Autopathogenic T Helper Cell Type 1 (Th1) and Protective Th2 Clones Differ in Their Recognition of the Autoantigenic Peptide of Myelin Proteolipid Protein

By Mercy Prabhu Das,* Lindsay B. Nicholson,* Judith M. Greer,‡ and Vijay K. Kuchroo*

*From the *Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115; and the ‡Department of Medicine, Royal Brisbane Hospital, University of Queensland, Herston Q4029, Australia*

Summary

We previously generated a panel of T helper cell 1 (Th1) clones specific for an encephalitogenic peptide of myelin proteolipid protein (PLP) peptide 139–151 (HSLGKWLGHPDKF) that induces experimental autoimmune encephalomyelitis (EAE) upon adoptive transfer. In spite of the differences in their T cell receptor (TCR) gene usage, all these Th1 clones required W144 as the primary and most critical TCR contact residue for the activation. In this study, we determined the TCR contact residues of a panel of Th2/Th0 clones specific for the PLP peptide 139–151 generated either by immunization with the PLP 139–151 peptide with anti–B7-1 antibody or by immunization with an altered peptide Q144. Using alanine-substituted peptide analogues of the native PLP peptide, we show that the Th2 clones have shifted their primary contact residue to the NH_2-terminal end of the peptide. These Th2 cells do not show any dependence on the W144, but show a critical requirement for L141/G142 as their major TCR contact residue. Thus, in contrast with the Th1 clones that did not proliferate to A144-substituted peptide, the Th2 clones tolerated a substitution at position 144 and proliferated to A144 peptide. This alternative A144 reactive repertoire appears to have a critical role in the regulation of autoimmune response to PLP 139–151 because preimmunization with A144 to expand the L141/G142-reactive repertoire protects mice from developing EAE induced with the native PLP 139–151 peptide. These data suggest that a balance between two different T cell repertoires specific for same autoantigenic epitope can determine disease phenotype, i.e., resistance or susceptibility to an autoimmune disease.

Experimental autoimmune encephalomyelitis (EAE)[1], is an example of an organ-specific autoimmune disease that can be induced in experimental animals by immunization with several different myelin proteins, including myelin basic protein (MBP) and myelin proteolipid protein (PLP). EAE is mediated by myelin antigen-reactive helper T cells because elimination of MBP reactive $Ly1^+$, but not $Ly2^+$, cells prevents transfer of EAE (1, 2), and monoclonal antibodies specific for CD4 can inhibit and/or reverse ongoing disease (3, 4). Direct evidence for the role of $CD4^+$ T cells in EAE induction has come from adoptive transfer studies in which MBP- and PLP-reactive $CD4^+$ T cell lines or clones were shown to induce chronic relapsing encephalomyelitis and paralysis after transfer (2, 5, 6).

Subpopulations of $CD4^+$ helper T cells (Th) produce distinct patterns of cytokines, and this has led to the concept of functional heterogeneity among Th cells (7, 8). Upon antigenic stimulation, naive $CD4^+$ T cells (Th precursor cells) differentiate into at least two populations: Th1 and Th2. Th1 produce IL-2 and/or IFN-γ, elicit delayed type hypersensitivity (DTH) responses, activate macrophages, and have been implicated in inducing tissue injury in many organ-specific autoimmune diseases. Th2, on the other hand, produce IL-4, IL-5, and IL-10, are especially important for IgE production and eosinophilic inflammation, and may suppress cell-mediated immunity. Different roles of these two subsets of Th in various immunopathological conditions like leprosy, leishmaniasis, and schistosomiasis, has been demonstrated (9–11). All encephalitogenic T cell clones examined thus far have been of the Th1 type

[1]*Abbreviations used in this paper:* DTH, delayed type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; LNC, lymph node cells; MBP, myelin basic protein; PLP, proteolipid protein; Q, glutamine; W, tryptophan.

M. Prabhu Das and L.B. Nicholson contributed equally to this work.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/97/09/867/10 $2.00
Volume 186, Number 6, September 15, 1997 867–876
http://www.jem.org

(12, 13). In contrast, the transfer of myelin antigen-reactive Th2 cells do not induce disease, and in some cases, have been shown to transfer protection against EAE (14–16).

Immunization of SJL mice with the encephalitogenic PLP peptide 139–151 leads to the generation of Th1 cells, and these clones recognize tryptophan in the center of the peptide (W144) as the primary TCR contact residue (17). The initial observation that the PLP 139–151-specific encephalitogenic Th1 clones require W144 as the primary TCR contact residue and most critical residue for activation has now been confirmed by three other laboratories (18–20). We recently generated a panel of Th2/Th0 clones/lines specific for PLP 139–151 and transfer of these Th2 clones both prevented and reversed PLP 139–151-induced EAE (15, 16). Preimmunization with the altered peptide Q144 prevents development of EAE and transfer of T cell lines specific for the Q144 peptide confers protection against EAE induced with the native PLP 139–151 peptide. In analyzing the cellular basis for this protection, we generated T cell clones from the Q144-specific line. These clones have two important properties: they (*a*) produce Th2/Th0 cytokines and (*b*) they cross-react with the native PLP 139–151 peptide (21). The PLP 139–151-specific Th2 clones included in this study were generated in three different ways. SJL mice were first immunized with PLP 139–151 and were injected simultaneously with anti-B7-1 antibody. For the second set of clones mice were immunized with an altered form of the encephalitogenic peptide 139–151 in which the primary TCR contact residue was changed from tryptophan (W) to glutamine (Q) at position 144, and T cell clones were propagated with Q144 peptide in culture. In a third independent T cell cloning, SJL mice were immunized with Q144, and T cell clones were propagated with the native peptide in vitro. Thus, we cloned altered peptide-specific Th2 clones from Q144 immunized mice from two independent lines. In one case, Q144 peptide was used (Q1 clones), and in the second case, native peptide W144 (QW clones) for in vitro propagation and as the cognate ligands. In this study, we determined the fine epitope recognition of the panel of Th2/Th0 clones and compared it with a panel of encephalitogenic Th1 clones, both of which react with the PLP peptide 139–151. The data presented here show that in contrast with Th1 clones, Th2 clones have shifted their primary TCR contact residue to the NH_2-terminal end of the peptide. This difference in the fine epitopic recognition of encephalitogenic and protective repertoire has been exploited to regulate EAE.

Materials and Methods

Antigens. PLP 139–151 (HSLGKWLGHPDKF) peptide, altered Q144 peptide (HSLGKQLGHPDKF), and alanine-substituted peptides on the native backbone were synthesized in the laboratory of Dr. R. Laursen (Boston University, Boston, MA) using FMOC chemistry on a Milligen synthesizer (model 9050; PerSeptive Biosystems, Framingham, MA). The control peptide PLP 43–64 (EKLIETYFSKNYQDYEYLINVI) was synthesized by Dr. D. Teplow (Brigham and Women's Hospital, Boston, MA). An I-A^s–binding peptide ADLIAYLKAQTAK of PCC 81–104 (synthesized by Quality Controlled Biochemicals,, Hopkinton, MA) was used as a control in some of the experiments.

T Cell Clones and Hybridomas. The generation of PLP 139–151-specific Th1 (13) and Th2 (15) clones has been described. In brief, the Th1 clones were generated from the lymph nodes of SJL mice after immunization with the PLP peptide 139–151 in CFA (Difco, Detroit, MI). The lymph node cells were obtained 7–10 d after immunization, activated with PLP 139–151 peptide, and propagated in the presence of IL-2 as described previously (15). For the epitope analysis of the Th1 clones, T cell hybridomas were also generated by the fusion of Th1 clones with TCR$\alpha^-\beta^-$ BW1100 (17, 22). The hybridomas and the parent Th1 clones expressed identical TCRs and showed similar fine antigenic specificities.

Generation of Th2 clones specific for the PLP 139–151 peptide by conventional techniques have met with limited success. We generated PLP 139–151, reactive Th2 clones by three different techniques. In the first attempt, the PLP 139–151-specific Th2 clones were generated by immunizing SJL mice with the PLP peptide 139–151, followed by intraperitoneal administration of monoclonal anti-B7-1 antibody (15). Except for the anti-B7-1 administration in vivo, the PLP 139–151-specific Th2 clones were expanded and propagated like the Th1 clones described above (15). A control Th1 clone was derived the same way by administration of control hamster Ig.

The second and third independent panels of PLP 139–151-reactive Th2/Th0 clones were generated by immunizing SJL mice with the altered peptide Q144 in CFA (16) on separate occasions. In one case, lymph node cells from Q144-immunized SJL mice were propagated in culture with Q144 peptide, whereas in the other case, the lymph node cells were propagated with the native peptide. The lymph node cells were activated with the Q144 peptide and maintained in DMEM containing 0.6% T cell growth factor (T-STIM; Collaborative Biomedical Research, Bedford, MA) and 0.06% recombinant IL-2. Q144-reactive T cell lines were shown to mediate protection against EAE upon adoptive transfer (16). All the clones were fed every 2–3 d and stimulated with the immunizing antigen (20 μg/ml) plus irradiated SJL spleen cells as a source of APC every 3–4 wk. In a third attempt, PLP 139–151-reactive Th2 clones were generated by immunizing SJL mice with the altered peptide Q144 as described above, but the lymph node cells were activated with the native PLP 139–151 peptide in vitro to expand T cell clones that are cross-reactive with the native peptide. In total, 10 Th2/Th0 clones, generated by three different clonings that react with the PLP 139–151, were included in the study.

Activation of T Cell Clones and Hybridomas. T cell clones or hybridomas (5×10^4) were cultured in triplicate in 96-well plates with 5×10^5 irradiated SJL spleen cells and various concentrations of synthetic peptides in a final volume of 0.2 ml. After 24 h, 0.1 ml of the supernatant from the hybridomas was transferred to 96-well flat-bottomed plates to which 10^4 HT-2 (IL-2/IL-4–dependent) cells were added. The ability of HT-2 cells to proliferate was determined by thymidine incorporation. The plates with T cell clones were incubated for 48 h at 37°C and then pulsed with [^{3}H]thymidine for 16–18 h, after which they were harvested and the mean thymidine incorporation in triplicate wells was calculated. The [^{3}H]thymidine incorporation was determined in a scintillation counter (model LS 5000; Beckman Instrs., Fullerton, CA). The data are presented as mean cpm incorporated into triplicate wells.

To compare the effects of a particular substitution on T cell

proliferation and to make data readily comparable, the data has also been presented as percentage of control, in which case the mean T cell proliferation with the cognate antigen (native peptide) was taken as 100% and response to the substituted peptide was expressed as percent response of the cognate antigen (percent control). Proliferation of >10% of the native peptide is taken as positive.

In Vitro Proliferation Assays. Mice were injected subcutaneously at five sites with antigen emulsified in CFA (Difco) containing a total of 250 μg *Myobacterium tuberculosis* H37 RA. Mice immunized with a single peptide received a total of 100 μg of antigen (native PLP 139–151 or A144) or a mixture of these two peptides. On day 10, lymph nodes were removed and LNC prepared from them. LNC (4×10^5/well) were cultured in triplicate in 96-well round-bottomed plates (Falcon, Becton Dickinson, Lincoln Park, NJ), in the presence of antigen, for 48 h and then [^{3}H]thymidine (1 μCi/well) was added for the last 16 h before harvesting the cells. The [^{3}H]thymidine incorporation was determined in a scintillation counter (model LS 5000; Beckman Instr.). Supernatants from the lymph node cells activated with antigenic peptides were harvested at 40 h for determining the type and amount of cytokines accumulated in the culture supernatant.

Cytokine ELISA. T cell clones were activated with syngeneic spleen cells as APCs and 50 μg/ml of antigen. Supernatants were harvested 48 h later and tested for the presence of cytokines by ELISA as described (15). IL-2, IL-4, and IL-10 ELISA components (antibodies and cytokine standards) were obtained from PharMingen (San Diego, CA). In brief, ELISA plates were coated with purified rat mAb to mouse IL-2 (clone JES6-1A12), IL-4 (clone BVD4-1D11), and IL-10 (clone JES5-2A5). Biotinylated rat mAb to mouse IL-2 (clone JES5H4), IL-4 (BVD6-24G2), and IL-10 (SXC-1) were used as secondary detecting antibodies. IFN-γ ELISA components were obtained from Genzyme (Cambridge, MA). Monoclonal hamster anti–mouse IFN-γ was used as the primary antibody; polyclonal goat anti–mouse IFN-γ was the secondary antibody. Assays were developed with TMB Microwell peroxidase substrate (Kirkegaard and Perry Labs., Inc., Gaithersburg, MD) and read after the addition of stop solution at 450 nm using a model 3550 Microplate Reader (BioRad Labs., Hercules, CA).

In Vivo Treatment with PLP 139-151 Analogues. SJL mice obtained from Jackson Labs. (Bar Harbor, ME) were pretreated with 100 μg of each of the following peptide analogues in 100 μg CFA: A141, A142, A144, A147, and native W144. After 1 wk, these mice were immunized with 50 μg of the native PLP 139–151 peptide (W144) and pertussis vaccine as described (15). Mice were observed regularly for signs of disease. Clinical course of the disease is scored as follows: 1, tail atony; 2, hind limb weakness; 3, hind limb paralysis; 4, paralysis of all four limbs; 5, moribund.

Results

Cytokine Profile of PLP 139–151-specific T Cell Clones. We previously generated a panel of T cell clones specific for PLP 139–151 peptide, of which five clones (2E5, 4E3, 5B6, 7A5, and SPL1.1) were tested in detail for epitope specificity (17), cytokine production (13), and ability to transfer EAE (6) upon adoptive transfer. All of these clones were of the Th1 phenotype in that they produced IFN-γ, but not IL-4 or IL-10, upon activation with the PLP 139–151 peptide (13). The structure of the epitope recognized by the T cell clones was defined, and all of them showed a critical requirement for W144 in the center of the peptide as a TCR contact residue for activation (17). The finding that the encephalitogenic PLP 139–151-specific Th1 clones require W144 as the primary and the most critical TCR contact residue for activation has since been confirmed by other groups (18–20). We subsequently generated a panel of T cell clones by immunization with PLP 139–151 peptide by administration of anti-B7-1 antibody in vivo, and upon cytokine analysis, the vast majority of these clones turned out to be of the Th2 phenotype. We have now analyzed in detail the cytokine production and epitope specificity of four of the clones (1E3, 1E6, 1E10, and 1A8) generated with anti–B7-1 antibody treatment in vivo. As a control, we also used a T cell clone (1D9) that was generated in parallel by immunization of SJL mice with PLP 139–151, followed by administration of hamster Ig in vivo (15). In a third cloning, we generated PLP 139–151-reactive Th2/Th0 clones by immunizing SJL mice with the altered peptide Q144. Q144 altered peptide induces PLP 139–151-reactive Th2/Th0 cells, which mediate protection upon adoptive transfer. Three T cell clones (Q1.1B6, Q1.3F4, and Q1.4A11) that showed stable growth in culture were included in the study and compared with other PLP 139–151-reactive Th1 or Th2 clones. In a fourth independent T cell cloning, we generated Th2 clones by immunizing mice with Q144 altered peptide and the lymph node cells from the immunized mice were activated with the native peptide 139–151 to expand only the native peptide cross-reactive clones. Three clones from this cloning (QW.1D4, QW.6D10, and QW.9E1) were included in this study. Thus, 10 PLP 139–151-reactive Th2/Th0 clones generated by three independent cloning attempts were included in this study.

The cytokine profile of the panel of 139–151-reactive T cell clones is shown in Table 1. The 1D9 T cell clone produced large amounts of IFN-γ (>5 ng) and small amounts of IL-10 (<1 ng) upon activation with PLP 139–151. The clones 1E3, 1E6, 1E10, and 1A8 that were generated after immunization with PLP 139–151 together with the anti-B7-1 in vivo showed a typical Th2 phenotype, in that they produced large amounts of both IL-4 and IL-10. The only exception was that the clone 1E6 produced only IL-10, and not IL-4, after activation. The PLP 139–151-reactive T cell clones generated after immunization with Q144 and in vitro propagation with the Q144 peptide demonstrated a mixed cytokine profile of Th0 and Th2 cytokines when activated with the cognate antigen Q144. The clone Q1.1B6 (Th0) produced IL-4 and IFN-γ together; clone Q1.3F4 produced IL-4 (with low levels of IFN-γ), whereas Q1.4A11 clone produced both IL-4 and IL-10 together with IFN-γ in some experiments. The three clones (QW.1D4, QW.6D10, and QW.9E1) generated by immunization with Q144 peptide followed by in vitro propagation with the native PLP peptide 139–151 showed a typical Th2 cytokine profile in that they produced both IL-4 and IL-10 together except for clone QW.1D4, which also produced small amounts of IFN-γ (Table 1).

To compare further MHC and CD4 requirements of the

Table 1. *Cytokine Profile of PLP 139–151-specific Th1 and Th2 Clones*

Clone	Antigen	Antibody treatment	T helper phenotype	Cytokine concentration			
				IL-2	IFN-γ	IL-4	IL-10
				Δ pg/ml			
ID9	PLP 139–151	Hamster Ig	Th1	<80	>5,000	<80	840
1E3	PLP 139–151	Anti-B7-1	Th2	<80	<80	>5,000	>5,000
1E6	PLP 139–151	Anti-B7-1	Th2	<80	<80	200	>5,000
1E10	PLP 139–151	Anti-B7-1	Th2	<80	<80	3,430	>5,000
1A8	PLP 139–151	Anti-B7-1	Th2	<80	<80	2,400	2,380
Q1.1B6	Q144	None	Th0	<80	1,770	1,260	<100
Q1.3F4	Q144	None	Th0	220	580	1,090	<100
Q1.4A11	Q144	None	Th2	150	320	650	2,890
QW.1D4	PLP 139–151	None	Th2	<80	260	1,330	2,590
QW.6D10	PLP 139–151	None	Th2	<80	<80	740	400
QW.9E1	PLP 139–151	None	Th2	240	<80	910	640

T cell clones were generated by immunization of SJL mice with PLP 139–151 in the presence of anti–B7-1 or control hamster Ig, or by immunization with Q144. The clones were activated with peptide (50 μg/ml) and syngeneic APCs. Culture supernatants were collected 48 h after activation and tested by cytokine ELISA. The upper limit of detection in these assays was 5,000 pg/ml. The lower limit of detection was 80 pg/ml.

PLP-specific Th1 and Th2 clones, we added mAbs specific for I-A^s and CD4. Like the PLP 139–151-specific Th1 clones (13), the antigen-specific proliferative response of all clones was blocked significantly by addition of an antibody to I-A^s (10.2.16), but not by a control anti-I-E^k antibody (14-4-4S), suggesting that the clones recognize the antigen in the context of I-A^s. Furthermore, the addition of anti-CD4 antibody (GK 1.5) inhibited the proliferative responses of all the clones tested (data not shown). These data demonstrate that, like the Th1 clones, the Th2 clones require I-A^s and CD4 for activation.

Antigen Specificity and Cross-reactivity of the PLP 139–151-specific Th1 and Th2 Clones. The PLP 139–151-specific Th1 (2E5, 4E3, 1D9, and SPL 1.1) and Th2 (1E3, 1E6, 1E10, and 1A8), and three Th2/Th0 (Q1.1B6, Q1.3F4, and Q1.4 A11) clones generated by immunization with Q144 peptide were tested for their antigen specificity to the cognate ligands (PLP 139–151 or Q144) in an in vitro proliferation assay. In addition, the A144 peptide was included in the assay because our previous data showed an absolute requirement for W at position 144 for the activation of all Th1 clones and substitution of W at position 144 with any other residue including A144 resulted in a peptide that did not activate Th1 clones. The PLP peptide 43–64 was used as a negative control peptide in these experiments. The Th1 cells (1D9, 2E5, 4E3, and 5B6) demonstrated specific proliferation only to PLP 139–151 and did not respond to the altered peptides A144 or Q144, confirming a strict requirement for W at position 144 for activation of Th1 cells (Fig. 1). The Th2 clones (1E3, 1E6, 1E10, and 1A8) also demonstrated specific proliferative responses to the PLP 139–151 peptide. However, in contrast with PLP 139–151-specific Th1 clones, when A144 was used as the antigen in the proliferation assay, all the Th2 clones responded with varying levels of proliferation. In addition, the Th2 clone 1A8 also showed a significant proliferative response to Q144. These data demonstrate that the PLP 139–151-specific Th2 clones do not have an absolute requirement for W144 for activation (Fig. 1). The group of PLP 139–151-reactive Th2/Th0 clones generated by immunization with Q144 peptide tested similarly in the proliferative assay showed even greater degeneracy in their antigen recognition in that they showed a significant proliferative response to the cognate antigen (Q144) and the native PLP peptide 139–151, but also showed a significant proliferative response to A144 peptide (Fig. 1). It was interesting to note that reactivity pattern of some of the clones generated by PLP 139–151 plus anti-B7-1 antibody administration appeared similar to the clones generated by Q144 immunization (compare 1A8 with Q1.1B6). These data demonstrate that unlike Th1 clones, the Th2 clones do not have a critical requirement for W at position 144, because they all respond to A144.

Determination of Critical Residues for Activation of the Th1 and Th2 Clones. Because the PLP 139–151-specific Th1 and Th2 clones showed a difference in proliferative responses when position 144 peptide-substituted analogues (Q144 and A144) were used, the question was raised of whether PLP 139–151-specific Th1 and Th2 clones recognize the PLP 139–151 peptide differently. To address this issue, we tested the PLP 139–151-specific Th1 and Th2 clones with a panel of peptides synthesized with alanine substitutions at each residue on the PLP 139–151 backbone at concentrations of 0.1–100 μg/ml in a proliferative assay. Maximal T cell responses were generally achieved between 10–100 μg/ml of the antigen, and only the maximal T cell

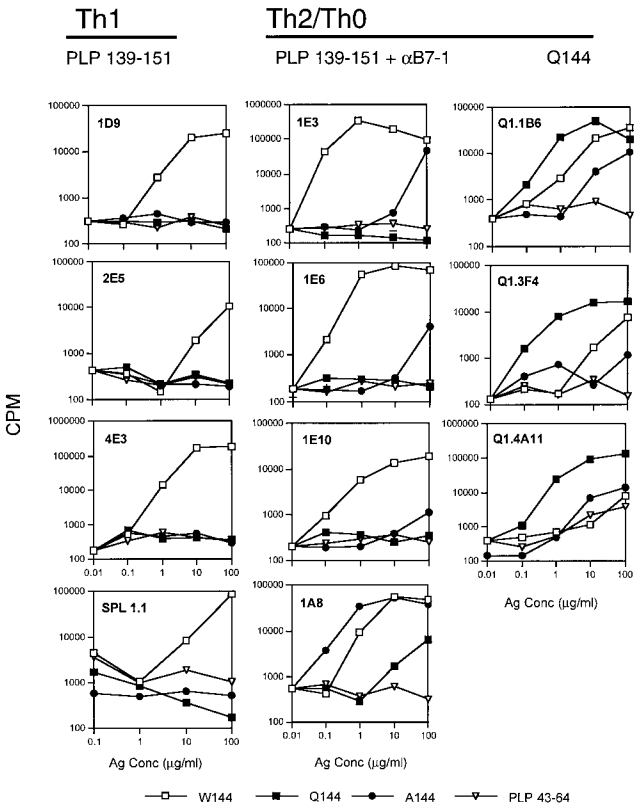

Figure 1. In vitro proliferative response of PLP peptide 139–151-specific Th1 and Th2 clones to the native PLP 139–151 (W144) and peptide analogs that were substituted at position 144 with glutamine (Q144) and alanine (A144). PLP 43–64 was used as a control antigen. The T cell clones were incubated with the syngeneic spleen cells as APCs with various concentrations of the peptides and 1 μCi of [^{3}H]thymidine was added during last 16–18 h of incubation. The data represent the mean CPM incorporated into insoluble DNA of triplicate wells. The standard deviations were <20%.

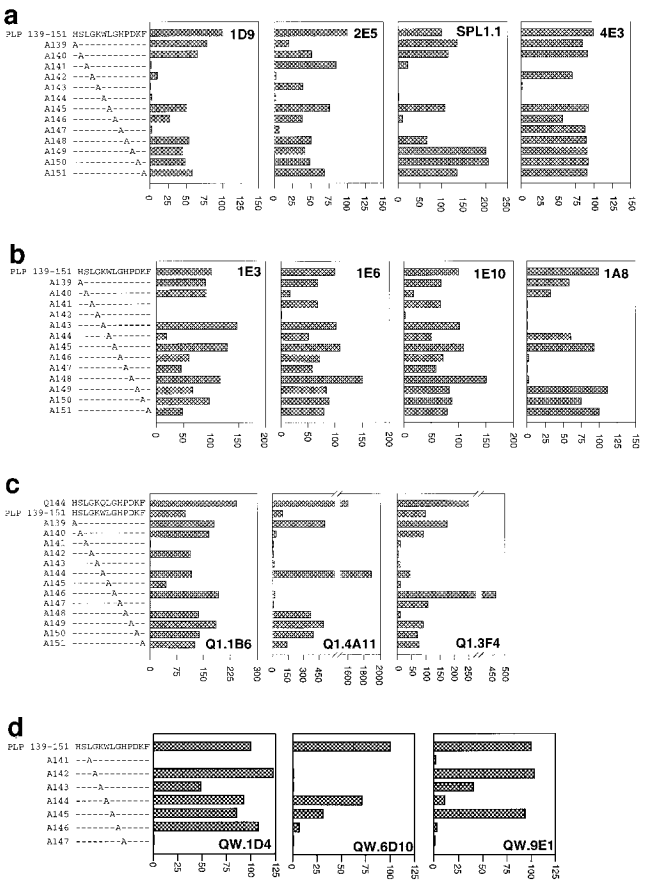

Figure 2. Proliferative responses of (*a*) Th1-, (*b*) Th2-, (*c*) Q1-reactive Th2/Th0 clones, and (*d*) QW-reactive Th2/Th0 clones to the PLP peptide 139–151, and panel of alanine-substituted peptides each position. The ability of each substituted peptide to induce proliferation was compared with that of the native peptide and the results expressed as the percentage of the response relative to the native peptide. Values are representative of 3–5 experiments. Maximal responses were obtained at peptide concentrations of either 10 or 100 μM.

responses at a single dose are shown (Fig. 2). The panel of Th1 clones reacted well to the native peptide, and various different clones were sensitive to alanine substitutions at positions 141, 142, 143, 144, and 147 (Fig. 2 *a*). In agreement with our previous results (17) and that of others (18–20), only W at position 144 was absolutely required for the activation of Th1 clones in that an alanine substitution at position 144 in the PLP peptide 139–151 led to abrogation of T cell proliferation of all the Th1 clones tested, including the newly derived Th1 clone 1D9. Histidine at position 147 and lysine at position 143 appear to be essential for three of the four Th1 clones, whereas two of the four Th1 clones had a requirement for L141 and G142 (Fig. 2 *a*).

Epitopic analysis of Th2 clones specific for PLP peptide 139–151 with the panel of alanine substituted peptides is shown in Fig. 2 *b*. The clones 1E3, 1E6, and 1E10 showed a decrease or a complete loss of reactivity when residues at positions 141, 142, and 143 were substituted with alanine in the native peptide. Clone 1A8 also failed to proliferate when alanine substitutions were made at positions 146, 147, and 148. In contrast with the Th1 clones, all Th2 clones showed significant reactivity to the A144 peptide (Fig. 2 *b*). The cumulative data with the four Th2 clones suggest that alanine substitution at position 142 completely abrogated responses to the peptide in all four clones tested, and an alanine substitution at position at 141 was not tolerated by three of the four clones.

The T cell clones generated by immunization with the altered peptide Q144 are generally of Th2/Th0 phenotype, and besides recognizing Q144 peptide, these clones cross-react with the native PLP 139–151 peptide (see Fig. 1). Because the Th2 clones specific for the PLP 139–151 show an epitope shift, it was of interest to determine whether PLP 139–151-reactive T cell clones generated by immunization with Q144 peptide show a similar epitope shift, which may explain both cross-reactivity with the native peptide and the Th2 phenotype. To address this issue, we tested the panels of alanine-substituted peptides in the native PLP 139–151 backbone (Fig. 2 *c*). Clone Q1.1B6 has a requirement for residues 141, 143, and 147. Clone Q1.4A11 was

not activated when substitutions were made at residues 140, 141, 142, 143, 145, 146, and 147. Q1.3F4 needed residues 141, 142, 143, 145, and 148 for activation. Similar to the Th2 clones described above, these clones also did not need W144 or Q144 for activation. However, the cumulative data suggested that Q144-specific T cell clones required residues 141 and 143 for activation, because alanine substitutions at these positions completely abrogated T cell activation of all the three clones. Glycine at position 142 and histidine at position 147 was critical for the activation of two out of three clones. The analysis of the third independent set of Th2 clones generated by immunization with the altered peptide Q144 and in vitro propagation with the native peptide also showed a pattern very similar to the Th2 clones described above (Fig. 2 *d*). Using alanine substitutions in the native peptide backbone, it was clear that this set of Th2 clones also did not show any critical requirement for W144, in that alanine substitution at this position induced significant proliferation of the clones. However, alanine substitution at positions 141 and 147 completely inhibited T cell proliferation in all the three clones and additional inhibitory effects were also seen by alanine substitution at positions 142, 143, and 146 for this set of Th2 clones. The analysis of fine epitopic specificity of the PLP 139–151-reactive Th1 versus Th2/Th0 clones, therefore, show striking differences in the requirement for critical residues for activation; while W144 is the primary and critical residue for the activation of all the Th1 clones, the Th2/Th0 clones do not need W144 for activation.

Using several different strategies to identify I-A^s binding residues in the PLP 139–151 peptide (17), we found that leucine at position 145 and proline at position 148 are the major anchor residues for binding to I-A^s.

Structure of the PLP 139–151 Peptide Recognized by the Th1 and Th2 Clones. From the cumulative data, using the panel of alanine-substituted peptide analogues to identify the structure of PLP 139–151 recognized by Th1 and Th2/Th0 clones, it appears that the two subsets differ significantly in their recognition of PLP 139–151 peptide. The epitope recognition data of the Th1 clones is consistent with our previous data (17) and those of three other independent laboratories (18–20). The data of the Th2/Th0 clones is based upon three different clonings and the generation of PLP 139–151-specific Th2/Th0 cells by different techniques. The responses of 4 PLP 139–151-specific Th1 clones and 10 Th2/Th0 clones to alanine-substituted peptides in the native peptide backbone is summarized in Fig. 3 *a*. The most striking difference is that all 4 Th1 clones require W144 for T cell activation, and an alanine substitution at this position completely abrogates the response. In contrast, all 10 Th2/Th0 clones showed a significant response to the A144 peptide, demonstrating that these clones do not require W144 as the primary contact residue. In the case of Th1 clones, W144 is the major TCR contact residue, and K143 and/or H147 the secondary TCR contact residues. L141/G142 are minor TCR-binding residues for the Th1 clones. 9 out of 10 Th2/Th0 clones seem to require L141, and 7 of 10 clones require G142 for activation; thus, the emphasis for the primary TCR contacts has been shifted to the NH_2-terminal end of the peptide (Fig. 3 *b*). Although 9 out of 10 Th0/Th2 clones require leucine at position 141, alanine at this position does activate some Th2 clones. Like the Th1 clones, these Th2 clones also appear to have a minor requirement for the residues K143 and H147. From these data, it appears that the Th2/Th0-reactive clones, generated by three different techniques, all do not require W144 for activation, but mostly seem to require L141/G142 for their activation. However, neither of the two residues (L141 or G142) appear to be absolutely critical for the clones tested. Furthermore, there appears to be subtle differences among the Th2 clones generated by the three techniques: anti–B7-1–derived clones show main emphasis on residues 142 and 141, Q144-specific clones require residues 141 and 143 with a minor requirement for 142 and 147 residues, and QW clones require 141 and 147. Unlike Th1 clones where W144 is absolutely required, alanine substitution at the position 141 or 142 does not lead to complete abrogation of PLP 139–151-reactive Th2 responses.

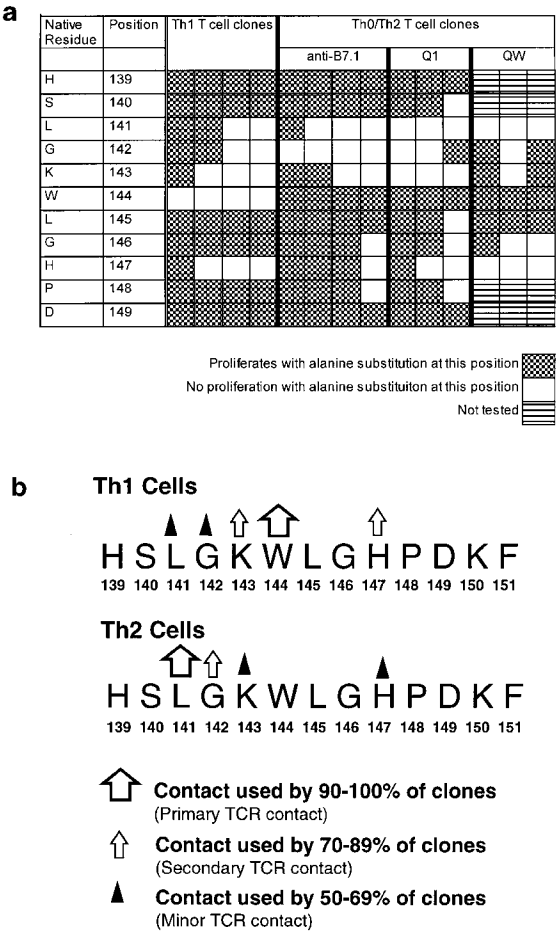

Figure 3. (*a*) Relative requirement of each residue in T cell activation and (*b*) structure of PLP 139–151 recognized by PLP 139–151-reactive Th1 and Th2/Th0 clones. This figure was compiled taking a proliferation of greater than 10% that of the cognate ligand as positive.

Effect of Preimmunization with A144 Analogue on EAE. Because encephalitogenic Th1 and protective Th2 cells

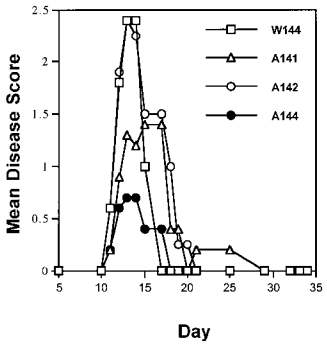

Figure 4. Preimmunization with A144 ameliorates EAE. Animals were preimmunized with the native peptide W144 (*open squares*), A141 (*open triangle*), A142 (*open circle*), or A144 (*closed circle*) and 7 d later reimmunized with the native PLP peptide 139–151 and assessed daily for signs of clinical disease.

show a dramatic difference in activation with A144 peptide in that A144 peptide activates PLP 139–151-specific Th2 clones but not the Th1 clones, we tested the effect of preimmunization with A144 peptide on the induction of EAE. Groups of SJL mice were preimmunized either with the native W144, A141, A142, or A144 peptides in CFA and then challenged with the encephalitogenic PLP 139–151 to induce EAE. As shown in Fig. 4, preimmunization with A144 protected mice from the development of EAE. In contrast, preimmunization with A142 did not show significant protection and A141 showed slight inhibition of disease, which was not significant. However, in other experiments preimmunization with A141 and A142 accelerated the day of onset and enhanced severity of disease (data not shown). These data suggest that preimmunization with A144 probably leads to the expansion of the A144-reactive protective repertoire that inhibited the disease induced with the native peptide.

Besides expanding a protective T cell repertoire, several other mechanisms could explain protective effects of preimmunization with A144 peptide; for example, A144 could mediate MHC blockade or antagonize PLP 139–151-specific Th1 cells and inhibit generation of an encephalitogenic T cell repertoire. A144 peptide does not have significantly higher binding for I-A^s than the native PLP 139–151 peptide. Furthermore, we coimmunized mice with A144 plus PLP 139–151 peptides and tested the lymph node cells for their ability to proliferate to PLP 139–151 and A144 peptides. Coimmunization of mice with A144 plus PLP 139–151 does not inhibit the generation of PLP 139–151-specific W144-reactive T cells (Fig. 5), suggesting that A144 does not mediate MHC blockade, thereby inhibiting the generation of W144-reactive cells. We have undertaken a detailed analysis to test whether A144 can act as a TCR antagonist of the PLP 139–151-specific Th1 repertoire. Our studies showed that the A144 does not antagonize PLP 139–151-specific T cell clones or PLP 139–151-specific T cell lines (17). To study whether A144 immunization expanded the protective Th2/Th0 repertoire, we immunized SJL mice with PLP 139–151 or A144 in CFA and tested the lymph node cells from these mice for proliferation and cytokine production by in vitro activation with native PLP 139–151 and A144 peptide (Table 2). The lymph node cells from PLP 139–151 immunized mice proliferated specifically to the native peptide and produced large amounts

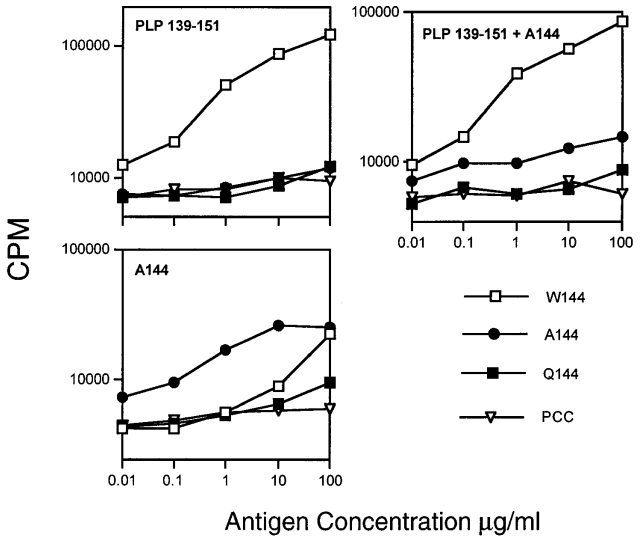

Figure 5. Proliferative response of lymph node cells of SJL mice immunized with 100 µg/mouse of PLP 139–151, A144, or a mixture of PLP 139–151 plus A144 peptides in CFA. Lymph node cells were activated 10 d after immunization with indicated peptides in a 3-d proliferation assay. Data represent mean CPM in triplicate wells.

of Th1 cytokines, particularly IL–2 and IFN-γ. Consistent with the Th1 clone data, PLP 139–151-immunized lymph node cells did not show a significant proliferation or produce significant cytokines in response to A144 peptide. In contrast, the lymph node cells from the A144-immunized mice proliferated equally well to both the A144 and PLP 139–151 peptides as has been detected with the Th2/Th0 clones. The lymph node cells from A144-immunized mice when activated with A144 produced little IFN-γ, but produced large amounts of IL-10. We could not detect significant amounts of IL-4 in the primary lymph node cultures after activation with A144, but small amounts of IL-4 were detected after activation with the native peptide. These data are consistent with the observation that in contrast with the native peptide that induces Th1 cells, immunization with A144 even in CFA activates the alternate PLP 139–151 cross-reactive Th2/Th0 repertoire, which may be responsible for protection against EAE. The data obtained with immunization with the A144 peptide are similar in many ways to those obtained with the Q144 peptide in which we have seen that preimmunization with Q144 protects mice for the development of EAE and lymph node cells of these mice produce IL-10 upon in vitro activation (21). However, after cloning these cells in vitro, IL-4–producing Th2 clones were uncovered (21). It is relatively difficult to detect IL-4 in primary lymph node cultures, which may be due to very low levels of IL-4, or to its consumption or inhibition by other cytokines.

Discussion

We have examined the structural requirements for activation of PLP 139–151-specific Th1 and Th2 clones. Although the autopathogenic Th1 clones recognize W144 as

Table 2. *Proliferation and Cytokine Production by the Lymph Nodes Cells of Mice Immunized with PLP 139–151 and A144 Peptide*

Immunization	Activation	Proliferation	Cytokine production			
			IL-2	IFN-γ	IL-4	IL-10
		Δ cpm		*Δ pg/ml*		
PLP 139–151	PLP 139–151	93,040	1,820	2,740	110	<80
	A144	4,490	<80	<80	<80	<80
A144	A144	15,620	<80	180	90	1,050
	PLP 139–151	16,430	<80	<80	180	<80

Groups of SJL mice were immunized with PLP 139–151 or A144 peptide (100 μg of peptide/mouse) in CFA. Lymph node cells were collected 10 d later and activated in vitro with four different dilutions (0.1–100 μg/ml) of PLP 139–151 or A144 peptides. Maximal proliferation and cytokine production was obtained at an antigen concentration of 10 μg/ml and this data is shown. Culture supernatants were collected 40 h after activation and tested by cytokine ELISA. Proliferation was assessed after 48 h as described.

the primary and critical TCR contact residue, the PLP 139–151-specific Th2 clones do not require this residue for activation. Instead, the Th2 clones have shifted their emphasis to L141/G142 at the NH_2-terminal end of the peptide. Analysis of additional PLP 139–151-reactive Th2/Th0 clones, generated by immunization with an altered peptide in which tryptophan was substituted by glutamine at position 144 (21), also showed a similar shift in the critical TCR residues towards the NH_2-terminal end of the peptide. These results suggest that Th1 and Th2 clones specific for the same peptide in the autoreactive repertoire can be dependent on different residues in the peptide for their induction and activation and that T cell differentiation may be influenced by the recognition of specific residues in an autoantigenic peptide.

The data presented here raise an important issue as to why Th1 and Th2 clones specific for PLP 139–151 should recognize different parts of the same peptide. It has already been shown that T cells from TCR transgenic mice of identical specificity can be driven to become either Th1 or Th2 cells, depending upon the cytokines used in the primary activation culture (23, 24) or the dose of antigen (25, 26) used in initial activation. Although cytokine milieu is the most dominant factor in determining the outcome of T cell differentiation (27), studies with antigen dose would suggest that strength of initial signal generated by the ligation of TCRs would also influence the outcome. Low dose antigen (lower T cell activation/lower strength of signal) generated Th2 cells; relatively higher amounts of antigen (presumably giving a higher strength of signal) promoted differentiation of T cells in the Th1 direction; further higher concentration led to the generation of Th2 cells (25, 26). Our data would suggest that in vivo, the PLP 139–151-specific T cell repertoire consists of at least two populations of T cells using either W144 or L141/G142 as the major contact residues. When these populations are exposed to PLP 139–151 under conventional immunizing conditions, the W144-dependent cells become dominant in the expanded repertoire. This may be because precursor cells of this type are present at higher frequency in the naive PLP 139–151-specific pool, or because W144-dependent TCRs have a higher avidity for antigen–MHC complex. In the latter case, such cells will receive a stronger signal at all antigen concentrations, leading to greater growth than the L141/G142-dependent cells and also tending to promote differentiation along a Th1 pathway. This may explain why immunization with the native PLP 139–151 routinely induces W144-specific Th1 clones when the cells are reactivated by the native peptide (13, 18–20). In the presence of anti–B7-1 antibody, the W144 costimulation-dependent Th1 clones are presumably not activated, allowing the growth of L141/G142-recognizing cells. These L141/G142 Th2 may belong to a memory population, stimulated in vivo by autoantigen or cross-reactive ligand and may, therefore, have a less stringent requirement for costimulation. It has been shown that memory cells that produce TGF-β or Th2 cytokines may play a critical role in maintenance of peripheral tolerance to self-antigens (28).

We (21) and others (29, 30) have recently shown that altered peptides generated by the substitution of TCR contact residues selectively affect T cell differentiation and alter cytokine balance. In our studies, replacement of the primary contact residue of tryptophan with glutamine at position 144 in the PLP 139–151 peptide leads to the induction of T cells that have two important properties: (*a*) the clones are of Th0/Th2 phenotype and (*b*) besides recognizing Q144, they also recognize the native PLP 139–151 peptide. Although it is possible that Q144-specific T cell clones could recognize Q at position 144 as the primary contact residue in the altered peptide and be Q144-specific, in that case the T cells would not cross-react with the native peptide. How does a single residue substitution in the primary T cell contact point of the encephalitogenic PLP peptide generate Th2/Th0 clones that cross-react with the native peptide? Our data on the shift of primary contact residue from W144 to L141/G142 begins to explain this data. The immunization with Q144 altered peptide does not activate W144-reactive Th1 cells, but promotes the

growth of L141/G142-reactive cells. Because of this epitope shift, the cells are of a Th2 phenotype, but maintain cross-reactivity with the native peptide.

The PLP 139–151-specific Th2 clones that we have reported here were generated after the administration with anti–B7-1 antibody in vivo, and one might argue that epitope shift is not unique to the PLP 139–151-specific Th2 cells but is due to the presence of anti–B7-1 antibody. Two lines of evidence argue against this hypothesis: (*a*) a PLP 139–151-specific Th1 clone generated in parallel by anti–B7-1 antibody administration in vivo used W144 and not L141/G142 as the primary TCR contact residue (data not shown), and (*b*) immunization with the altered peptides in which the primary TCR contact residue was substituted induces PLP 139–151-reactive Th2 cells with a similar shift in the primary contact residues, further suggesting that epitope shift is not specifically due to the administration of anti–B7-1 antibody in vivo.

Some studies have shown that Th2 cells are not sufficient to protect from autoimmune disease (16). Khoruts et al. (14) and Katz et al. (31) reported that Th1 populations effectively transferred autoimmunity, whereas Th2 did not. However, the Th2 did not suppress autoimmunity caused by Th1. In our previous studies (15, 21), we demonstrated the Th2 clones studied in detail here and Q144-specific lines mediated protection from EAE. These cells also have a shift in their TCR contact residue. Therefore, it is possible that epitope specificity together with Th2 cytokines may be necessary for the protective phenotype. Thus, because PLP 139–151-specific pathogenic Th1 and protective Th2 clones differ in their primary TCR contact residue, this provided us with a unique opportunity to generate altered peptides that can inhibit pathogenic Th1, selectively induce protective Th2 clones, and ameliorate autoimmunity.

We thank T. Howard and V. Turchin for their technical help and Dr. H. Weiner for support.

This work was supported by grants from the National Multiple Sclerosis Society, New York (RG2571A2 and RG2320B3) and the National Institutes of Health (R29-30843, R01NS35685, P01AI39671-01A1) to V.K. Kuchroo. L. Nicholson is a postdoctoral fellow of the National Multiple Sclerosis Society.

Address correspondence to Vijay K. Kuchroo, Center for Neurologic Diseases, Brigham and Women's Hospital, 77 Avenue Louis Pasteur, Boston, MA 02115. E-mail: Kuchroo@cnd.bwh.Harvard.edu

Received for publication 28 January 1997 and in revised form 30 June 1997.

References

1. Pettinelli, C.B., and D.E. McFarlin. 1981. Adoptive transfer of experimental allergic encephalomyelitis in SJL/J mice after in vitro activation of lymph node cells by myelin basic protein: requirement for Ly1+2− lymphocytes. *J. Immunol.* 127: 1420–1423.
2. Zamvil, S.S., and L. Steinman. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol.* 8:579–621.
3. Waldor, M.K., S.S. Hardy, R. Herzenberg, L.A. Herzenberg, L. Lanier, M. Lim, and L. Steinman. 1985. Reversal of experimental allergic encephalomyelitis with a monoclonal antibody to a T cell subset marker (L3T4). *Science (Wash. DC).* 227:415–417.
4. Brostoff, S.W., and D.W. Mason. 1984. Experimental allergic encephalomyelitis: successful treatment in vivo with a monoclonal antibody that recognizes T helper cells. *J. Immunol.* 133:1938–1942.
5. Ben-Nun, A., and I. Cohen. 1982. Experimental autoimmune encephalomyelitis (EAE) mediated by T cell lines: process of selection of line and characterization of the cells. *J. Immunol.* 129:303–308.
6. Kuchroo, V.K., R.A. Sobel, J.C. Laning, C.A. Martin, E. Greenfield, M.E. Dorf, and M.B. Lees. 1992. Experimental allergic encephalomyelitis mediated by cloned T cells specific for a synthetic peptide of myelin proteolipid protein. *J. Immunol.* 148:3776–3782.
7. Mosmann, T.R., and R.L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 7:145–173.
8. Mosmann, T.R., and R.L. Coffman. 1989. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv. Immunol.* 46:111–147.
9. Heinzel, F.P., M.D. Sadick, B.J. Holaday, R.L. Coffman, and R.M. Locksley. 1989. Reciprocal expression of interferon-γ or interleukin-4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J. Exp. Med.* 169:59–72.
10. Salagame, P., J.S. Abrams, C. Clayberger, H. Goldstein, J. Convit, R.L. Modlin, and B.R. Bloom. 1991. Differing lymphokine profiles of functional subsets of human CD4 and CD8 T cell clones. *Science (Wash. DC).* 254:279–282.
11. Romagnani, S. 1994. Lymphokine production by human T cells in disease states. *Annu. Rev. Immunol.* 12:227–257.
12. Baron, J.L., J.A. Madri, N.H. Ruddle, G. Hashim, and C.A. Janeway. 1993. Surface expression of α4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J. Exp. Med.* 177:57–68.
13. Kuchroo, V.K., C.A. Martin, J.M. Greer, S.T. Ju, R.A. Sobel, and M.E. Dorf. 1993. Cytokines and adhesion molecules contribute to the ability of myelin proteolipid protein-specific T cell clones to mediate experimental allergic encephalomyelitis. *J. Immunol.* 151:4371–4382.
14. Khoruts, A., S.D. Miller, and M.K. Jenkins. 1995. Neuroantigen-specific Th2 cells are inefficient suppressors of experimental autoimmune encephalomyelitis induced by effector Th1 cells. *J. Immunol.* 155:5011–5017.

15. Kuchroo, V.K., M. Prabhu Das, J.A. Brown, A.M. Ranger, S.S. Zamvil, R.A. Sobel, H.L. Weiner, N. Nabavi, and L.H. Glimcher. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell*. 80:707–718.
16. Nicholson, L.B., and V.K. Kuchroo. 1996. Manipulation of the Th1/Th2 balance in autoimmune disease. *Curr. Opin. Immunol*. 8:837–842.
17. Kuchroo, V.K., J.M. Greer, D. Kaul, G. Ishioka, A. Franco, A. Sette, R.A. Sobel, and M.B. Lees. 1994. A single TCR antagonist peptide inhibits experimental allergic encephalomyelitis mediated by a diverse T cell repertoire. *J. Immunol*. 153:3326–3336.
18. Franco, A., S. Southwood, T. Arrhenius, V.K. Kuchroo, H.M. Grey, A. Sette, and G.Y. Ishioka. 1994. T cell receptor antagonist peptides are highly effective inhibitors of experimental allergic encephalomyelitis. *Eur. J. Immunol.* 24:940–946.
19. McRae, B.L., K. Nikcevich, S. Hurst and S.D. Miller. 1995. Differential recognition of peptide analogs by naive verses activated PLP 139–151-specific CD4+ T cells. *J. Neuroimmunol*. 60:17–28.
20. van der Veen, R.C., P. Chen, and M. McMillan. 1995. Myelin proteolipid protein–induced Th1 and Th2 clones express TCR with similar fine specificity for peptide and CDR3 homology despite diverse Vβ usage. *Cell. Immunol*. 166:291–295.
21. Nicholson, L.B., J.M. Greer, R.A. Sobel, M.A. Lees, and V.K. Kuchroo. 1995. An altered peptide ligand mediates immune deviation and prevents experimental autoimmune encephalomyelitis. *Immunity*. 3:397–405.
22. Kuchroo, V.K., J.K. Steele, R.M. O'Hara, S. Jayaraman, P. Selvaraj, E. Greenfield, R.T. Kubo, and M.E. Dorf. 1990. Relationships between antigen specific helper and inducer suppressor T cell hybridomas. *J. Immunol*. 145:438–448.
23. O'Garra, A., and K. Murphy 1994. Role of cytokines in determining T-lymphocyte function. *Curr. Opin. Immunol*. 6: 458–466.
24. Janeway, C.A., and K. Bottomly. 1994. Signals and signs for lymphocyte responses. *Cell*. 76:275–285.
25. Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, and K. Bottomly. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive $CD4^+$ T cells. *J. Exp. Med*. 182:1591–1596.
26. Hosken, N.A., K. Shibuya, A.W. Heath, K.M. Murphy, and A.O. O'Garra. 1995. The effect of antigen dose on $CD4^+$ T helper cell phenotype development in a T cell receptor–transgenic model. *J. Exp. Med*. 182:1579–1584.
27. Paul, W.E., and R.A. Seder. 1994. Lymphocyte responses and cytokines. *Cell*. 76:241–251.
28. Fowell, D., A.J. McKnight, F. Powrie, R. Dyke, and D. Mason. 1991. Subsets of CD4+ T cells and their roles in the induction and prevention of autoimmunity. *Immunol. Rev*. 123: 37–64.
29. Pfeiffer, C., J. Stein, S. Southwood, H. Ketelaar, A. Sette, and K. Bottomly. 1995. Altered peptide ligands can control CD4 T lymphocyte differentiation in vivo. *J. Exp. Med*. 181: 1569–1574.
30. Brocke, S., K. Gijbels, M. Allegretta, I. Ferber, C. Piercy, T. Blankenstein, R. Martin, U. Utz, N. Karin, D.J. Mitchell, et al. 1996. Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. *Nature (Lond.)*. 379: 343–346.
31. Katz, J.D., C. Benoist, and D. Mathis. 1995. T helper cell subsets in insulin-dependent diabetes. *Science (Wash. DC)*. 268:1185–1188.
32. Pakala, S.V., M.O. Kurrer, and J.D. Katz. 1997. T helper 2 (Th2) T cells induce acute pancreatitis and diabetes in immune-compromised nonobese diabetic (NOD) mice. *J. Exp. Med*. 186:299–306.
33. Lafaille, J.J., F. Van de Keere, A.L. Hsu, J.L. Baron, W. Haas, C.S. Raine, and S. Tonegawa. 1997. Myelin basic protein–specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from disease. *J. Exp. Med*. 186:307–312.