considered essential in the pathogenesis of multiple sclerosis. The myelin basic protein peptide (a.a. 83–99) represents one candidate antigen; therefore, it was chosen to design an altered peptide ligand, CGP77116, for specific immunotherapy of multiple sclerosis. A magnetic resonance imaging-controlled phase II clinical trial with this altered peptide ligand documented that it was poorly tolerated at the dose tested, and the trial had therefore to be halted. Improvement or worsening of clinical or magnetic resonance imaging parameters could not be demonstrated in this small group of individuals because of the short treatment duration. Three patients developed exacerbations of multiple sclerosis, and in two this could be linked to altered peptide ligand treatment by immunological studies demonstrating the encephalitogenic potential of the myelin basic protein peptide (a.a. 83–99) in a subgroup of patients. These data raise important considerations for the use of specific immunotherapies in general.

Multiple sclerosis (MS) is the most frequent demyelinating disease[1] and is characterized by inflammatory demyelinating foci in the brain white matter with variable axonal damage[2–4]. Its etiology remains unknown, but the composition of plaques, immunogenetic background, response to immunomodulation and data from animal models support the idea that MS is an autoimmune disease mediated by myelin-specific CD4+ T cells[1,5–8], although there is considerable heterogeneity in clinical characteristics, magnetic resonance imaging (MRI) findings and pathological patterns[9,10]. Certain myelin epitopes that are encephalitogenic in experimental allergic encephalomyelitis (EAE) are also immunodominant in humans[11–15] in the context of MS-associated human leukocyte antigen (HLA) DR molecules[12,16,17]. The myelin basic protein (MBP) peptide of amino acids 83–99 (called MBP$_{(83–99)}$ here) is the best examined and is considered one candidate antigen in MS (refs. 1,18). However, the evidence described above is indirect, and the function of autoreactive T cells for disease pathogenesis has remained controversial.
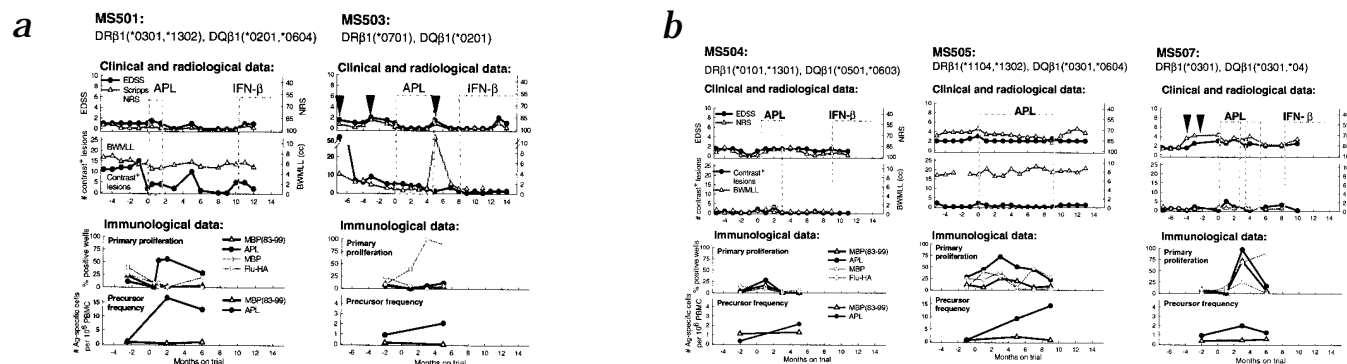
Specific immunotherapies of T cell-mediated autoimmune diseases have been successful in animal models with known target antigens[19–23]. Consequently, there is a strong interest in adopting such approaches for human autoimmune diseases. One strategy uses altered peptide ligand (APL) peptides with amino-acid substitutions in T-cell receptor (TCR) contact positions[24–26]. APLs may block T-cell responses by acting as partial agonists, TCR antagonists or by inducing regulatory T-cell populations that mediate 'bystander' suppression[24,26–30]. We sought to alter disease activity in patients with active relapsing–remitting MS using an APL of MBP$_{(83–99)}$, CGP77116. Here we report the results of our MRI-controlled, single center, baseline-to-treatment cross-over phase II clinical trial as well as complementary immunological studies to elucidate the mechanism of action of APL.

Safety and tolerability of APL CGP77116

We sought to assess the safety, tolerability and efficacy on MRI and immunological parameters of 50 mg CGP77116 given subcutaneously weekly. The study design stipulated enrollment of 24 patients with relapsing–remitting MS and six monthly clinical and MRI examinations as baseline, followed by 9 months of treatment. The adverse events encountered in patients MS501–MS507 led to a modification of the dose regimen (from 50 mg weekly to 5 mg weekly for 4 weeks and 5 mg monthly thereafter). Only patient MS601 was enrolled in this lower-dose protocol, and when he suffered an exacerbation that was considered treatment-related, the trial was halted. During the baseline, treatment and post-APL phases, we monitored the course of the neurological status using the expanded disability status scale (EDSS; ref. 31) and the Scripps neurological rating scale (Scripps NRS; ref. 32), and monitored MRI findings by total gadolinium contrast-enhancing lesions and T2 bulk white matter lesion load (BWMLL) (Fig. 1). Only patient MS505 completed the full treatment. All other patients discontinued therapy, for a variety of reasons: exacerbations of MS (patients MS502, MS503 and MS601), systemic hypersensitivity reaction

Fig. 2 Cytokine profile and cross-reactivity of TCL specific for MBP$_{(83–99)}$ and CGP77116 **a**, Cytokine phenotype of T cells specific for MBP$_{(83–99)}$ (triangles) and APL (circles) isolated before (left) and during (right) APL therapy. Regression lines demonstrate phenotypic changes of T cells specific for MBP$_{(83–99)}$ (dotted lines) or APL (solid lines). **b**, Changes in cross-reactivity between native MBP$_{(83–99)}$ and APL inTCL specific for MBP$_{(83–99)}$ (**b**) and CGP77116 (**c**) induced by APL therapy, tested in avidity assays. Left, number of TCL: non-cross-reactive, ■; cross-reactive, ▨. Right, percentage of cross- reactive TCL; symbols indicate individual patients.

react with the native peptide and secrete T_H2 cytokines would be the most desirable mechanism of action, we assessed the cross-reactivity of TCL specific for APL and MBP$_{(83–99)}$ at baseline and during treatment across a wide concentration range with both peptides (0.001–100 μg/ml) (Fig. 2b). Before treatment, only 3 of 47 TCL specific for MBP$_{(83–99)}$ (6.4%) were cross-reactive (Fig. 2b,). APL immunization significantly increased the percentage to 86 of 123 (69.9%; $P = 0.017$). After therapy, the percentage decreased again (17 of 75 T cells; 22.7%), but remained higher than at baseline. We obtained similar results for TCL specific for APL (Fig. 2c). The percentage of cross-reactive T cells increased from 6.9% (4 of 58) before treatment to 36.5% (74 of 203; $P = 0.031$) during APL therapy and remained stable (53 of 141; 37.6%) after treatment.

As described above, there was no substantial improvement or worsening in the clinical and MRI parameters in the whole cohort of 8 patients. To determine whether a composite of clinical and MRI measures allows the discernment of a trend in individual patients and linking of this to the immunological measures, we ranked the changes in the five disease activity parameters (EDSS, Scripps NRS, contrast-enhancing MRI lesions, BWMLL and exacerbation rate) and assigned arbitrary values as follows: 1, if the parameter improved by at least 10%; 0, if it remained unchanged; or –1, if it worsened by at least 10% over baseline. We considered disease activity had improved with a positive score of +2 or greater, had worsened

BWMLL during APL treatment (marginal increase of 0.004 ± 0.88 ml) or during both post-treatment phases.

The study was 'powered' to detect an influence of APL treatment on the primary outcome (contrast-enhancing lesions), but not to assess clinical efficacy. As study drop-outs shortened treatment in all but one patient (Fig. 1), we did not expect to detect more than clinical trends. The clinical scores remained stable or improved in three of eight or two of eight patients, respectively, with an overall small increase in the EDSS score during APL therapy (0.22 ± 0.73) and a decrease during follow-up (of 0.33 ±0.5). There was a similar trend in the Scripps NRS results[32]. The exacerbation rate, estimated from the number of exacerbations per patient-years, was 1.25 during baseline, 1.38 during treatment, 1.64 after treatment and 0.52 during therapy with IFN-β-1a.

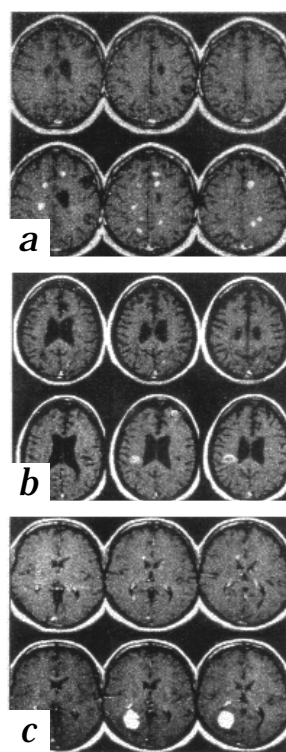### Correlation of immunological, clinical and MRI data

We monitored several immunological parameters to determine the *in vivo* mechanism of action of APL. We estimated the precursor frequencies of T cells specific for APL and MBP$_{(83–99)}$ from limiting dilution assays during baseline and APL therapy (Table 1). At baseline, these were in the range previously reported for autoreactive CD4$^+$ T cells[12]. APL therapy resulted in a significant increase in the precursor frequencies of T cells specific for APL ($P = 0.008$) in every patient (from 2-fold to 1,943-fold) , whereas the frequencies of T cells specific for MBP$_{(83–99)}$ varied with increases seen in patients MS502 and MS506 and less in patient MS601 and no significant changes or decreases in the others.

As we hoped to induce T cells specific for APL with a type 2 helper T-cell (T_H2) cytokine phenotype, we evaluated IFN-γ and interleukin (IL)-4 production after maximal stimulation with MBP$_{(83–99)}$ and APL in T cell lines TCL specific for these peptides. We classified each TCL according to its ratio of IFN-γ to IL-4: type 1 helper T cells (T_H1) had a ratio of more than 100; type 0 helper T cells (T_H0) had a ratio between 0.01 and 100; and T_H2 had a ratio of less than 0.01. Before therapy, T cells specific for APL were mainly of the T_H1/T_H0 phenotype (mean ratio, 272.35) (Fig 2a), and a further skewing toward T_H1 (mean ratio, 394.31; $P = 0.007$) was induced by APL treatment, with both higher production of IFN-γ (mean, 495.2 pg/ml at baseline and 635.6 pg/ml during therapy) and lower production of IL-4 (157.8 pg/ml at baseline and 68.7 pg/ml during therapy). The phenotype of T cells specific for MBP$_{(83–99)}$ remained stable (mean ratio, 225.12 at baseline and 227.56 during treatment). Among all 237 cross-reactive TCL, there were no phenotypic changes after stimulation with the respective other antigen.

As 'bystander' suppression by T cells specific for APL that cross-

Fig. 3 Axial brain MRI images before and during exacerbations of MS. In each patient, there is a substantial or atypical increase in brain inflammatory MRI activity. **a**, Patient MS502. Contrast-enhanced T1W images of three consecutive representative MRI slices at week 4 of APL therapy (top row) and during exacerbation 1 week later (bottom row). **b**, Patient MS601. Contrast-enhanced T1W images of three consecutive representative MRI slices at month 2 of APL therapy (top row) and during exacerbation 2 weeks later (bottom row). **c**, Patient MS503. Top row, At month 4 of APL therapy, there are two contrast-enhancing lesions in Patient MS503, adjacent to the frontal horn ventricle and to the occipital horn of the right lateral ventricle. Bottom row, One month later, the frontal lesion is no longer contrast-enhancing, whereas the occipital lesion has expanded into an unusually large tumor-like lesion with associated edema and mass effect. Within the next 3 months, the lesion contracted to a small MS plaque (less than 2 × 2 mm in size) without accompanying T1W hypointensity (data not shown).

ARTICLES

Table 2 Precursor frequencies of T cells specific for MBP$_{(83-99)}$ and APL in patients MS502 and MS601 during APL trial

MS patient	Ex vivo selecting antigen	Specificity and cross-reactivity	Pre-trial baseline	MS exacerbation during APL therapy	
			PBMC	PBMC	CSF
MS502	MBP$_{(83-99)}$	Specific for MBP$_{(83-99)}$ only	1 of 3.3 x 10^6	1 of 840	1 of 571
		Cross-reactive with APL	0	1 of 1400	1 of 800
	APL CGP771	Specific for APL only	1 of 1.2 x 10^6	1 of 620	1 of 1,200
		Cross-reactive with MBP$_{(83-99)}$	0	1 of 1320	1 of 2,400
MS601	MBP$_{(83-99)}$	Specific for MBP$_{(83-99)}$ only	1 of 3.0 x 10^6	1 of 0.4 x 10^6	1 of 667
		Cross-reactive with APL	0	1 of 1.2 x 10^6	NT
	APL CGP771	Specific for APL only	1 of 0.79 x 10^6	1 of 1.5 x 10^6	1 of 2,000
		Cross-reactive with MBP$_{(83-99)}$	1 of 2.8 x 10^6	1 of 3 x 10^6	NT

PBMC, peripheral blood mononuclear cell; NT, not tested.

were no significant changes in precursor frequency of MBP$_{(83-99)}$ in patients with relatively stable disease activity (Fig. 1 and Table 1).

## Treatment with APL may lead to exacerbations of MS

The three exacerbations of MS during APL treatment were characterized by atypical clinical and MRI features, raising concerns about a relationship to therapy (Fig. 3). Patient MS502 suffered from gait imbalance necessitating unilateral support after the fifth APL injection. There was also an unusual increase of brain inflammation in this patient (91 contrast-enhancing lesions; Fig. 3). Such MRI activity was very atypical for this patient, who had been monitored monthly for more than 9 years with more than 100 MRI scans; the number of enhancing lesions never exceeded 30 lesions. Patient MS601 was treated with a lower dose regimen (5 mg subcutaneously weekly for 4 weeks, followed by 5 mg monthly), and at month 3 developed an exacerbation with left hemiparesis and atypical flaccid lower extremity weakness rendering him non-ambulatory. The brain MRI showed increased inflammatory activity (increase in contrast-enhancing activity by 13 lesions between the two time points; Fig. 3) and the cord MRI demonstrated swelling and clumping of lumbosacral roots (data not shown). The latter was most likely due to an underlying diffuse but clinically silent demyelinating polyneuropathy. Patient MS503 reported the disappearance of her chronic symptoms during APL therapy, and her clinical and MRI measures improved substantially (Fig. 1). At her 5-month visit, an unusually large contrast-enhancing, right parieto-occipital lesion was found in this subjectively asymptomatic patient (Fig. 3). Neurological examination revealed a left superior homonymous quadrant anopia. All three patients recovered to their pre-exacerbation baseline after a course of standard intravenous steroid therapy.

To determine whether the exacerbations described above were treatment-related, we studied in detail T-cell reactivity to

with a negative value of –2 or less, and was stable between –1 and +1 (Table 1). As T cells specific for APL were induced in all patients (Fig. 1a) and given the very short treatment in patients MS501, MS502 and MS506, we included disease activity in the post-treatment phase before initiation of interferon therapy in the composite score (Table 1). Even when considered separately, the scores from treatment and post-treatment phases were consistent for each patient. Using these criteria, patients MS501 and MS503 had improved (Fig. 1 and Table 1; patient MS503 improved in all clinical and MRI measures except BWMLL, even though she suffered an exacerbation during treatment). Disease activity scores worsened in patients MS502, MS506 and MS601 and remained relatively stable in patients MS504, MS505 and MS507. We reached similar conclusions using only the primary outcome measure (the number of contrast-enhancing MRI lesions), with a previously confirmed cut-off of 60% for the significance of change[33] (Table 1). In comparing these data with immunological measures, only the change in precursor frequency of T cells specific for MBP$_{(83-99)}$ showed a significantly positive association ($P < 0.05$) with the composite score. All patients with significant increases in disease activity showed an increase in precursor frequency (Table 1). In contrast, there was improved disease activity in patients with decreases in precursor frequencies of T cells specific for MBP$_{(83-99)}$ (patients MS501 and MS503), and there

Fig. 4 T-cell reactivity to multiple myelin epitopes in patients MS502 (left) and MS601 (right). *a*, Primary proliferation assays. Vertical axes, sum of stimulation indices (Σ of SI) of all 'positive' cultures for a given antigen specificity. Inset, The CSF profile (assessed after the third *in vitro* re-stimulation) closely resembles that of PBLs. For patient MS601, both pre-treatment baseline (filled bars) and exacerbation (hatched bars) data are available. *b*, Cytokine phenotype of antigen-specific T cells based on the ratio of IFN-γ to IL-4: T$_H$0, hatched bars; T$_H$1, filled bars; T$_H$2, red hatched bars. Insets, CSF phenotypes. *c*, Functional antigen avidity of myelin-specific T cells. Left Patient MS601 only): baseline, peripheral blood mononuclear cells, □; exacerbation, peripheral blood mononuclear cells, ●; exacerbation, CSF cells, ▲.

a

b

c

d
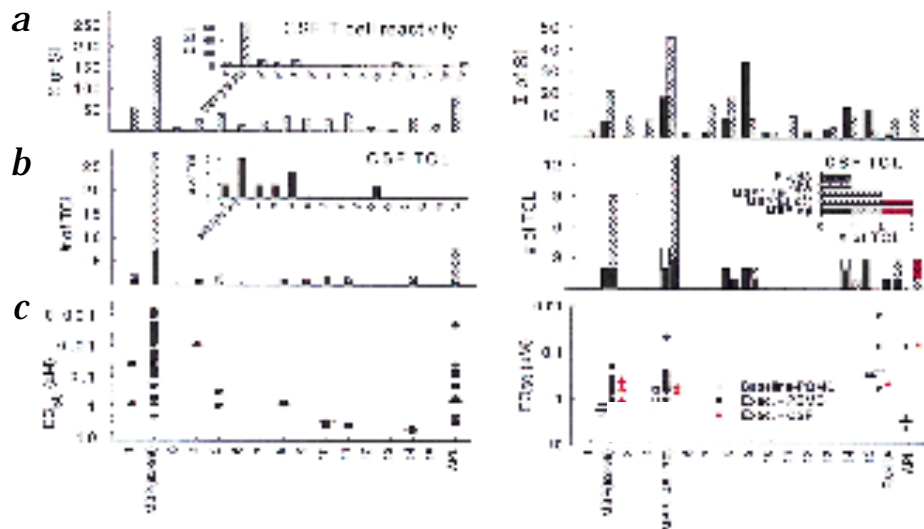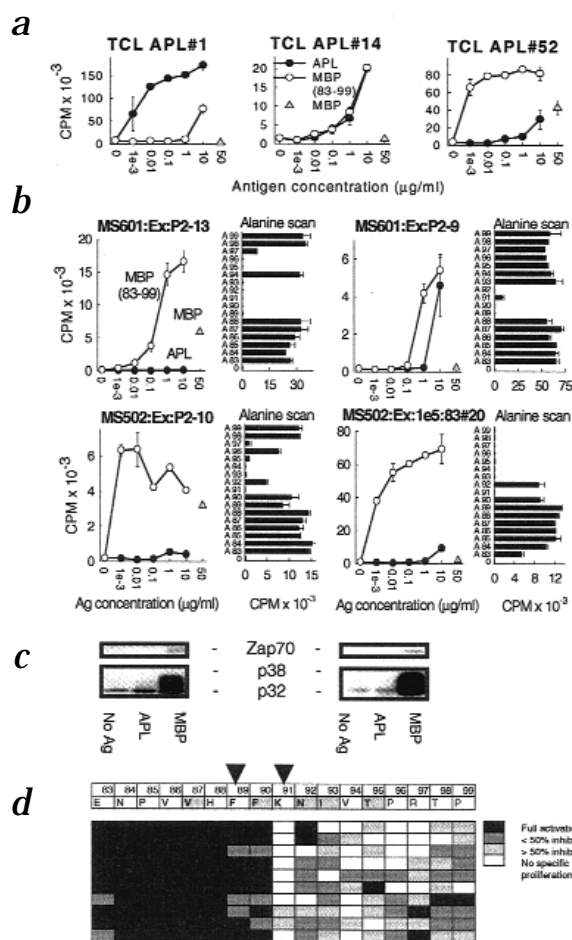
Fig. 5 Cross-reactivity patterns between MBP$_{(83-99)}$ and CGP77116 and delineation of the fine specificity of autoreactive T cells from patients MS502 and MS601. *a*, Regardless of the *in vitro* selecting stimulus (APL CGP77116, MBP$_{(83-99)}$ or IL-2), there are three different patterns of cross-reactivity between APL and MBP$_{(83-99)}$ in patient MS502 (data represent only APL-selected T cells): T cells with high avidity for the selecting antigen and low avidity for the cross-reactive antigen (TCL APL#1); T cells with intermediate, but similar avidities for both antigens (TCL APL#14); and T cells with low avidity for the selecting antigen but high avidity for the cross-reactive antigen (TCL APL#52). *b*, Fine specificity of selected T cells from patients MS502 and MS601 to alanine-substituted peptides (1 μM) scanning the MBP$_{(83-99)}$ sequence (alanine scan). Data represent two T cell clones from patient MS601 (top) and two from patient MS502 (bottom), showing the proliferation to both MBP$_{(83-99)}$ and CGP77116 in avidity assays (left) and to the alanine-substituted peptides (right) for each. CPM (*a* and *b*), counts per minute. *c*, Proximal TCR signaling for T-cell clones MS502:Ex:P2-10 (clonal population by TCR sequencing) (left) and MS502:Ex:1e5:83#20 (right). MBP$_{(83-99)}$ delivers a full agonist signal to both of these T cells. Ag, antigen. *d*, Top, predicted preferred binding motif of MBP$_{(83-99)}$ to HLA DRB1*0404. Downward arrowheads indicate positions 89 and 91 that are modified in the CGP 77116; HLA-DR contact positions filling pockets P1, P4, P6-7 and P9 are shaded. Bottom, fine-specificity testing of 10 high-avidity T cells specific for MBP$_{(83-99)}$ from patient MS502 (alanine scan); shades of grey, magnitude of response. Each line represents one T cell.

CGP77116 and MBP$_{(83-99)}$ as well as to 14 immunodominant myelin epitopes in detail both in peripheral blood and cerebrospinal fluid (CSF). In patient MS502, the precursor frequencies to both APL and MBP$_{(83-99)}$ increased more than 1,000-fold during exacerbation (1 of 840 for MBP$_{(83-99)}$ and 1 of 620 for APL; table 2) after *in vitro* stimulation with antigen or with IL-2 to expand *in vivo*-activated cells (1 of 920 for MBP$_{(83-99)}$ and 1 of 970 for CGP77116; data not shown). More than 50% of these T cells were cross-reactive (Table 2). T cells specific for MBP$_{(83-99)}$ were even further enriched in the CSF (1 of 571), whereas the precursor frequency of T cells specific for APL was slightly lower in the CSF compared to peripheral blood (1 of 1,200). Two weeks after treatment with intravenous steroids, the frequencies of T cells specific for MBP$_{(83-99)}$ and APL had dropped to 2-fold and 13-fold above baseline (Fig. 1*c*). There were also similarly high precursor frequencies of CSF T cells specific for APL and MBP$_{(83-99)}$ in patient MS601 at exacerbation (1 of 667 for MBP$_{(83-99)}$ and 1 of 2,000 for APL; Table 2), whereas T cells specific for MBP$_{(83-99)}$ in the PBLs had only increased about 7-fold. Of the PBLs specific for MBP$_{(83-99)}$, 35% cross-reacted with APL. Unlike the results in those patients, there was only a twofold increase in T cells specific for APL in patient MS503. T cells specific for MBP$_{(83-99)}$ completely disappeared (Table 1), and antigen-specific T cells could not be cloned from the CSF.

To determine whether myelin reactivity was directed mainly against MBP$_{(83-99)}$ or other myelin peptides, we tested PBLs and CSF lymphocytes against a panel of 15 myelin peptides derived

from MBP (*n* = 5), proteolipid protein (PLP) (*n* = 5), myelin oligodendrocyte glycoprotein (MOG) (*n* = 3) and 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNP) (*n* = 2). We chose these because of their immunodominance in humans and/or encephalitogenicity in EAE. The main myelin reactivity of PBLs and CSF T cells in patient MS502 was directed against MBP$_{(83-99)}$ (Fig. 4*a*, left), with some intramolecular spreading against other MBP peptides in the CSF response (Fig. 4*a*, inset). The relevance of the T-cell response against MBP$_{(83-99)}$ was further supported by the high antigen avidity (response at low antigen concentrations) and strong cytokine response of T_H0/T_H1 (PBL) and pure T_H1 (CSF) phenotype (Fig. 4*b* and *c*, left). TCL that were generated at the time of exacerbation from PBLs by limiting dilution were also strongly skewed toward T_H1 (mean IFN-γ:IL4 ratios, 183.7 and 451.3 for T cells specific for MBP$_{(83-99)}$ and APL, respectively; data not shown) and had significantly higher antigen avidities than did other autoreactive T cells and T cells specific for APL (*P* < 0.05), with 7 of 28 CD4$^+$ T cells specific for MBP$_{(83-99)}$ showing very high antigen avidities (EC$_{50}$ (dose leading to half-maximal stimulation), less than 0.001 μM) comparable to those typically seen for virus-specific T-cell responses[34] (Fig. 4*c*, left .

Although the antigen-specificity pattern was not as uniform in patient MS601 (Fig. 4*a*, right), the reactivity to MBP (MBP$_{(83-99)}$, MBP$_{(146-170)}$ and PLP$_{(89-106)}$) increased significantly compared with baseline, whereas the reactivities to all other autoantigens were marginal or decreased (Fig. 4*a*, right). The relevance of the two MBP epitopes was further supported by the fact that the primary proliferative responses were confirmed in long-term TCL, they mounted the strongest cytokine response (T_H0/T_H1; Fig. 4*b*, right), had the highest antigen avidities (Fig. 4*c*, right ; particularly CSF T cells), and only reactivities to MBP$_{(83-99)}$, MBP$_{(146-170)}$, whole MBP protein, APL and Influenza hemagglutinin peptide (Flu-HA) could be found in the CSF. Finally, MBP$_{(83-99)}$ was the dominant reactivity in the CSF compartment (Fig. 4*c*, right, inset). Whether the differences in cytokine phenotype and antigen avidities between patients MS502 and MS601 were due to the lower-dose regimen given to patient MS601 and/or to the pre-existing heterogeneity in T-cell reactivity between these patients is not clear.

ARTICLES

Although the data described above indicate a correlation between APL therapy and exacerbations in patients MS502 and MS601, patient MS503 was different in that she did well during 5 months of APL therapy compared with study baseline and the previous 2 years of monthly MRI follow-up, until she developed a large, tumor-like lesion with edema and mass effect (Fig. 3) compared with study baseline and the previous 2 years of monthly MRI follow-up. Furthermore, T cells specific for $MBP_{(83–99)}$ disappeared during APL therapy (Table 1 and data not shown), and the determination of fine specificities demonstrated an expansion of T cells specific for APL and those epitopes that were immunodominant during baseline: $PLP_{(89–106)}$ and $MBP_{(146–170)}$ (data not shown). T cells specific for $MBP_{(83–99)}$ could not be demonstrated by primary proliferation or by limiting dilution. All autoreactive TCL and TCL specific for APL at the time of exacerbation expressed a T_H1 phenotype, and CSF T cells produced very large amounts of IFN-γ after primary bulk *ex vivo* stimulation with APL (10864.69 ± 476.01 pg/ml) and PLP epitopes (8721.34 ± 3809.41 pg/ml), but less than 30 pg/ml IL-4 (not shown).

Cross-reactivity between APL and $MBP_{(83–99)}$

For all patients, cross-recognition of $MBP_{(83–99)}$ and APL was readily induced by treatment with APL (Fig. 2). For the exacerbations of MS, we sought to determine whether cross-reactivity had contributed to unwanted activation of pathogenic T cells. In patient MS502, regardless of the *in vitro* selecting stimulus (that is, whether cells had been expanded with $MBP_{(83–99)}$ or APL), there were cross-reactive TCL with higher avidity for the selecting antigen (Fig. 5a, APL#1), comparable reactivity to either antigen (Fig. 5a, APL#14), or even stronger responsiveness to the cross-reactive antigen (Fig. 5a, APL#52). We readily identified the first two types of cross-reactivity during APL therapy in all patients except patient MS503, whereas the third pattern, and particularly the occurrence of T cells with very high avidity for $MBP_{(83–99)}$, was rare and only occurred in patients MS502, MS506 and MS507 (data not shown for patients MS506 and MS507). We confirmed the clonality of cross-reactive T-cell cultures by TCR variable region chain staining using flow cytometry, by single-stranded conformational polymorphism analysis and by Vα/Vβ-TCR sequencing in selected clones (data not shown).

As such strong cross-reactivity was not expected from extensive *in vitro* testing of many APLs, particularly as two important TCR contacts (residues F89 and K91) had been altered in CGP77116, we sought to delineate the epitope core for selected clones from patients MS502 and MS601 with L-Ala-substituted peptides scanning the $MBP_{(83–99)}$ sequence (Fig. 5b). The regions of the peptide in which substitutions were not tolerated varied for each of the four representative clones. In clone MS601:P2-13, which did not cross-react with APL CGP77116, both F89A and K91A completely abrogated T-cell recognition. However, in the other cross-reactive clones one of the essential substitutions (F89A or K91A) was at least partially recognized, particularly when the L-Ala-substituted peptides were tested not only at a concentration of 1 μM (Fig. 5b) but also with a wide range of concentrations (10–100μM). In this case, clones MS502:P2-10 and MS502:83#20 proliferated well in response to both substituted peptides (F89A and K91A; data not shown), indicating that high concentrations of CGP77116 may fully activate such T-cell clones.

Further evidence that supported the biological relevance of T cells expanded by CGP77116 were the very high antigen avidi-

ties of some clones (Fig. 4c and 5b). Full titration with $MBP_{(83–99)}$ down to a concentration of 0.01 pM demonstrated an EC_{50} below 1 pM (data not shown) for clones MS502:P2-10 and MS502:83#20. Both were T_H1/T_H0, and stimulation with $MBP_{(83–99)}$ was able to deliver a full TCR agonist signal; that is, fully phosphorylated TCR ζ-chain p38 and recruitment and phosphorylation of Zeta associated protein 70 (Zap70) kinase[35] (Fig. 5c), which is usually only seen with clones specific for foreign antigen, but not with autoreactive clones[36]. APL stimulation, in contrast, delivered a partial agonist signal to these T cells; that is, low-molecular-weight isoform of TCR ζ-chain, p32 and no Zap70 recruitment (Fig. 5c).

The heterogeneity of the response of T cells specific for $MBP_{(83–99)}$ after treatment with CGP 77116 is underscored by data depicted in figure 5d. We tested 10 additional T cells isolated from patient MS502 at exacerbation against L-Ala-substituted $MBP_{(83–99)}$ peptides presented by DRB1*0404 transfectants, as most clones were restricted by DRB1*0404 (data not shown). We determined the preferred HLA-DRB1*0404-binding motif for $MBP_{(83–99)}$ (ref. 37) (Fig. 5d, top; amino acids filling the pockets P1, P4, P6–7 and P9 are highlighted). Testing the 10 clones with L-Ala scanning peptides indicated that the epitope core of each was shifted toward the C terminus of $MBP_{(83–99)}$ (amino acids 91–99). Only the K91A substitution significantly altered the recognition of most T cells; F89A was generally well tolerated. Thus, in agreement with previous data, K91 represents the TCR contact for most of these T cells, but their cross-recognition of CGP77116 indicates that modifications of this position do not completely abrogate recognition. We conclude that CGP 77116 contains sufficiently long stretch of unmodified sequence of the $MBP_{(83–99)}$ that allowed in patient MS502 the cross stimulation of DRB1*0404-restricted T cells specific for the native peptide $MBP_{(83–99)}$.

Discussion

This phase II clinical trial of APL in humans showed the following: The selected dosing regimen was poorly tolerated. APL treatment in this small cohort did not generally improve or worsen disease, although there was a positive association between clinical/MRI outcomes and the changes in precursor frequencies of T cells specific for $MBP_{(83–99)}$. In contrast to *in vitro* studies, CGP77116 was very immunogenic *in vivo* and resulted in substantial expansion of T cells specific for this APL that were cross-reactive with the native autoantigen. De novo-activated T cells specific for APL often expressed a pro-inflammatory phenotype. Finally, in two of three patients, an exacerbation of MS could be linked to an APL-induced expansion of T cells specific for $MBP_{(83–99)}$, demonstrating its encephalitogenic potential.

As for the mode of action of APL, several mechanisms have been shown in EAE. These include influences on the pathogenic T-cell population at the level of the TCR (partial agonism, antagonism and T-cell anergy)[27,29,38] [26,30] and the induction of immunoregulatory T cells specific for APL (ref. 24). The latter are able to produce anti-inflammatory cytokines after cross-activation with autoantigen, i.e. 'bystander' suppression[24]. In this clinical trial, the induction of anergy or TCR antagonism should have decreased the precursor frequencies of T cells specific for $MBP_{(83–99)}$. This occurred in only two of eight patients (Table 1), and given the considerable flexibility of TCR recognition (ref. 39 and Fig. 5d), it seems unlikely that a single APL will antagonize all T cells specific for one epitope *in vivo*. Furthermore, structurally related ligands may exert a broad spectrum of functional

effects *in vivo*, such as decreasing the antigen sensitivity of T cells during maturation[40] or enhancing the responsiveness to native antigen after expansion with APL (ref. 41). This phenomenon may explain cross-reactivity pattern 3 (Fig. 5*a*), with heteroclitic responses to the native peptide despite the fact that these T cells had been expanded *in vitro* with the weaker agonist (the APL). Here, *in vivo* vaccination resulted in the activation of T cells with a wide range of antigen-avidities, fine specificities and restriction patterns even within one individual (patient MS502; Fig. 5). This flexibility of antigen recognition indicates that there may always be T cells that cross-react with the native autoantigen, which may preclude the use of APL without careful consideration of the HLA background and pre-existing T-cell repertoire to myelin antigens in individual patients.

Thus, the remaining effector mechanism for APL therapy is 'bystander' suppression. We believe that this is the only mechanism that may be achievable *in vivo* in humans based on the following considerations. Here, there was *in vivo* expansion of T cells specific for APL in every treated patient. Furthermore, a large fraction of these cells was cross-reactive with the native peptide. Although this important prerequisite for 'bystander' suppression (the de novo expansion of cross-reactive T cells specific for APL) was achieved, these cells often did not express the therapeutically desired anti-inflammatory phenotype, but were T_H1 instead. The inability of CGP77116 to induce cross-reactive immunomodulatory T cells *in vitro* (refs. 38,42) unpublished data) led to our selection of the high dose for treatment. Contrary to these expectations, CGP77116 proved to be very immunogenic and induced a state of 'hyperimmunization', emphasizing that *in vitro* data cannot easily be extrapolated to the *in vivo* situation. Consistent with experimental data that high antigen doses skew a developing immune response toward T_H1 (ref. 43), the high-dose regimen (50 mg weekly) is likely to account for the T_H1 bias of T cells specific for APL. Patient MS601, who was immunized with a lower dose, had mainly a T_H0 response; furthermore, the immunological data from phase I testing indicate a trend toward T_H2 in individuals treated with this low dose of APL (P. Crowe, personal communication). It will therefore be useful in the future to identify the optimal dosing regimen, consider alternative routes of administration (intranasal or oral) or use T_H2-skewing adjuvants to minimize the chances of inducing a pro-inflammatory response.

Although evidence from animal models indicates certain myelin peptides as targets in EAE (refs. 1,7,8,44), their relevance in MS is still controversial. At the beginning of this century, rabies vaccination provided the first indication that myelin components may induce an encephalomyelitis[45] in some vaccinees, which, as documented 30 years later, probably resulted from a vaccine contaminant by rabbit spinal cord components[46]. These studies became the foundation for the development of EAE as an animal model for MS (refs. 47,48). The current pathogenetic concept of MS is based on parallels to EAE and assumes that autoreactive T_H1 cells are activated in susceptible individuals most likely by cross-reactive foreign agents[49]. The inadvertent results of our study indicate that T cells specific for $MBP_{(83–99)}$ may become encephalitogenic and induce exacerbations in certain patients with MS. This conclusion is based on the temporal coincidence of clinical relapses, a considerable and atypical increase of brain inflammation and an expansion of more than 1,000-fold of T cells specific for $MBP_{(83–99)}$ and APL, with a high percent of them cross-reacting with each other in patients MS502 and MS601. With the isolation of these cells from the CSF

at even higher precursor frequencies, their high antigen avidity, T_H1 phenotype and full agonist response to $MBP_{(83–99)}$, there remains little doubt that the administration of APL activated disease-mediating T cells.

Despite these results, which initially seem disappointing, our data indicate that T cells specific for $MBP_{(83–99)}$ in MS are a valid target for antigen-specific immunomodulation at least in some patients with MS. The immunological data from the third patient, MS503, demonstrated further that $MBP_{(83–99)}$ is clearly only one of the relevant autoantigens in MS and is not responsible for all MS attacks. Given the heterogeneity of MS in immune function and genetic background[1], which translates into different pathological disease patterns[10] and also differences in MRI and clinical phenotypes[9], the observations in patient MS503 could indicate that different effector mechanisms and reactivity to different autoantigens contributed to her exacerbation compared with those of patients MS502 and MS601.

The finding of the encephalitogenic potential of $MBP_{(83–99)}$ in MS has relevance for many aspects of autoimmune disease research. It emphasizes that the extrapolations from EAE and human *in vitro* studies are valid for MS and that target antigens identified in these systems are important. Our observations, however, also indicate that a better understanding of the complexities of the pathogenesis of T cell-mediated autoimmune diseases and their heterogeneity in individual patients is required before specific immunotherapies can be applied both effectively and safely.

Methods

Study design and subjects. The trial design stipulated a 6-month baseline followed by 9 months treatment with monthly clinical and MRI examinations and immunological assessments. Patients 18–55 years of age with clinically definite relapsing–remitting MS and an EDSS score (ref. 31) of 7.0 were enrolled. Inclusion criteria required one or more relapses in the 2 preceding years and a mean of at least 0.5 gadolinium-enhancing lesions during baseline. Relapses were defined according to Schumacher criteria[50]. The primary outcome measure was the change in mean number of contrast-enhancing MRI lesions. The secondary outcome measures included the change in mean T2 BWMLL, change in EDSS, relapse rate and precursor frequency of T cells specific for $MBP_{(83–99)}$ and CGP77116.

The trial was approved by the National Institute of Neurological Disorders and Stroke institutional review board. Patients were not pre-selected for certain HLA-DR haplotypes, as $MBP_{(83–99)}$ is a 'promiscuous' HLA-DR binder[16,17], and each patient was expected to express at least one DR allele to which the APL would bind. CGP77116 was administered weekly at a dose of 50 mg by subcutaneous injections in aqueous solution. After reviewing the data from the first seven patients (identified as MS501–MS507), the dosing regimen was changed to 5 mg weekly for the first 4 weeks followed by 5 mg monthly. When patient MS601, the only patient enrolled in this regimen, suffered an exacerbation that was considered treatment-related, the trial was halted.

MRI. MRI scans were acquired at 1.5 Tesla with proton density/T2-weighted spin echo-, fluid attenuation inversion recovery-, and T1-weighted pulse sequences before and after contrast administration (Magnevist 0.1 mmol/kg; Berlex Laboratories, Wayne, New Jersey). Contrast-enhancing lesions were recorded on hard-copy films after a consensus of two neuroradiologists. T2 BWMLL was determined using a semi-automated thresholding technique [51].

Antigens. MBP was prepared according to a published procedure[52]. Peptides $MBP_{(83–99)}$, Flu-$HA_{(306–318)}$ and APL CGP77116 were synthesized by Merrifield's solid phase methodology as described[53]; CGP77116 (provided by N. Ling, Neurocrine Biosciences, San Diego, California) contains sequence modifications in the positions indicated by X (lower case, substitution by a D-amino acid): xXPVVHXFXNIVTPRTP (native sequence, ENPVVHFFKNIVTPRTP). A panel of 15 immunodominant peptides derived

ARTICLES

from MBP, PLP, MOG or CNP were synthesized by Merrifield's solid-phase approach in conjunction with the method of simultaneous multiple peptide synthesis[54] (provided by R. Houghten, Mixture Sciences, San Diego, California) and were characterized by reverse-phase high-performance liquid chromatography and mass spectrometry. Peptides are coded as follows: P1=MBP$_{(13-32)}$,2=MBP$_{(83-99)}$,P3=MBP$_{(111-129)}$,P4=MBP$_{(131-155)}$,P5=MBP$_{(146-170)}$P6= PLP$_{(40-60)}$,P7=PLP$_{(89-106)}$,P8=PLP$_{(139-154)}$,P9=PLP$_{(178-197)}$,P10=PLP$_{(190-209)}$,P11=MOG$_{(1-20)}$,P12=MOG$_{(11-30)}$,P13=MOG$_{(35-55)}$,P14=CNPase$_{(343-373)}$,P15=CNPase$_{(356-388)}$,Flu-Ha=Flu-Ha$_{(305-318)}$ and APL=CGP77116.

Primary proliferation and limiting dilution analysis. For limiting dilution analysis[55], PBLs were seeded at a density of 1×10^3–2×10^5 PBLs/well and stimulated with MBP$_{(83-99)}$ or CGP77116 (10 µg/ml), and were re-stimulated periodically[15]. Precursor frequency estimates were based on the number of positive wells/seeded cells as described[55]. To estimate the frequencies of in vivo-activated T cells[56], PBLs were first expanded with 20 international units/ml IL-2, and after 7 d were tested for reactivity to both APL and MBP$_{(83-99)}$.

CSF cells were seeded at a density of 100–200 cells/well, with 10–20 wells per antigen (depending on the number of cells available), with 2×10^5 irradiated (3,000 R) peptide-pulsed (1 µM) PBLs. After periodical re-stimulation, T cells were tested for antigen specificity. Precursor frequencies were estimated based on the number of positive wells/seeded cells.

T-cell reactivity to other myelin proteins (15 immunodominant peptides derived from MBP, PLP, MOG and CNP) was tested by IL-7-modified primary proliferation using 10 ng/ml recombinant IL-7 (Pepro-Tech, Rocky Hill, New Jersey), which also expands in vivo-activated T cells[34]. Cells (1×10^5 cells/well, 60 wells/antigen, at a concentration of µM) were seeded and incubated for 7 d, before cultures were split and pulsed with ^{3}H-thymidine. Positive cultures (with a stimulation index greater than 2 and thymidine incorporation values (counts per minute) 3 s.d. above the mean counts per minute of 60 negative control wells) were periodically re-stimulated.

Functional antigen avidity and cross-reactivity. Antigen-specific TCL were tested with a range of antigen concentrations from 0.001 to 10 µg/ml in 3-day proliferative assays[57]. The EC$_{50}$ was calculated from these dose–response curves. Culture supernatants were collected at 36 h and frozen at –30 ºC for further analysis.

Cytokine analysis. Secretion of IFN-γ and IL-4 was detected by enzyme-linked immunosorbent assay (Cyto-Sets; BioSource International, Camarillo, California). Based on the ratio of IFN-γ to IL-4, cells were categorized as T$_H$1 (ratio, > 100), T$_H$0 (ratio, between 0.01 and 100) and T$_H$2 (ratio, < 0.01). This categorization was modified from published studies that used a ratio of greater than 10 to classify the T$_H$1 phenotype[57]. However, as most T cells produce nanogram quantities of IFN-γ, but usually only 100-pg quantities of IL-4 (refs. 34,57), our strict criterion for T$_H$1 categorization better reflects the functional characterization and potential encephalitogenicity of T cells.

T-cell receptor signaling. T cells (1×10^6) were added to peptide-pulsed autologous B cells transformed by Epstein-Bar virus (2×10^6 cells) and, after centrifugation (10 s at 300g),were incubated at 37 °C for 5 min, washed once with PBS and incubated for 25 min on ice in lysis buffer (1% Nonidet P-40, 10mM Tris-HCl, pH 7.2, 140 mM NaCl, 2 mM EDTA, 5 mM iodoacetamide, 1 mM Na$_3$VO$_4$ and complete protease inhibitor 'cocktail'; Boehringer). After removal of nuclear debris, lysate supernatants were subjected to immunoprecipitation by incubation at 4°C for 12 h with rabbit antibody against Zap70. Samples were separated by SDS–PAGE and immunoblotted with 4G10, a mouse monoclonal antibody against phosphotyrosine (Upstate Biotechnology, Lake Placid, New York).

Statistical analysis. A commercial software package (Sigma-Stat 2.03; SPSS Science, Chicago, IL) was used. Because of the small number of patients and the non-Gaussian distribution, non-parametric tests were applied for most analyses. Changes in clinical and MRI parameters were evaluated by the Mann Whitney rank sum test; primary proliferation, precursor frequency and cross-reactivity, by the Wilcoxon signed rank test (two time points) or by one-way repeated measures analysis on ranks in conjunction with the Student-Newman-Keuls method of pair-wise multiple comparisons (more than two time points); and differences in cytokine production, by the t-test. The significance of association between clinical outcome (composite score) and change in precursor frequency of T cells specific for MBP$_{(83-99)}$ was evaluated by the chi-square test. The EC$_{50}$ between different antigen-specific T cells was evaluated by the Mann Whitney rank sum test. Results were considered statistically significant at $P < 0.05$.

1. Martin, R., McFarland, H.F. & McFarlin, D.E. Immunological aspects of demyelinating diseases. *Annu. Rev. Immunol.* **10**, 153–187 (1992).
2. Lassmann, H. Neuropathology in multiple sclerosis: new concepts. *Mult. Scler.* **4**, 93–98 (1998).
3. Raine, C.S. in *Multiple Sclerosis* (eds. Hallpike, J.F., Adams, C.W. & Tourtellotte, W.W.) 413–478 (Williams & Wilkins, Baltimore, 1983).
4. Trapp, B.D. *et al.* Axonal transection in the lesions of multiple sclerosis. *N. Engl. J. Med.* **338**, 278–285 (1998).
5. Steinman, L. Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* **85**, 299–302 (1996).
6. Hafler, D.A. & Weiner, H.L. MS: a CNS and systemic autoimmune disease. *Immunol. Today* **10**, 104–107 (1989).
7. Wekerle, H., Linington, C., Lassmann, H. & Meyermann, R. Cellular immune reactivity within the CNS. *Trends. Neuro. Sci.* **9**, 271–277 (1986).
8. Madsen, L.S. *et al.* A humanized model for multiple sclerosis using HLA-DR2 and a human T-cell receptor. *Nature Genet.* **23**, 343–347 (1999).
9. Lassmann, H., Raine, C.S., Antel, J. & Prineas, J.W. Immunopathology of multiple sclerosis: report on an international meeting held at the Institute of Neurology of the University of Vienna. *J. Neuroimmunol.* **86**, 213–217 (1998).
10. Lucchinetti, C.F., Brueck, W., Rodriguez, M. & Lassmann, H. Multiple sclerosis: lessons from neuropathology. *Semin. Neurol.* **18**, 337–349 (1998).
11. Zamvil, S.S. & Steinman, L. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol.* **8**, 579–621 (1990).
12. Ota, K. *et al.* T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis. *Nature* **346**, 183–187 (1990).
13. Martin, R. *et al.* Fine specificity and HLA restriction of myelin basic protein-specific cytotoxic T cell lines from multiple sclerosis patients and healthy individuals. *J. Immunol.* **145**, 540–548 (1990).
14. Fritz, R.B. & McFarlin, D.E. in *Antigenic Determinants and Immune Response. Chem. Immunol.* Vol **46** (ed. Sercarz, E.E.) 101–125 (Karger, Basel, 1989).
15. Pette, M. *et al.* Myelin autoreactivity in multiple sclerosis: recognition of myelin basic protein in the context of HLA-DR2 products by T lymphocytes of multiple sclerosis patients and healthy donors. *Proc. Natl. Acad. Sci. USA* **87**, 7968–7972 (1990).
16. Valli, A. *et al.* Binding of myelin basic protein peptides to human histocompatibility leukocyte antigen class II molecules and their recognition by T cells from multiple sclerosis patients. *J. Clin. Invest.* **91**, 616–628 (1993).
17. Martin, R. *et al.* A myelin basic protein peptide is recognized by cytotoxic T cells in the context of four HLA-DR types associated with multiple sclerosis. *J. Exp. Med.* **173**, 19–24 (1991).
18. Krogsgaard, M. *et al.* Visualization of myelin basic protein (MBP) T cell epitopes in multiple sclerosis lesions using a monoclonal antibody specific for the human histocompatibility leukocyte antigen (HLA)-DR2-MBP 85-99 complex. *J. Exp. Med.* **191**, 1395–1412 (2000).
19. Vandenbark, A.A., Hashim, G. & Offner, H. Immunization with a synthetic T-cell receptor V-region peptide against experimental autoimmune encephalomyelitis. *Nature* **341**, 541–544 (1989).
20. Howell, M.D. *et al.* Vaccination against experimental allergic autoimmune encephalomyelitis with T cell receptor peptides. *Science* **246**, 668–670 (1989).
21. Wraith, D.C., McDevitt, H.O., Steinman, L. & Acha-Orbea, H. T cell recognition as the target for immune intervention in autoimmune disease. *Cell* **57**, 709–715

(1989).

22. Acha-Orbea, H. *et al.* Limited heterogeneity of T cell receptors from lymphocytes mediating autoimmune encephalomyelitis allows specific immune intervention. *Cell* **54**, 263–273 (1988).

23. Hohlfeld, R. Biotechnological agents of the immunotherapy of multiple sclerosis. Principles, problems and perspectives. *Brain* **120**, 865–916 (1997).

24. Nicholson, L.B., Greer, J.M., Sobel, R.A., Lees, M.B. & Kuchroo, V.K. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity* **3**, 397–405 (1995).

25. Karin, N., Mitchell, D.J., Brocke, S., Ling, N. & Steinman, L. Reversal of experimental autoimmune encephalomyelitis by a soluble peptide variant of a myelin basic protein epitope: T cell receptor antagonism and reduction of interferon γ and tumor necrosis factor α production. *J. Exp. Med.* **180**, 2227–2237 (1994).

26. Brocke, S. *et al.* Dynamics of autoimmune T cell infiltration: reversal of paralysis and disappearance of inflammation following treatment of experimental encephalomyelitis with a myelin basic protein peptide analog. *Nature* **379**, 343–346 (1996).

27. De Magistris, M.T. *et al.* Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell* **68**, 625–634 (1992).

28. Sloan-Lancaster, J., Shaw, A.S., Rothbard, J.B. & Allen, P.M. Partial T cell signaling: Altered phospho-ζ and lack of zap70 recruitment in APL-induced T cell anergy. *Cell* **79**, 913–922 (1994).

29. Madrenas, J. *et al.* ζ phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science* **167**, 515–518 (1995).

30. Windhagen, A. *et al.* Modulation of cytokine patterns of human autoreactive T cell clones by a single amino acid substitution of their peptide ligand. *Immunity* **2**, 373–380 (1995).

31. Kurtzke, J.F. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). *Neurology* **33**, 1444–52 (1983).

32. Sharrack, B. & Hughes, R.A. Clinical scales for multiple sclerosis. *J. Neurol. Sci.* **135**, 1–9 (1996).

33. Stone, L.A. *et al.* Characterization of MRI response to treatment with interferon beta-1b: contrast-enhancing MRI lesion frequency as a primary outcome measure. *Neurology* **49**, 862–869 (1997).

34. Bielekova, B. *et al.* Preferential expansion of autoreactive T lymphocytes from the memory T-cell pool by IL-7. *J. Neuroimmunol.* **100**, 115–123 (1999).

35. Germain, R.N. & Stefanova, I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annu. Rev. Immunol.* **17**, 467–522 (1999).

36. Hemmer, B., Stefanova, I., Vergelli, M., Germain, R.N. & Martin, R. Relationships among TCR ligand potency, thresholds for effector function elicitation, and the quality of early signaling events in human T cells. *J. Immunol.* **160**, 5807–5814 (1998).

37. Sturniolo, T. *et al.* Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nature Biotechnol.* **17**, 555–561 (1999).

38. Vergelli, M. *et al.* Differential activation of human autoreactive T cell clones by altered peptide ligands derived from myelin basic protein peptide (87-99). *Eur. J. Immunol.* **26**, 2624–2634 (1996).

39. Mason, D. A very high level of crossreactivity is an essential feature of the T- cell receptor. *Immunol. Today* **19**, 395–404 (1998).

40. Lucas, B., Stefanova, I., Yasutomo, K., Dautigny, N. & Germain, R.N. Divergent changes in the sensitivity of maturing T cells to structurally related ligands underlies formation of a useful T cell repertoire. *Immunity* **10**, 367–376 (1999).

41. Nicholson, L.B., Anderson, A.C. & Kuchroo, V.K. Tuning T cell activation threshold and effector function with cross-reactive peptide ligands. *Int. Immunol.* **12**, 205–213 (2000).

42. Hemmer, B. *et al.* Human T-cell response to myelin basic protein peptide (83–99): Extensive heterogeneity in antigen recognition, function, and phenotype. *Neurology* **49**, 1116–1126 (1997).

43. Constant, S.L. & Bottomly, K. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu. Rev. Immunol.* **15**, 297–322 (1997).

44. Tuohy, V.K., Sobel, R.A. & Lees, M.B. Myelin proteolipid protein-induced experimental allergic encephalomyelitis. Variations of disease expression in different strains of mice. *J. Immunol.* **140**, 1868–1873 (1988).

45. Remlinger, J. Accidents paralytiques au cours du traitment antirabique. *Ann. Inst. Pasteur* **19**, 625–646 (1905).

46. Rivers, T.M., Sprunt, D.H. & Berry, G.P. Observations on attempts to produce acute disseminated encephalomyelitis in monkeys. *J. Exp. Med.* **58**, 39–53 (1933).

47. Paterson, P.Y. Transfer of allergic encephalomyelitis in rats by means of lymph node cells. *J. Exp. Med.* **111**, 119–133 (1960).

48. Pettinelli, C.B. & McFarlin, D.E. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vivo activation of lymph node cells by myelin basic protein: requirement for Lyt-1+2- T lymphocytes. *J. Immunol.* **127**, 1420–1423 (1981).

49. Fujinami, R.S. & Oldstone, M.B.A. Amino Acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science* **230**, 1043–1045 (1985).

50. Schumacher, G.A. Problems of experimental trials of therapy in multiple sclerosis: Report by the panel on the evaluation of experimental trials in multiple sclerosis. *Ann. New York Acad. Sci.* **112**, 552–568 (1965).

51. DeCarli, C. *et al.* Method for quantification of brain, ventricular, and subarachnoid CSF volumes from MR images. *J. Comput. Assist. Tomogr.* **16**, 274–284 (1992).

52. Deibler, G.E., Martenson, R.E. & Kies, M.W. Large scale preparation of myelin basic protein from central nervous system tissue of several mammalian species. *Prep. Biochem.* **2**, 139–165 (1972).

53. Ling, N. *et al.* Isolation, primary structure, and synthesis of human hypothalamic somatocrinin: growth hormone-releasing factor. *Proc. Natl. Acad. Sci. USA* **81**, 4302–4306 (1984).

54. Houghten, R.A. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. *Proc. Natl. Acad. Sci. USA* **82**, 5131–5135 (1985).

55. Lefkovits, I. & Waldmann, H. Limiting dilution analysis of the cells of immune system. The clonal basis of the immune response. *Immunol. Today* **5**, 265–277 (1984).

56. Zhang, J. *et al.* Increased frequency of interleukin-2-responsive T cells specific for myelin basic protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J. Exp. Med.* **179**, 973–984 (1994).

57. Muraro, P.A., Pette, M., Bielekova, B., McFarland, H.F. & Martin, R. Human autoreactive CD4+ T cells from naive CD45RA+ and memory CD45RO+ subsets differ with respect to epitope specificity and functional antigen avidity. *J. Immunol.* **164**, 5474–5481 (2000).