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Th1 and Th2 Deviation of Myelin-Autoreactive T Cells by Altered Peptide Ligands Is Associated with Reciprocal Regulation of Lck, Fyn, and ZAP-70

Rana A. K. Singh,† Ying C. Q. Zang,‡ Anju Shrivastava,§ Jian Hong,† George T. Wang,† Sufang Li,† Maria V. Tejada-Simon,* Milena Kozovska,* Victor M. Rivera,* and Jingwu Z. Zhang‡§

Th0 clones recognizing an immunodominant peptide of myelin basic protein (residues 83–99) were derived from patients with multiple sclerosis. We demonstrate that analogue peptides with alanine substitution at Val86 and His88 had a unique partial agonistic property in inducing Th0 →Th1 and Th0 →Th2 deviation of the myelin basic protein-reactive T cell clones, respectively. Th0 to Th1 deviation induced by peptide 86V→A correlated with up-regulation of Fyn and ZAP-70 kinase activities. Conversely, Th0 to Th2 deviation induced by peptide 88H→A was associated with complete failure to activate Fyn and ZAP-70 kinases. The observed Th1 and Th2 shift also correlated, to a lesser extent, with Lck kinase activity that was down-regulated with Th1 deviation and increased with Th2 deviation in some T cell clones. We demonstrated that the Th1 and Th2 shift induced by the analogue peptides was a reversible process, as the T cell clones previously exposed to either 86V→A or 88H→A peptide could revert to an opposite phenotype when rechallenged reciprocally with a different analogue peptide. The study has important implications in our understanding of regulation of TCR-associated tyrosine kinases by altered peptide ligands and its role in cytokine regulation of autoreactive T cells. The Journal of Immunology, 1999, 163: 6393–6402.

The current paradigm of T cell activation proposes that signals essential for triggering T cell activation are generated as a consequence of the interactions of TCR-αβ dimers with the peptide-MHC displayed on the surface of the APC (1). It has been well documented that TCR engagement with the peptide-MHC complexes results in tyrosine phosphorylation of TCR-associated CD3, γ, δ, ε, and ζ-chain subunits, leading to inositol phospholipid hydrolysis, protein kinase C activation, and [Ca2++] changes (2, 3). An early step in this process is the activation of src family of protein tyrosine kinases (Lck and Fyn), an event that governs the phosphorylation of the CD3 and ζ-chain (4). The coreceptor-associated kinase Lck is initially recruited into the interaction site by TCR coaggregation with CD4 or CD8 molecules, while Fyn is constitutively associated with the TCR (5). Phosphotyrosines of the CD3 and ζ-chains act as recognition sites for downstream signaling components. Subsequently, ZAP-70, a Syk family kinase associated with the phospho-ζ-chain, is itself phosphorylated by one or both of the receptor-associated Src family kinases. This biochemical change further activates ZAP-70 and generates new sites for attachment of additional signaling proteins in this phosphorylation cascade (6, 7). These signaling cascades ultimately converge on the nucleus, resulting in the alteration of gene expression required for T cell activation.

Implicit in this paradigm is the assumption that similar level of TCR occupancy by different ligands will lead to identical signal transduction events. However, recent evidence indicates that the TCR is not a simple off-on signal transduction complex; rather, it is able to sense structural differences in its ligand and transduce different signaling patterns, resulting in qualitatively and quantitatively distinct effects on subsequent activation and function of T cells (8, 9). Structural modification in a TCR ligand can render it to act as a partial agonist or an antagonist, leading to a cascade of characteristic intracellular changes that in turn translate functionally into inhibition or alteration of cell proliferation and cytokine production (10, 11). Such signaling alteration can be achieved by altered peptide ligands (APLS), in which single amino acid substitution of an antigenic peptide at a given position alters its interaction with the TCR (12). It has been demonstrated that single alanine substitution of an immunodominant peptide (residues 83–99) of myelin basic protein (MBP) at certain positions not only leads to complete inhibition or partial activation of human MBP-specific T cell clones, but also alters their ability to produce Th1 cytokines (IFN-γ, TNF-α, IL-2) or Th2 cytokines (IL-4, IL-5, IL-10) (10–14).

The physiological and clinical consequences of differentiation of memory Th0 cells into Th1 or Th2 cells are significant in health and in various autoimmune conditions. In particular, the balance between Th1 and Th2 immune responses plays an important role in the pathogenesis of autoimmune diseases. For example, in addition to the recognition of encephalitogenic epitopes, the ability to

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3 Abbreviations used in this paper: APL, altered peptide ligand; MBP, myelin basic protein; MS, multiple sclerosis.
produce Th1 cytokines is an important functional requirement for MBP-reactive T cells to mediate experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis (MS) (15, 16). On the other hand, there is an increasing body of evidence suggesting Th2 cells secreting IL-4 and IL-10 can suppress autoimmune inflammation in various experimental animal models (17–20). Similar role of Th1 and Th2 cytokines has also been implicated in the pathogenesis of MS, in which T cell responses to MBP are potentially involved in the disease process (21–23). The beneficial effect of IFN-β on the clinical course of MS is at least partially attributable to its ability to promote the production of IL-4 and IL-10 (24). These findings provide rationale for further investigations in defining the molecular mechanisms underlying Th1 and Th2 deviation and its implication in designing an effective peptide-based immunotherapy for MS.

The present study was undertaken to address whether amino acid substitution at certain position(s) within the immunodominant 83–99 region of MBP can induce a consistent pattern of Th1 and Th2 deviation among MBPp83–99-specific T cell clones (MBPp83–99), and whether regulation of the cytokine profile by altered peptides correlates with TCR-associated tyrosine kinase activity (Lck, Fyn, and ZAP-70). For this purpose, Th0 MBPp83–99-specific T cell clones were generated from patients with MS and examined for their reactivity and cytokine profile in response to a complete panel of analogue peptides with a single alanine substitution at successive positions. We report in this work that two alanine-substituted peptides, Val at position 86 and His at position 88, exhibited characteristic partial agonist properties in driving Th1 and Th2 deviation of the Th0 MBPp83–99-specific T cell clones. Th1 and Th2 deviation induced by the two analogue peptides were found to correlate reciprocally with the activity of TCR-associated tyrosine kinases Fyn, ZAP-70, and, to a lesser extent, with the Lck kinase activity. The findings described in this study suggest that down-regulation of Lck tyrosine kinase activity and up-regulation of Fyn and ZAP-70 tyrosine kinase activity are required for Th0 to Th1 deviation. Conversely, Th0 to Th2 deviation is associated with an increased Lck kinase activity and complete absence of detectable Fyn and ZAP-70 activities. The study provides new experimental evidence regarding the molecular mechanism involved in the regulation of TCR-associated tyrosine kinases by APLs and its role in the activation and cytokine production of MBP-reactive T cells in MS.

**Materials and Methods**

**Peptides**

MBPp83–99 (ENPVVHFFKNIVTPRTP) and a panel of 17 analogue peptides substituted at subsequent positions with alanine (hereafter APLs) were synthesized by the Merrifield solid-phase method and purified by HPLC (courtesy of Dr. Stefan Boheme, Neurocine Biosciences, San Diego, CA). The purity of all peptides used in this study was greater than 98%.

**Generation of MBPp83–99-reactive T cell clones from patients with MS**

To generate specific T cell lines, PBMCs were plated out at 2 × 10^6 cells/well in U-bottom plates (Costar, Cambridge, MA) in the presence of the 83–99 peptide (10 μg/ml). Seven days later, all cultures were restimulated with irradiated autologous PBMCs pulsed with the peptide as a source of APCs. After another week, each culture was examined for specific proliferation to the 83–99 peptide in proliferation assays. Briefly, each well was split into four aliquots (~1 × 10^6 cells/ aliquot) and cultured in duplicate in the presence of 1 × 10^5 APCs pulsed with the 83–99 peptide or a control peptide, respectively. Cells were cultured for 72 h and pulsed with [3H]thymidine (Amersham, Arlington Heights, IL) at 1 μCi/well during the last 16 h of the culture. Cells were then harvested and [3H]thymidine incorporation was measured in a Betaplate counter (Wallac, Turku, Finland). A T cell line was considered to be specific for the 83–99 peptide when the cpm were greater than 1500 (in the presence of the peptide) and exceeded the reference cpm (in the absence of the peptide) by at least 3-fold (25, 26).

To establish stable MBPp83–99-reactive T cell clones, the resulting T cell lines were cloned by PHA (Sigma, St. Louis, MO) in the presence of autologous PBMC as accessory cells (26). Briefly, T cells were plated out at 0.3 cell/well under limiting dilution condition and cultured with 10^5 irradiated autologous PBMC and 2 μg/ml of PHA. Cultures were fed with fresh medium containing 50 IU/ml of rIL-2 every 3–4 days. After ~10–12 days, growth-positive wells became visible and were tested in proliferation assays for specific responses to the 83–99 peptide.

**Proliferation assays with peptides of MBP and analogue peptides**

For subsequent studies, DBR1*1501 (DR2a)- and DBR1*0101 (DR2b)-transfected L cells were used as APCs. Irradiated L cells (2 × 10^5 cells/well) were pulsed with peptides (20 μg/ml) for 3 h, washed, and cocultured with a given T cell clone (5 × 10^5 cells/well). Cells were set up in duplicate for each peptide. In all cases, cell proliferation was measured after 72 h by [3H]thymidine incorporation assays, as described above.

**Cytokine quantification**

For measurement of IFN-γ, TNF-α, IL-4, and IL-10, supernatants recovered from duplicate cultures were collected 48 h after stimulation using identical conditions as for proliferation assay. Culture supernatants were diluted 1:4 with PBS before assays. Cytokines were determined quantitatively using ELISA kits obtained from Pharmingen (San Diego, CA). The kits were used according to the manufacturer’s instructions. Briefly, 96-well flat-bottom microtiter plates (Nunc, Nalge Nunc International, Naperville, IL) were coated overnight at 4°C with 2 μg/ml of respective mouse-capturing mAbs in PBS. Wells were then blocked at 37°C for 2 h with 2% BSA-PBS and washed three times with cold washing solution, containing 0.02% Tween 20. A total of 50 μl of each sample and its control were added to the adjacent wells and incubated for 2 h at ambient temperature simultaneously with 50 μl of biotinylated detecting Ab (0.25 μg/ml of each mAb) in 2% BSA/PBS/Tween 20. Plates were washed and incubated for 30 min with streptavidin-conjugated HRP. A total of 100 μl of 0.0125% TMB and 0.008% H₂O₂ in citrate buffer was used as substrate, and color development was stopped using 100 μl of 1 N HCl. The concentration of each cytokine in a given sample was calculated using a double standard curve of corresponding recombinant cytokine (Pharmingen) in each ELISA plate, which also served as a quality control. The detection limits for all cytokine measurements were <35 pg/ml in all assays.

**PCR amplifications and direct sequencing of PCR-amplified DNA products**

Total RNA was extracted from 10^6 cells of each MBPp83–99-reactive T cell clone using RNeasy mini kit (Qiagen, Santa Clara, CA). TCR α- and β-chain genes were amplified and directly sequenced, as previously described (27, 28). Briefly, extracted RNA was reverse transcribed into first-strand cDNA using an oligo(dT) primer and reverse transcriptase (Life Technologies, Gaithersburg, MD). cDNA was then subject to PCR amplification with a set of primers specific for TCR Vα and Vβ gene families whose sequences were published previously (27, 28). PCR was performed with 1 μl of cDNA in the following amplification mixture: 5 μl of 10 × PCR buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 3 μl of 25 mM magnesium chloride, 1 μl of 10 mM dNTP mix, 0.3 μl of Taq polymerase (5 U/μl; AmpliTaq Gold; PerkinElmer, Norwalk, CT), 20 pmol of each 5’ Vα or 5’ Vβ primer as the forward primer, and 20 pmol of 3’ Co or 3’ Cβ primer as the reverse primer. For each reaction, Co or Cβ gene was amplified simultaneously with 5’ Co and 5’ Cβ to control the integrity of TCR cDNA. The PCR amplification profile used was 1 min at 95°C for denaturation, 20 s at 56°C for annealing, and 40 s at 72°C for extension in a total of 35 cycles. The amplified PCR products were separated in a 1% agarose gel by electrophoresis and stained with ethidium bromide. The visualized PCR products were cut and purified subsequently using a QIAquick gel extraction kit (Qiagen) before sequence analysis. The purified PCR products were directly sequenced with the T7 sequencing kit (Pharmacia, Uppsala, Sweden). A total of 1.5 μg of template was sequenced with 2 pmol of the corresponding V gene primer using the method of dyeoxy chain termination (29).

**Immune complex kinase assay**

T cell clones (5–7 × 10^6 cells) were incubated at room temperature in 24-well flat-bottom culture plates with adherent L cells (1 × 10^5 cells) pre pulsed with 20 μg/ml relevant peptide. After 10 min, T cells were lysed.
Materials and Methods

A/G-Sepharose beads (Santa Cruz Biotechnology) were blocked in a solution of 10% nonfat dry milk in PBS for 1 h at room temperature. The membrane was then washed with 100 ml PBS-Tween for 1 h at room temperature. The membrane was rinsed three times with 100 ml of 0.1% Tween 20 in PBS and incubated with the appropriate dilutions of the specific Abs in PBS-Tween for 1 h at room temperature. The membrane was rinsed three times with 100 ml of 0.1% Tween 20 in PBS and incubated with the appropriate dilutions of the specific Abs in PBS-Tween for 1 h at room temperature. The membrane was then washed five times with 100 ml PBS-Tween and subsequently incubated with a peroxidase-labeled anti-rabbit Ig (Santa Cruz) in PBS-Tween then washed five times with 100 ml PBS-Tween and subsequently incubated in a buffer (pH 7.4) containing 20 mM HEPES, 250 mM NaCl, 0.1% Nonidet P-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSEF, 0.5 mM benzamidine, and 1 mM EDTA with 250 μg of anti-Lck, anti-Fyn, anti-ZAP-70, or anti-CD3-ζ (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4°C. Immune complexes were collected by incubation with protein A/G-Sepharose beads (Santa Cruz Biotechnology) for 30 min at 4°C. The beads were collected by centrifugation and washed extensively with lysis buffer (4 × 400 μl) and kinase buffer (2 × 200 μl: 20 mM HEPES, pH 7.4, 1 mM DTT, 25 mM NaCl). Kinase assays were performed for 15 min at 37°C in 20 μl of kinase assay buffer [20 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM DTT, and 10 μCi [γ-32P]ATP (Amersham)] in the absence of an exogenous substrate (autophosphorylation) or in the presence of tyrosine kinase substrate (Santa Cruz Biotechnology). Reactions were stopped with 20 μl SDS sample buffer, boiled for 5 min, and subjected to SDS-PAGE. The incorporation of [32P]phosphate was quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis

The immunoprecipitates were subjected to SDS-PAGE, and the electrophoresed proteins were transferred to a nitrocellulose membrane (Hybond; Amersham, Piscataway, NJ) by standard procedures. The membrane was blocked in a solution of 10% nonfat dry milk in PBS for 1 h at room temperature. The membrane was rinsed three times with 100 ml of 0.1% Tween 20 in PBS and incubated with the appropriate dilutions of the specific Ab in PBS-Tween for 1 h at room temperature. The membrane was then washed five times with 100 ml PBS-Tween and subsequently incubated with a peroxidase-labeled anti-rabbit Ig (Santa Cruz) in PBS-Tween for 1 h. After six washes with 100 ml PBS-Tween, the membrane was processed by the enhanced chemiluminescence (ECL) method according to the manufacturer’s specifications (Amersham).

Results

Recognition pattern and cytokine profile of Th0 MBPp83–99-specific T cell clones in response to analogue peptides with single alanine substitutions

DR2-restricted T cell clones specific for the 83–99 peptide of MBP were analyzed for the structural requirements in recognition of MBPp83–99 using a panel of 17-mer analogue peptide with single alanine substitutions at successive positions. DRB1*1501 (DR2a)- and DRB1*0101 (DR2b)-transfected mouse fibroblasts were used as the APC (24). Four independent T cell clones originally derived from three patients with MS were selected for this study. All four T cell clones were found to express distinct TCR Vα and Vβ genes with completely unrelated CDR3 sequences (Table I). As shown in Fig. 1, the analogue peptides could be divided into three functional categories according to their ability to stimulate the T cell clones in relation to the wild-type peptide. Although each clone had individual requirements in recognition of the 83–99 peptide, in general, analogue peptides with alanine substitutions at the central positions, such as positions 90 and 91, acted as antagonists and were able to completely abolish the activation and cytokine production of the MBPp83–99-specific T cell clones (Figs. 1 and 2). The finding is consistent with previous studies that indicate the critical role of the central amino acids within the 83–99 region of MBP as primary TCR contact residues (24, 30, 31). In contrast, analogue peptides with alanine substitution at flanking regions had an agonistic property, suggesting that these residues were less critical for interacting with the TCR. The third category of the analogue peptides, such as 86A and 88A, had unique properties in inducing minimal activation of the T cell clones and modulating their cytokine production, as compared with the wild-type peptide (Figs. 1 and 2).

Most interestingly, the two partial agonist peptides 86A and 88A exhibited a consistent pattern of cytokine regulation. Peptide 86A selectively down-regulated the production of Th2 cytokines (IL-4 and IL-10) in all clones without affecting their ability to produce Th1 cytokines (IFN-γ and TNF-α). In contrast, peptide 88A had an

![FIGURE 1](http://www.jimmunol.org/)

Proliferative responses of MBPp83–99-specific T cell clones to a panel of alanine-substituted peptides. CD4+ T cell clones MS1-2E8#9 (A), MS1-1C#7 (B), MS2-1D9 (C), and MS3-2E4 (D) generated from subjects with MS were stimulated with DR2a-transfected L cells pulsed with various peptides. Proliferative responses of the T cell clones were determined by [3H]thymidine incorporation, as described in Materials and Methods. Values shown are representative of three independent experiments performed in triplicates, SE in all experiments was less than 10%. Med, Medium alone as a control.

<table>
<thead>
<tr>
<th>T cell clone</th>
<th>V Region</th>
<th>N/Junctional Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS1-2E8#9</td>
<td>Vα14</td>
<td>LHSGSGSNYLPPGTGKTTLYVNP</td>
</tr>
<tr>
<td></td>
<td>Vβ18</td>
<td>PSTDQYQPGTRTLV</td>
</tr>
<tr>
<td>MS1-1C#7</td>
<td>Vα3</td>
<td>PEFLKDQYTGSTGGLGKGRLP</td>
</tr>
<tr>
<td></td>
<td>Vβ18</td>
<td>TSLAQDQYGPFRTTVL</td>
</tr>
<tr>
<td>MS2-1D9</td>
<td>Vα3</td>
<td>DAGGTYYIFGTRKLVL</td>
</tr>
<tr>
<td></td>
<td>Vβ7</td>
<td>QDAPQYQFGTGTLLVL</td>
</tr>
<tr>
<td>MS3-2E4</td>
<td>Vα15</td>
<td>NKLAGAQLGFPQGTRTLTINP</td>
</tr>
<tr>
<td></td>
<td>Vβ14</td>
<td>KGRGNKELFSGSGTQLSVL</td>
</tr>
</tbody>
</table>

*a TCR V gene usage and CDR3 sequences were determined as described in Materials and Methods.
opposite modulatory effect on the T cell clones in inhibiting Th1 but not Th2 cytokine production (Fig. 2). The results suggest that the two partial agonist peptides may have unique properties in triggering distinct signaling events leading to specific alteration of the cytokine production.

Modulation of the Lck kinase activity in MBPp83–99-specific T cell clones by partial agonist peptides 86A and 88A

Protein tyrosine kinases associated with the TCR complex represent the early/proximal signal culminating into a particular response. TCR engagement by an agonist peptide ligand leads to efficient recruitment and/or activation of Lck kinase at the T cell contact cap (32). In contrast, TCR interaction with an APL may induce a distinct signaling event characterized by changes in the Lck kinase activity (33), resulting in an entirely different T cell phenotype. We first examined whether T cell phenotype induced by peptides 86A and 88A is consistently associated with the Lck kinase activity. To this end, T cell clones were exposed to DR2-transfected L cells prepulsed with the wide-type peptide 83–99, 83A (an agonist), 86A or 88A (partial agonist), or 90A (an antagonist), respectively. The Lck kinase activity was measured subsequently in the T cell clones by immune complex kinase assays. As shown in Fig. 3, a significantly increased Lck kinase activity was detected in all T cell clones after exposure to the wild-type peptide or the agonist peptide 83A, suggesting that activation of Lck kinase is required for the Th0 response.

The two partial agonist peptides, 86A and 88A, appeared to exhibit distinct characteristics in regulating the Lck kinase activity as compared with the wild-type peptide. Although both peptides stimulated the T cell clones and significantly enhanced the Lck activity over the basal level, the Lck kinase activity induced by peptide 86A was markedly lower than that induced by the wild-type peptide in the T cell clones, with the exception of clone MS-C7#1 (Fig. 3). In contrast, peptide 88A overall exhibited an opposite modulating effect on the T cell clones by enhancing the Lck kinase activity (Fig. 3). Additional experiments with autophosphorylation revealed that the Lck autophosphorylation pattern correlated with its kinase activity toward exogenous substrate (Fig. 3, autophosphorylation). It is likely that the observed change in the Lck kinase activity after stimulation with different peptide ligands may be due to altered Lck protein level. To address this issue, the Lck protein level in peptide-stimulated T cell clones was examined.
by Western blotting. The results showed that the Lck protein level remained unchanged in given T cell clones, irrespective of the peptide ligands used (Fig. 3, Lck protein). Taken together, the findings indicate that analogue peptide with alanine substitution at position 86 down-regulated the Lck kinase activity and rendered the T cell clones to produce Th1 but not Th2 cytokines, whereas alteration at position 88 induced a distinct signaling event characterized by increased Lck kinase activity and the production of Th2 cytokines.

Fyn tyrosine kinase is another important member of Src family and is required for successful T cell activation (34). It has been shown that mature CD4⁺ thymocytes from fyn⁻/⁻ mice are severely impaired in TCR signaling, indicating the importance of Fyn in the phosphorylation cascade of T cell signaling and activation (35, 36). We examined whether the two partial agonist peptides are capable of modulating the Fyn kinase activity and whether the change in the Fyn kinase activity is reflected at the protein level. The results revealed that none of the peptides examined above could alter the Fyn protein level (Fig. 4, Fyn protein), suggesting that the characteristic changes in the Fyn kinase activity and autophosphorylation are not due to the variation in their protein level. Thus, the findings provide further evidence indicating that peptide 86A induces a distinct signaling process characterized by a substantially increased Fyn kinase activity in the MBPp83–99-specific T cell clones, which leads to the production of Th1 but not Th2 cytokines.

**Association of ZAP-70 kinase activity and its autophosphorylation with Th1 and Th2 deviation induced by 86A and 88A**

The observed regulation of Lck and Fyn kinases by 86A and 88A and its association with cytokine deviation prompted us to further investigate whether the signaling changes in the tyrosine kinase activity are carried over by the next signaling molecule, ZAP-70.
in this phosphorylation cascade. For this purpose, the kinase activity of ZAP-70 was examined in an immune complex kinase assay in terms of phosphorylation of an exogenous substrate and its autophosphorylation. As shown in Fig. 5, a basal level of ZAP-70 kinase activity and autophosphorylation was observed in the untreated T cell clones. Exposure of the T cell clones to the wild-type peptide or the agonist peptide 83A resulted in a significantly increased ZAP-70 kinase activity and autophosphorylation over the basal level. Stimulation with peptide 86A further enhanced the ZAP-70 kinase activity and their autophosphorylation. The kinase activity induced by peptide 86A was at least doubled in some clones as compared with that induced by the wild-type peptide (Fig. 5, A and B). Similar to the antagonist peptide 90A, stimulation of the T cell clones with peptide 88A resulted in a marked reduction or complete absence of ZAP-70 kinase activity and its autophosphorylation (Fig. 5, lane 5). Western blot experiments revealed that the protein level of ZAP-70 remained almost unchanged. Furthermore, as the activation level of ZAP-70 is a measure of its physical association with the $\zeta$-chain of TCR complex (7), we investigated the physical interaction of ZAP-70 with the $\zeta$-chain in the T cell clones after stimulation with various peptides. ZAP-70 and $\zeta$-chain were coprecipitated from the T cell clones using polyclonal Abs directed at the $\zeta$-chain. As illustrated in Fig. 6, the results confirmed a positive correlation between ZAP-70 kinase activity and ZAP-70/$\zeta$-chain association.

As summarized in Fig. 7, activation of all three tyrosine kinases, Lck, Fyn, and ZAP-70, is required for the MBPp83–99-specific T cell clones to produce both Th1 and Th2 cytokines. Th0 to Th1 deviation induced by peptide 86A (production of Th1 but not Th2 cytokines) is associated with a substantially enhanced Fyn and ZAP-70 kinase activity. Interestingly, peptide 88A appears to induce a partial signaling event characterized by complete absence of detectable Fyn and ZAP-70 activities, indicating that Fyn and ZAP-70 are not required for the T cell clones to produce only Th2 cytokines. Although the Lck kinase activity is less well correlated with the Th1 and Th2 deviation, stimulation of the T cell clones with the analogue peptides appears to alter its activity reciprocal to Fyn and ZAP-70 in some T cell clones.

Reversible Th1 and Th2 phenotypic shift in response to the analogue peptides

It is of interest to further examine whether the observed Th1 and Th2 shift is a differentiation process or whether it represents a reversible phenotypic switch induced by the analogue peptides. In a representative experiment, the Th0 clones were first treated with peptide 86A or peptide 88A, respectively, in the presence of L cells expressing DRB1*1501 to induce Th0 $\rightarrow$ Th1 and Th0 $\rightarrow$ Th2 shift. The T cells were then washed and allowed to rest for 5 days in culture before being rechallenged reciprocally with different analogue peptides to determine the cytokine profile. As shown in Fig. 8, the results indicate that the T cell clones induced by peptide 86A to produce Th1 cytokines could revert to secreting IL-4 and IL-10, but not TNF-α and IFN-γ upon second stimulation with peptide 88A. Conversely, T cell clones previously exposed to the Th2-inducing peptide 88A were able to produce Th1 cytokines when restimulated with peptide 86A. The finding suggests that the
Th1 and Th2 shift induced by the analogue peptides is not a differentiation process and the MBP-reactive Th0 cells can be rendered to produce either Th1 or Th2 cytokines, depending upon the nature of the analogue peptides.

Discussion

Autoreactive T cells recognizing MBP are thought to play an important role in the pathogenesis of MS (22, 37). Two immunodominant regions of human MBP, spanning the 83–99 residues and the 149–170 residues, have been identified in humans (21, 26, 38, 39). The immunodominant 83–99 region is of particular interest for its high binding affinity to the DRB1*1501 molecule, an HLA gene product preferentially expressed in the MS population, and its association with T cell responses to MBP in patients with MS (21, 40). Although MBP-reactive T cells generally express a heterogeneous TCR Vα and Vβ gene usage in different patients with MS,
the V gene rearrangements are limited in individual MS patients as a result of in vivo clonal activation and expansion (26, 27, 41). Furthermore, some restricted structural features have been found, including a preferential Va3 rearrangement and a common CDR3 sequence motif, among the T cell clones that are derived from different MS patients and uniformly recognize the 83–99 peptide in the context of DRB1*1501 (28, 42).

APLs can provide an incomplete signal to specific T cells, resulting in the inhibition of T cell proliferation and alteration of the cytokine profile (43). The findings described in this work demonstrate that some amino acids within the 83–99 region of MBP act as primary TCR contact residues critical for the T cell recognition, with Phe90 and Lys91 being the most critical TCR contact residues. Alanine substitutions at these positions will lead to a complete abolishment of the T cell responses to the MBPp83–99. However, alanine substitutions at Val86 and His88 rendered the peptides to unique partial agonists capable of inducing Th0 → Th1 and Th0 → Th2 deviation, respectively, in MBPp83–99-specific T cell clones. In contrast to the primary TCR contact points, both residues Val86 and His88 function as so-called secondary contact points in which alteration leads to incomplete inhibition of T cell proliferation and modulation of their cytokine production. As both peptides, 86A and 88A, were shown to bind DRBI*1501 with a high affinity (30), the functional changes (inhibition of cell proliferation and deviation of cytokine profile) are most likely due to their specific engagement with the TCR. The study also indicates that the observed Th1 and Th2 phenotypic shift of the MBP-reactive T cell clones is a reversible process induced by the analogue peptides and is not a differentiation process, which may have important implications in considering the therapeutic use of the analogue peptides.

In this study, DR2-transfected L cells were used as APC (24) to avoid the costimulatory signals that could have been delivered through ligation of T cell surface molecules such as CD28, CD2, LFA-1, VLA-4, or heat-stable Ag. Although costimulation clearly plays an important role in T cell signaling and activation, there is mounting evidence that certain conditions may render T cells less dependent on the costimulatory molecules. Recently, Lovett-Racke et al. (44) and Scholz et al. (45) have shown that activation and expansion of MBP-reactive T cells derived from patients with MS are independent of costimulatory signals delivered by the B7-CD28 ligation.

The mechanisms by which subtle changes in the exogenous binding to the αβ recognition unit lead to different signaling events and altered functional responses are unknown. A possible explanation may be related to either failure to induce a required conformational change in the TCR or failure to assemble all of the necessary molecules in the T cell “contact cap”(43). Consistent with this possibility is the notion that upon ligand binding, both receptor cross-linking and subsequent conformational change must occur for successful activation (46). There is evidence suggesting that members of Src family of nonreceptor protein tyrosine kinases, such as Lck and Fyn proteins, are intimately associated with TCR-CD3 complex and play an important role in T cell activation.
In addition to Lck and Fyn, the overall phosphorylation within the TCR-CD3 complex is controlled by either of the two tyrosine kinases of Syk family, namely, Syk and ZAP-70 (49). The present study suggests that activation of all three protein tyrosine kinases (Lck, Fyn, and ZAP-70) is required for a Th0 response, and that an equilibrium in the activation of these tyrosine kinases is a critical factor in determining the nature of an ensuing response. This possibility is strengthened by the finding that when the balance of Lck and Fyn is tilted in favor of Fyn, it leads to enhanced activation of ZAP-70 and an increased association with the ζ-chain. These changes correlate characteristically with a Th1 phenotype. Conversely, if the balance is tilted toward Lck, the production of IFN-γ and TNF-α is diminished, while the production of IL-4 and IL-10 is unaffected. The finding indicates that Th0→Th2 deviation induced by the analogue peptides requires selective activation of Lck kinase, but not the activation of Fyn and ZAP-70 kinases. The observation is consistent with several previous studies that attempted to address the role of TCR-associated protein tyrosine kinases in T cell activation using differentiated Th1 and Th2 clones. Tamura and coworkers reported that the production of IL-2 in Th1 cells correlated with up-regulation of Fyn and ZAP-70, while the production of IL-4 in Th2 cells was associated with decreased activities of Fyn and ZAP-70 (50). Furthermore, deficient expression of Lck in Th2 cells was found to result in partial TCR signaling and changes in mRNA levels of various cytokines (51). Taken together, the results described in this work demonstrate that the TCR-CD3 complex can engage selective intracellular signaling pathways, depending upon the nature of the peptide ligands. The alteration of the initial signals results in modulation of tyrosine kinase activity and subsequent T cell function, highlighting the exquisite sensitivity of the TCR to subtle changes in its recognition unit.

The finding that a single alanine substitution at a given position within the immunodominant peptide of MBP leads to the production of Th2 cytokines and the inhibition of the Th1 cytokines has important therapeutic implications for MS. It has been demonstrated that the Th1 cell responses to the 83–99 region of MBP and ZAP-70, while the production of IL-4 in Th2 cells was associated with decreased activities of Fyn and ZAP-70 (50). Furthermore, deficient expression of Lck in Th2 cells was found to result in partial TCR signaling and changes in mRNA levels of various cytokines (51). Taken together, the results described in this work demonstrate that the TCR-CD3 complex can engage selective intracellular signaling pathways, depending upon the nature of the peptide ligands. The alteration of the initial signals results in modulation of tyrosine kinase activity and subsequent T cell function, highlighting the exquisite sensitivity of the TCR to subtle changes in its recognition unit.

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