Cross-Reactive TCR Responses to Self Antigens Presented by Different MHC Class II Molecules

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Autoreactive T cells represent a natural repertoire of T cells in both diseased patients and healthy individuals. The mechanisms regulating the function of these autoreactive T cells are still unknown. Ob1A12 is a myelin basic protein (MBP)-reactive Th cell clone derived from a patient with relapsing-remitting multiple sclerosis. Mice transgenic for this human TCR and DRA and DRB1*1501 chains develop spontaneous experimental autoimmune encephalomyelitis. The reactivity of Ob1A12 is reported to be restricted to recognition of MBP peptide 85–99 in the context of DRB1*1501. DRA/DRB1*1501 and the patient's other restriction element, DRA/DRB1*0401, differ significantly in their amino acid sequences. In this study we describe an altered peptide ligand derived from $MBP_{85–99}$ with a single amino acid substitution at position 88 (Val to Lys; 88V→K), that could stimulate the Ob1A12.TCR in the context of both DRA/DRB1*1501 and DRA/DRB1*0401. Analysis of a panel of transfected T cell hybridomas expressing Ob1A12.TCR and CD4 indicated that Ob1A12.TCR cross-reactivity in the context of DRA/DRB1*0401 is critically dependent on the presence of the CD4 coreceptor. Furthermore, we found that activation of Ob1A12.TCR with MBP altered peptide ligand 85–99 88V→K presented by DRB1*1501 or DRB1*0401 resulted in significant differences in TCRζ phosphorylation. Our data indicate that injection of altered peptide ligand into patients heterozygous for MHC class II molecules may result in unexpected cross-reactivities, leading to activation of autoreactive T cells. ***The Journal of Immunology*****, 2004, 173: 1689–1698.**

The definition of self-epitopes for human T cells has helped in understanding the immune response of human autoimmune diseases, including multiple sclerosis (MS)[3] (1). The search for the specific T cell autoantigens in MS has yielded several potential autoantigens, predominantly myelin proteins, including myelin basic protein (MBP), proteolipid protein, and myelin oligodendrocyte glycoprotein (2, 3). For the purpose of defining autoantigens in human diseases, T cell specificity has been characterized as the ability of individual T cells, expressing a single TCR, to discriminate among peptide Ags and to respond specifically to Ag presented in the context of the cognate MHC from the subject (4). In contrast, T cell degeneracy, defined as a capacity to react with many disparate Ags (peptide-MHC complexes) was discovered as a challenge for the concept of T cell specificity (5). Although T cell degeneracy was believed to be associated with the increased risk of induction of autoimmune responses, it may represent a fundamental mechanism for maintenance of the diversity of TCRs (6). Furthermore self-peptide-MHC molecule recognition seems to be important for the survival of a peripheral pool of T cells, particularly for the homeostasis of naive T cells (7). Thus, T cell degeneracy and self-reactivity may be two different facets of the same phenomenon aimed at the maintenance of diversity of T cells in the periphery.

Analysis of the human immune response to myelin Ags has focused primarily on human MBP. We and others have shown that human MBP peptide 85–99 is immunodominant for human MBP-specific T cells; in particular, in patients with the HLA-DR2 (DRA/DRB1*1501, DRB5*0101) haplotype (8–10). Recent experiments indicate that mice transgenic for human TCRs recognizing $MBP_{85–99}$ and DRA and DRB1*1501 can develop spontaneous experimental autoimmune encephalomyelitis (EAE), even in the presence of RAG genes (S. Ellmerich, M. Mycko, D. A. Hafler, V. Kuchroo, and D. Altmann, unpublished observations). Moreover, there is evidence indicating that MBP-specific T cells are activated and clonally expanded in patients with MS. Clonal expansion of MBP-specific T cells was demonstrated by TCR sequence analysis of independent MBP-specific T cell clones from MS patients (9, 11). It was also shown that MBP-reactive T cells are less dependent on costimulation for proliferation in MS patients (12, 13). Ex vivo analysis of $MBP_{85–99}$-associated TCR chain transcripts suggested frequencies of MBP-reactive T cells in MS patients as high as 1 in 300 (14).

TCR degeneracy is tightly linked to the strength of the signal delivered through this receptor, which can determine which cytokines are secreted by the T cell (15). The T cell apparently measures affinity in part by timing the engagement between the TCR and the peptide/MHC complex. With longer engagement, a total, rather than partial, TCR complex has time to form, and the extent of ζ-chain phosphorylation increases correspondingly. Altered peptide ligands (APLs), which bind with lower affinity to the TCRs and change the cytokine program of a T cell from a Th1 to a Th2 response, have been used as a therapy for autoimmune disease (16). Using the murine EAE model of MS, it was shown that APLs can activate IL-4 secretion by both encephalitogenic T cells and naive T cell clones cross-reactive with self Ags.

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[3] Abbreviations used in this paper: MS, multiple sclerosis; APL, altered peptide ligand; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein.

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Injection of APLs is of clear therapeutic value in treating different models of EAE (17), and autoreactive human T cell clones can also be induced to secrete the anti-inflammatory cytokines IL-4 and TGF-β after TCR engagement by APLs (18). However, we previously observed that whereas APLs can induce Th2 cytokine secretion of MBP-reactive T cells isolated from the peripheral blood T cell of MS patients, they can also induce an unexpected response in some patients, activating these MBP-reactive T cells against the patient's own tissues (19).

Together, these data provided strong rationale for the use of APLs as a therapeutic approach for patients with autoimmune disease. They also raised the issue that in some instances, highly degenerate TCRs may recognize APLs as self Ags. A recently published phase II clinical trial on the value of an altered $MBP_{85–99}$ peptide confirms these findings. At the higher peptide dosage tested, two of seven patients developed remarkably high frequencies of MBP-reactive T cells, and these responses were associated with significant increases in lesions detected by magnetic resonance imaging. In contrast, patients treated with lower doses of the APL did not appear to have such disease flare-ups and may have indeed exhibited some degree of immune deviation toward increases in IL-4 secretion of MBP-reactive T cells (20–22). Thus, APLs represent a classic double-edged sword. In our outbred population, given the high degree of degeneracy in the immune system, will it be possible to find APLs of self peptides that do not pose a risk of cross-reactivity with self?

Previous studies concentrated on the specificity of T cell clones recognizing MBP in the context of the single MHC class II molecule. Because the majority of patients are heterozygous for the HLA-DR locus and to better understand the bounds of autoreactive CD4 T cell cross-reactivity in the context of different MHC class II molecules, we analyzed the recognition patterns of T cell receptors generated against MBP peptide 85–99 and its altered peptide ligands, which differed from the native peptide by a single amino acid substitution. We also examined the modulation of T cell responses induced by stimulation with different MHC class II/Ag complexes. Our data demonstrate the existence of significant cross-reactivity of CD4 T cells in the context of the distinct MHC class II molecules. These investigations indicate that injection of APL into patients heterozygous for MHC class II molecules may result in unexpected cross-reactivities, leading to activation of autoreactive T cells.

Materials and Methods

Ags and T cell clone

MBP peptide 85–99 (ENPVVHFFKNIVTPR) and altered peptides were synthesized in the Biopolymer Laboratory, Harvard Medical School, by automated solid phase methods, and human MBP protein was purified as previously described in detail (9). MBP-reactive T cell clone Ob1A12, generated from PBMC from a patient with MS by limited dilution cloning, has been described and characterized previously (8, 9, 18). The Ob1A12 T cell clone was maintained by weekly restimulation with 2 μg/ml PHA (Murex, Dartford, U.K.) in RPMI 1640 supplemented with 10% human serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2 mM glutamine, and 10% T-Stim (Collaborative Biomedicine Products, Bedford, MA) using irradiated human PBMC as feeder cells.

Generation of T cell hybridomas

PCR primers with flanking restriction sites were designed to amplify the rearranged VJα and VDJβ segments from genomic DNA of the Ob1A12 T cell clone, based on previously published sequences of the clone's TCRα and TCRβ cDNAs (9, 23). The PCR products were subcloned into the appropriate restriction sites of the genomic TCR expression cassettes pTαCass and pTβCass (24). The constructs were electroporated together with pPink2 vector encoding both full-length human CD4 and the neomycin resistance marker gene (gift from Dr. Reinherz, Dana-Farber Cancer Institute, Boston, MA), into $58\alpha^{-}\beta^{-}$ hybridomas (25). After selecting the transfectants in medium supplemented with G418 (1 mg/ml), resistant cells were screened by flow cytometry for the expression of CD3, CD4, or Vβ2. Selected Ob1A12.TCR transfectants were further cultured in DMEM supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, and 2 mM glutamine.

T cell proliferation assay

T cell proliferation was assessed by [^{3}H]thymidine incorporation assay. The homozygous B cell line MGAR (DRA/DRB1*1501), PREISS (DRA/DRB1*0401), and the autologous heterozygous Ob EBV line (DRA/DRB1*1501/DR*0401) were used as APCs and treated with mitomycin C (50 μg/ml, 20 min). T cell proliferation assays were set up in triplicate in 96-well, U-bottom plates with 5×10^4 T cells/well (Ob1A12 transfectants), 5×10^4 APCs/well, and Ag at different concentrations. After 48 h of culture, [^{3}H]thymidine was added (1 μCi/well = 37 GBq/well). After an additional 16 h, cells were harvested onto glass-fiber filters (Wallac, Gaithersburg, MD), and radioactivity was counted in a beta scintillation counter (Wallac). The background proliferation levels of the unstimulated T cell cultures ranged from 1900–3900 cpm. Proliferation data are expressed as Δcpm, which is equal to the mean cpm cultures with Ag subtracted by the mean cpm of culture medium alone.

Ag presentation by DR transfectants

L cells transfected with DR1501 (DRA/DRB1*1501), DR0401 (DRA/DRB1*0401), DR0404 (DRA/DRB1*0404), 71R86G (DRA/DRB1*0404 single amino acid mutant 86V→G), and 71K86V (DRA/DRB1*0404 single amino acid mutant 71R→K) were used as APCs (gift from Dr. Sette, Epimmune, San Diego, CA). Ag presentation by DR transfectants assays were set up in triplicate in 96-well, U-bottom plates with 1×10^5 Ob1A12.TCR transfectants/well and 1×10^5 APCs/well plus Ag at different concentrations. After 24-h culture, 100 μl of supernatants were harvested and assayed for the presence of IL-2 using the IL-2-dependent cell line HT-2. The supernatants were added to 1×10^4 HT-2 cells and incubated at 37°C for 20 h. IL-2-dependent proliferation of HT-2 cells was tested by the uptake of [^{3}H]thymidine as described above.

FACS analysis of surface Ags

To screen the stable transfected lines, Ob1A12.TCR hybridomas were stained with FITC-labeled anti-mouse CD3ϵ (clone 145-2C11; BD Pharmingen, San Diego, CA), anti-mouse TCRβ (clone H57-597; BD Pharmingen), anti-human Vβ2-FITC (clone MPB2D5; Coulter, Miami, FL), PE-labeled anti-human CD4 (clone 13B8.2; Coulter), and isotype-matched control Abs: FITC-labeled hamster IgG1 (clone A19-3; BD Pharmingen), FITC-labeled hamster IgG2 (clone Ha4/8; BD Pharmingen), and PE- and FITC-labeled mouse IgG1 (clone MOPC-21; BD Pharmingen). DR-transfected L cells and B cell lines were stained with PE-labeled anti-HLA-DR (clone B8.12.2; Coulter). Stained cells were analyzed by flow cytometry on FACScan using CellQuest software (BD Biosciences, San Diego, CA).

Immunoprecipitations and Western blots

Ob1A12.TCR transfectants (10^7 cells) were washed and resuspended in RPMI 1640, incubated on ice for 15 min, mixed with peptide-pulsed APCs (10^7 cells), and warmed to 37°C for the time indicated. The cells were then washed twice with cold RPMI 1640 containing 1 μM sodium orthovanadate (Sigma-Aldrich, St. Louis, MO) and subsequently lysed for 15 min on ice in cold lysis buffer (1% Brij 96, 150 mM NaCl, 25 mM HEPES (pH 7.5), 1 mM EDTA, 1 μM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Detergent extracts were cleared by centrifugation at 14,000 rpm for 10 min at 4°C. The resulting supernatants were subjected to immunoprecipitations by incubating them with anti-mouse CD3ϵ (clone 145-2C11; BD Pharmingen) and 25 μl of protein A-agarose for at least 2 h at 4°C. The samples were washed three times in lysis buffers and subsequently electrophoresed through 10% SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), immunoblotted with anti-phosphotyrosine (clone 4G10; Upstate Biotechnology, Lake Placid, NY), and detected by ECL (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's instructions.

Results

Generation and analysis of Ob1A12.TCR hybridomas

To generate $MBP_{85–99}$-specific T cell hybridomas, we isolated genomically rearranged TCRα and TCRβ genes from the $MBP_{85–99}$-specific T cell clone Ob1A12 (1–3). The rearranged TCR genes of

Ob1A12, which used Vα3.1Jα40 and Vβ2.1Jβ2.1 (9, 23), were subcloned into cosmid cassette vectors containing murine TCR-specific regulatory elements (24). The transgenic constructs were electroporated together with plasmids containing human CD4 and the neomycin resistance marker gene into the murine 58$\alpha^-\beta^-$ T cell hybridoma (25). Thus, the resulting hybrid TCR consisted of the human Ob1A12-derived variable region and the murine constant region (Fig. 1*A*). To confirm TCR complex surface expression, G418-resistant transfectants were screened for the expression of CD3 and TCR by flow cytometry (Fig. 1*B*). The selected transfectants expressed mouse CD3ϵ-chain and TCRβ constant regions as well as human Vβ2, indicating that the T cell hybridoma expressed the hybrid human VDJ/mouse C TCR complex. To investigate the functional property of the hybrid Ob1A12VDJ TCR, we analyzed the Ag specificity of Ob1A12.TCR hybridomas by thymidine incorporation assays (Fig. 1*C*). The Ob1A12.TCR transfectants were responsive to MBP peptide 85–99 presented in the context of HLA-DRA/DRB1*1501 (9, 23). The Ob1A12.TCR hybridomas were also able to recognize processed

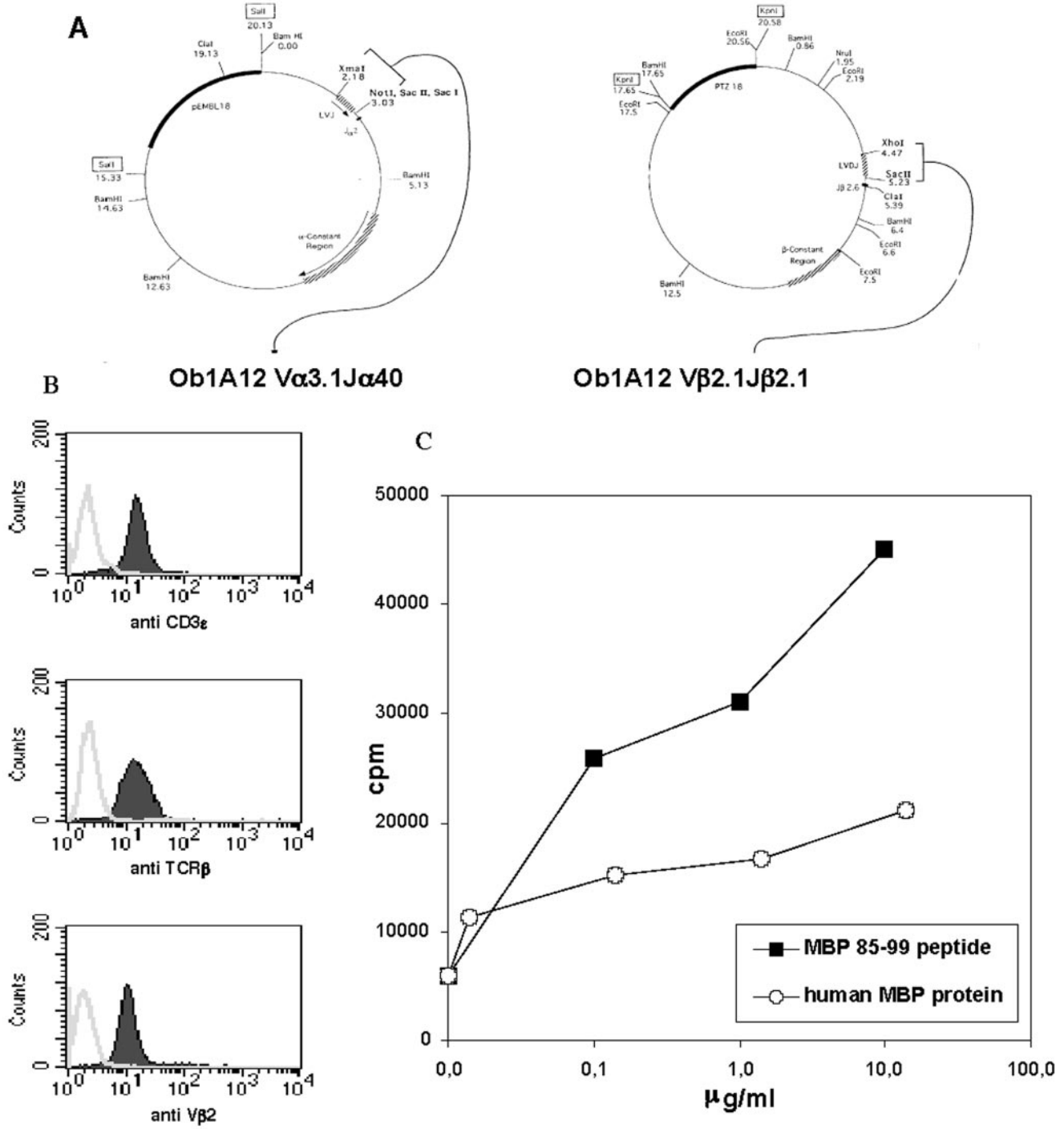

FIGURE 1. The Ob1A12.TCRα and TCRβ expression vectors were generated using pTαCass and pTβCass expression vectors (*A*). The expression of OB1A12.TCR in 58$\alpha^-\beta^-$ transfectants was confirmed by FACS analysis with anti-mouse CD3ϵ, anti-mouse TCRβ, and anti-human Vβ2 Abs vs isotype-matched control Abs (*B*). The OB1A12.TCR-positive hybridomas showed specific recognition of both MBP peptide 85–99 and human MBP in the context of HLA-DRA/DRB1*1501, as measured by IL-2 production HT.2 assay (*C*). SDs were <10%.

human MBP protein presented by the homozygous B cell line MGAR (DRA/DRB1*1501). Thus, the reconstituted hybrid human/mouse surface TCR/CD3 complex was functional, maintained Ag specificity to $MBP_{85–99}$, and processed human MBP protein presented by DRA/DRB1*1501-expressing APCs.

Fine T cell specificity of Ob1A12.TCR

To inspect the fine specificity of the Ob1A12.TCR, its response to a panel of APLs with single amino acid substitutions to $MBP_{85–99}$ peptides was examined. Similar to what we previously reported for the Ob1A12 T cell clone, the Ob1A12.TCR transfectants recognized MBP peptide 85–99 in the context of DRA/DRB1*1501; 90H, 91F, and 93K were identified as TCR contact residues, whereas 89V and 92F were identified as major DRA/DRB1*1501 anchor residues (23, 26). The pattern of MBP peptide recognition in the context of DRA/DRB1*1501, presented by DR-transfected L cells was identical with that of the EBV-transformed B cell line (Fig. 2*A*). Thus, the fine specificity of Ob1A12.TCR recognition in the transfectant in the context of DRA/DRB1*1501 mimics the previously described Ob1A12 T cell clone responses (23, 26, 27) (Fig. 2). Because the patient is HLA-DR heterozygous, expressing DRA1, DRB1*1501 and DRA/DRB1*0401 (8, 9), we examined the responses of MBP peptide 85–99 and the panel of MBP peptide 85–99 with single amino acid substitutions in the context of the other self DR allele, DRA/DRB1*0401 (Fig. 2). As we previously reported, MBP peptide 85–99 presented in the context of DRA/DRB1*0401 does not lead to activation of Ob1A12.TCR (23). Surprisingly, the $MBP_{85–99}$ 88V→K APL (88K) was recognized by Ob1A12.TCR in the context of DRA/DRB1*0401 (Fig. 2B).

Role of CD4 coreceptor in recognition of $MBP_{85–99}$ by Ob1A12.TCR$^+$ transfectants

The crystal structure of the MBP/DRA, DR1*1501 complex revealed that residue 88 (P-1 position) is solvent-exposed and may thus contribute to TCR recognition (28). For TCR recognition of MBP peptide 85–99, P2 His, P3 Phe, and P5 Lys pockets were previously shown to be essential (18, 23, 26). To explore the possibility that amino acid substitution of $MBP_{85–99}$ at position 88V to lysine (88K) may strengthen the TCR signal alone, we investigated the role of CD4 in the presentation of 88K peptide. 58$\alpha^-\beta^-$ cells were transfected with plasmids encoding the Ob1A12.TCR and human CD4. Ob1A12.TCR$^+$ transfectants were selected by FACS

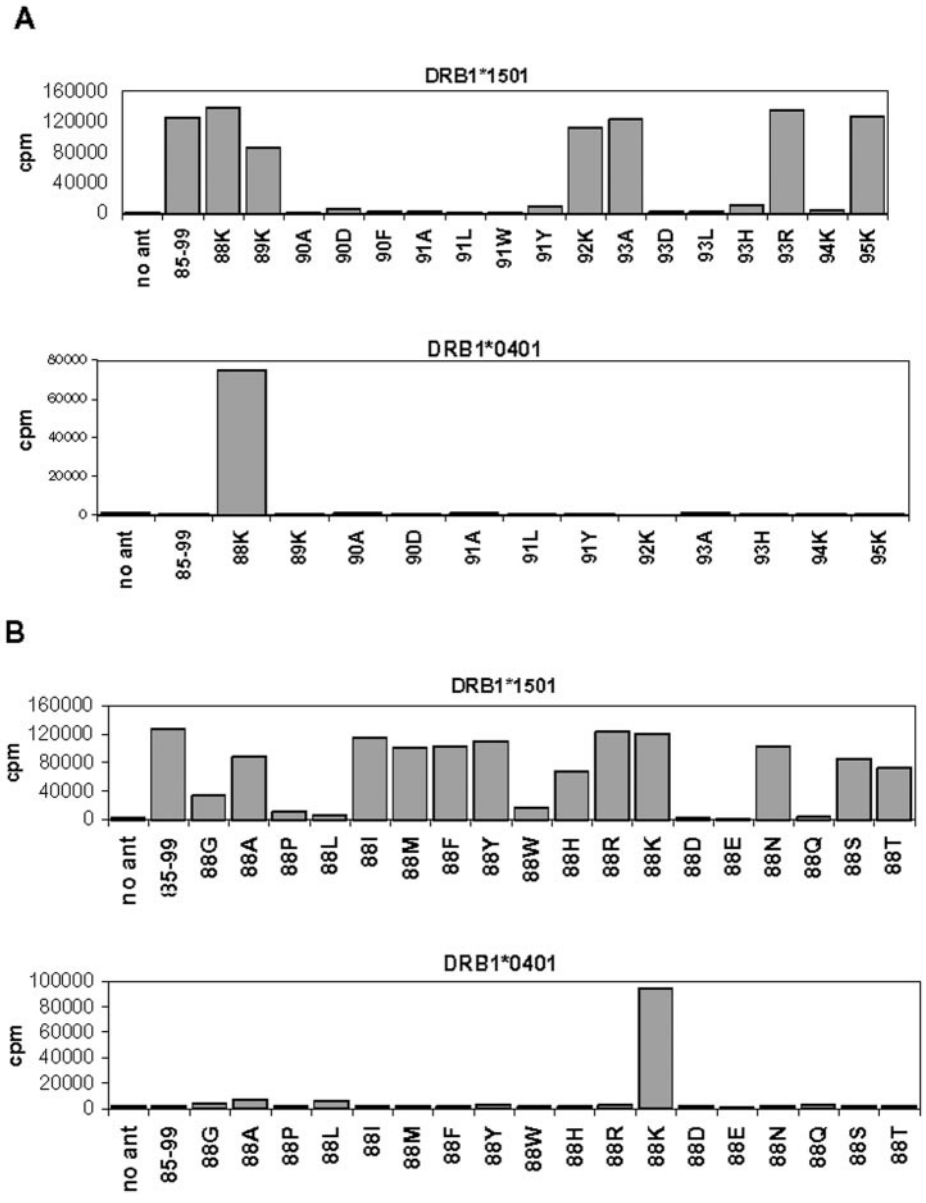

FIGURE 2. Ob1A12.TCR hybridoma recognition of MBP peptide 85–99 APLs. *A*, The $MBP_{85–99}$ peptide was substituted in the core recognition region and presented by either DRB1*1501 or DRB1*0401. *B*, As the MBP 88K peptide was recognized in the context of DRB1*0401, a series of $MBP_{85–99}$ peptides with substitutions at position 88 was synthesized. Proliferation was measured by IL-2 production using the HT.2 assay. SDs were $<10\%$.

analysis in lines that showed the expression or lack of expression of human CD4 ($CD4^+$ lines and $CD4^-$ lines, respectively) and were subcloned (data not shown). $CD4^+$ and $CD4^-$ lines of Ob1A12.TCR^+ transfectants were examined for recognition of MBP peptide 85–99 and 88K APL in the context of DRA/DRB*1501 (Fig. 3*A*) and DRA/DRB*0401 (Fig. 3*B*). The proliferative response of Ob1A12.TCR transfectants to $MBP_{85–99}$ presented by DRA/DRB1*1501 was similar, albeit increased, in lines that expressed human CD4 compared with lines that did not (Fig. 3*A*). However, when $MBP_{85–99}$ 88K APL was presented by DRA/DRB1*0401, only the lines that expressed human CD4 responded by proliferation (Fig. 3*B*). Thus, we demonstrate a crucial role for the CD4 coreceptor in the presentation of MBP peptide 85–99 88V→K APL in the context of DRA/DRB1*0401.

*MBP peptide 85–99 and APL presentation in the context of DRA/DRB1*0404*

The amino acid sequences of DRB1*1501 and DRB1*0401 differ by 10 residues (22), including residues 71 and 86, which are critical for the binding and positioning of the peptides in the DR binding groove (23, 27, 28). To examine whether presentation of $MBP_{85–99}$ or its APL (88K) by DRB1*1501 or DRB1*0401 affects the recognition of Ob1A12.TCR, we analyzed IL-2 production by Ob1A12.TCR transfectants to MBP peptide 85–99 presented by DRA/DRB1*0404. DRB1*0404 differs from DRB1*0401 by two amino acids at positions 71 and 86, which are critical for peptide presentation. DRB1*0404 shares the same amino acid at position β86 (valine) with DRB1*1501, which shapes the P1 pocket crucial for peptide binding in the groove (28). Changes in the P1 pocket, which is occupied by the 89V residue of MBP peptide 85–99, could influence the final positioning of the neighboring 88 residue of MBP peptide 85–99 and thereby affect its recognition by Ob1A12.TCR. We examined whether sequence differences between DRB1 1501 and DRB1 0404 affected the recognition of $MBP_{85–99}$ by the Ob1A12.TCR by determining IL-2 production by Ob1A12.TCR transfectants to $MBP_{85–99}$ and its APL (88K) in the context of either DR molecule (Fig. 4). At low Ag concentrations, the native MBP peptide 85–99 presented to Ob1A12.TCR transfectants elicited a weaker response when presented by L cells expressing DRA/DRB1*0404 compared with L cells expressing DRA/DRB1*1501 (Fig. 4*A*). The stimulation by 88K APL resulted in a similar proliferative response to $MBP_{85–99}$ when presented by DRB1 1501 molecules (Fig. 4*B*). In contrast, recognition of the APL by DRB1 0404 resulted in a significantly increased response at low Ag concentrations (0.1–10 μg/ml) (Fig. 4*B*). Moreover, the presentation of a panel of different $MBP_{85–99}$ single amino acid substitutions at various peptide positions (Fig. 4*C*) were the same in the context of DRA/DRB1*1501 as in the DRB1 0404 context.

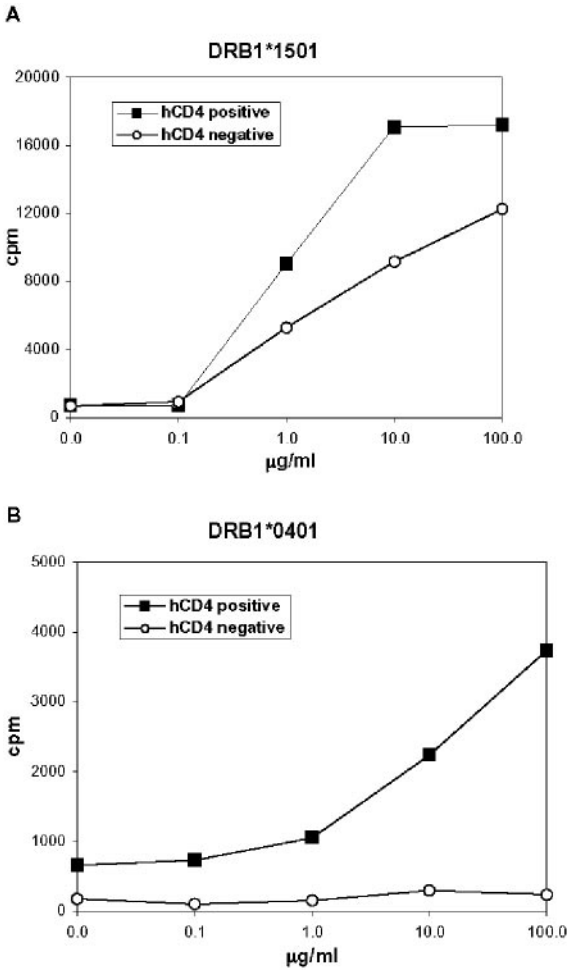

FIGURE 3. Ob1A12.TCR hybridomas can recognize MBP peptide 85–99 in the context of DRA/DRB1*1501 regardless of the presence of human CD4 (*A*), but MBP peptide 85–99 APL 88K recognition in the context of DRA/DRB1*0401 is critically dependant on the CD4 coreceptor (*B*), as measured by IL-2 production using the HT.2 assay. The most representative results of the several different Ob1A12.TCR line experiments are presented. SDs were <10%.

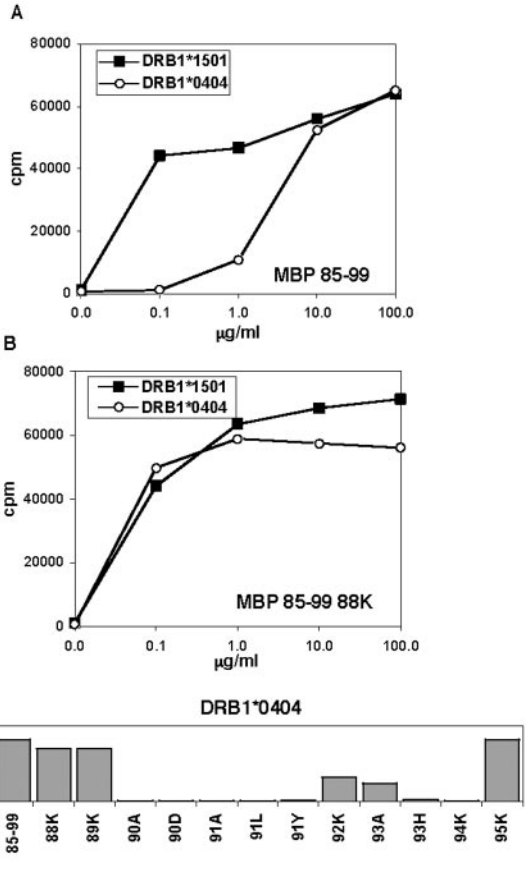

FIGURE 4. Ob1A12.TCR recognizes MBP peptide 85–99 (*A*) and MBP peptide 85–99 APL 88K (*B*) in the context of either DRA/DRB1*1501 or DRA/DRB1*0404. Recognition of the panel of MBP peptide 85–99 APLs by Ob1A12.TCR hybridomas is similar in the context of DRA/DRB1*0404 (*C*) and DRA/DRB1*1501 (Fig. 2*A*). SDs were <10%.

*Comparison of MBP peptide 85–99 presentation in the context of DRA/DRB1*0404 and DRA/DRB1*0401*

Because MHC residues 71 and 86 are the only two differences between the DRβ-chain sequences of DRB1*0404 and DRB1*0401 involved in peptide binding, we analyzed the pattern of $MBP_{85–99}$ presentation to Ob1A12.TCR by L cells transfected with either DRA/DRB1*0404 or DRA/DRB1*0401. DR*0401 expresses a lysine at position 71 and a glycine at position 86, whereas DR*0404 expresses an arginine and a valine, respectively. We also used L cells expressing single amino acid mutants with either a lysine at position 71 and a valine at 86 (71K86V) or with a arginine at 71 and a glycine at 86 (71R86G). The DR-transfected L cells used for this analysis had comparable levels of surface DR molecules in flow cytometric analysis (data not shown). The greatest responses to $MBP_{85–99}$, as expected, were associated with peptide presentation in the context of DRA/DRB1*0404, whereas modifications of amino acids at position 71 or 86 significantly diminished peptide recognition in the following order of importance: modification at 86 < modification at 71 < modification at both 71 and 86. This hierarchy of the Ob1A12.TCR responses was similar regardless of whether the CD4 coreceptor was present in Ob1A12.TCR transfectants (Fig. 5). Of interest, Ob1A12 responses to MBP peptide 85–99 88K APL, although generally better than recognition of the native $MBP_{85–99}$, also showed the same hierarchy of responses to MHC modifications at positions 71 and 86 (Fig. 6). The hierarchy of recognition by Ob1A12.TCR of 88K APL was also not modified by CD4 cotransfection (Fig. 6). Thus, this series of experiments using four different DR molecules with single amino acids modifications at DRβ positions 71 and 86 suggests that the positioning of MBP peptide 85–99 and that of MBP peptide 85–99 88V→K APL in the DR binding groove are not significantly different between DRA/DRB1*1501, DRA/DRB1*0404, and DRA/DRB1*0401.

*Ob1A12 T recognition of MBP peptide 85–99 and MBP peptide 85–99 88V→K APL in the context of DRA/DRB1*1501 and DRA/DRB1*0401*

To confirm the observation made in the Ob1A12.TCR transfectants, we examined the responses of MBP peptide 85–99 to the original Ob1A12 T cell clone. As APCs, we used EBV-transformed B cell lines expressing different DR molecules. All the B cell lines used were found by flow cytometry to express comparable levels of HLA-DR (not shown). We observed that the Ob1A12 T cell clone, in a similar manner to OB1A12.TCR transfectants, was stimulated by the MBP peptide 85–99 88K APL in the context of both DRA/DRB1*1501 and DRA/DRB1*0401 (Fig.

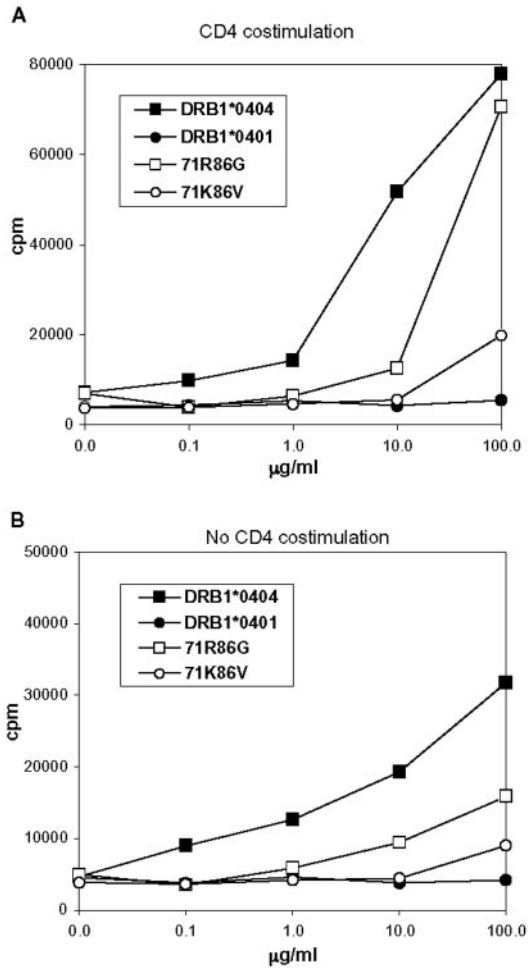

FIGURE 5. Recognition of $MBP_{85–99}$ peptide by Ob1A12.TCR in the context of DRA/DRB1*0404, DRA/DRB1*0401, and single mutants of DRB1*0401 at positions 71R86G and 71K86V. Stimulation was measured for transfectants expressing (*A*) or not expressing (*B*) CD4. SDs were <10%.

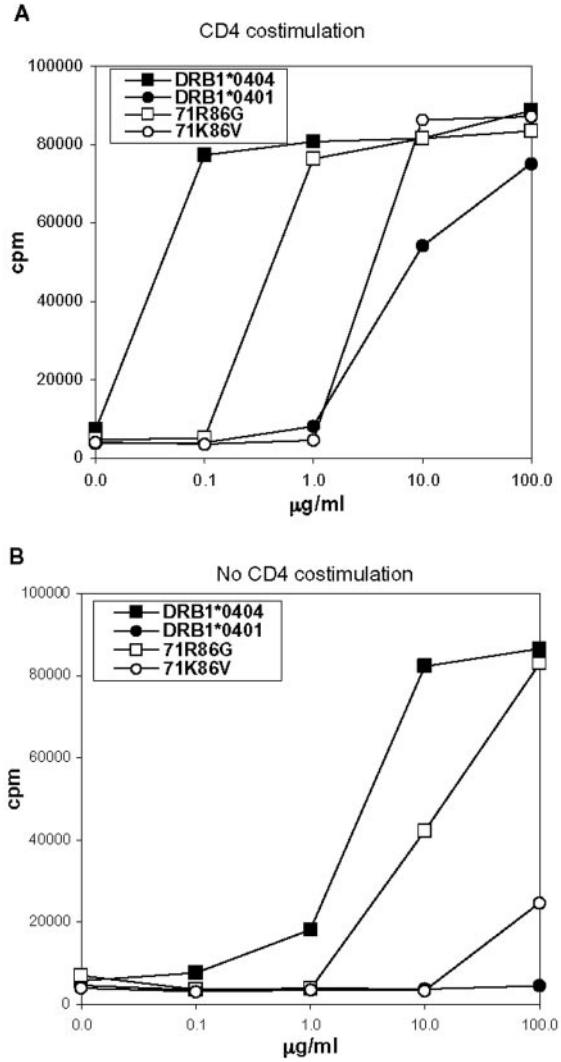

FIGURE 6. Recognition of $MBP_{85–99}$ 88K APL by Ob1A12.TCR in the context of DRA/DRB1*0404, DRA/DRB1*0401, and single mutants of DRB1*0401 at positions 71R86G and 71K86V. Stimulation was measured for transfectants expressing (*A*) or not expressing (*B*) CD4. SDs were <10%.

7). In contrast, no significant response was found when native MBP peptide 85–99 was presented by DRA/DRB1*0401 (Fig. 7*B*). Thus, the cross-reactivity demonstrated in the Ob1A12.TCR transfectants was confirmed in the Ob1A12 T cell clone in the context of DRB1 1501 and 0401. We also examined cytokine secretion induced by EBV cell lines coexpressing both DRB1*0401 and DRB1*1501 and found that the cytokine profile of T cells stimulated by the native peptide was not changed (data not shown).

*Analysis of the difference in TCR proximal signaling of Ob1A12.TCR in antigenic stimulation by DRA/DRB1*1501 or DRA/DRB1*0401*

TCRζ-chain phosphorylation was shown to be among the first critical events following TCR engagement that leads to subsequent T cell activation (29–31). To investigate whether $MBP_{85–99}$ presentation by DRA/DRB1*1501 or DRA/DRB1*0401 resulted in differences in Ob1A12 T cell activation, we determined whether the TCRζ-chains were tyrosine phosphorylated after Ag stimulation, because tyrosine phosphorylation of TCRζ-chains is an important step leading to downstream signaling events. We coimmunoprecipitated the TCRζ-chain with the TCR-CD3 complex from $MBP_{85–99}$-stimulated, Ob1A12.TCR-transfected T cell hybridomas and determined the degree of its phosphorylation using a specific anti-phosphotyrosine Ab. TCRζ phosphorylation resulted in the formation of the two classical phospho-TCRζ isoforms: p23 and p21 (Fig. 8). Phosphorylation of the p23 isoform represents one of the major events during activation of the TCR complex (30, 31). Phospho-TCRζ p23 isoform was detected only when Ob1A12.TCR was stimulated with MBP peptide 85–99 in the context of DRA/DRB1*1501 or with MBP peptide 85–99 88K APL presented by DRA/DRB1*1501 or DRA/DRB1*0401. In contrast, phospho-TCRζ p23 was not detected when Ob1A12.TCR was stimulated with MBP peptide 85–99 in the context of DRA/DRB1*0401 (Fig. 8*B*), thus confirming data from IL-2 secretion measurements. Furthermore, the duration of TCRζ-chain phosphorylation was visibly prolonged, up to 10 min after the initial T cell/APC contact, when Ob1A12.TCR was stimulated by 88K APL compared with $MBP_{85–99}$ stimulation (Fig. 8, *C* and *D*). The extended kinetics of 88K stimulation of TCRζ phosphorylation were found to be associated with the presence of the CD4 coreceptor, because the TCRζ phosphorylation kinetics in CD4-negative Ob1A12.TCR hybridomas were significantly shortened to $<$10 min (Fig. 8*E*). These data provide further evidence for CD4 involvement in the mechanism of the MBP peptide 85–99 88V→K APL presentation to the Ob1A12.TCR.

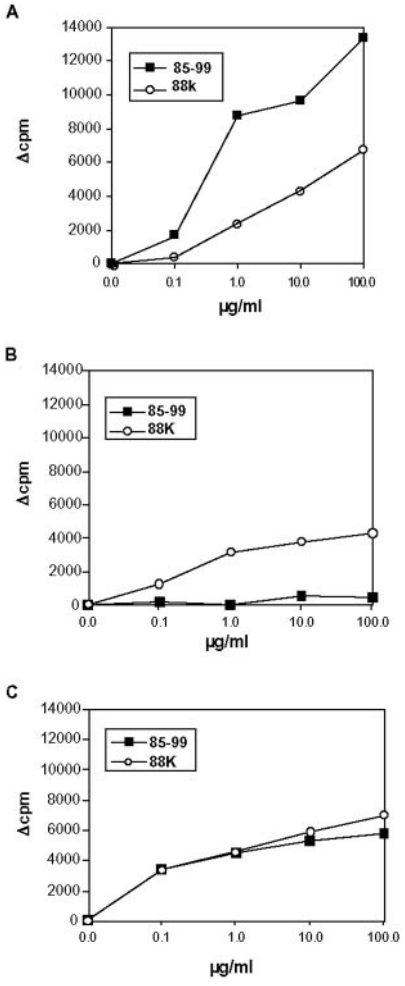

FIGURE 7. Ob1A12 T cell clone recognition of MBP peptide 85–99 and MBP peptide 85–99 APL 88K in the context of homozygous DRB1*1501 (*A*), homozygous DRB1*0401 (*B*), and heterozygous DRB1*1501/DRB1*0401 (*C*). Proliferation data are expressed as Δcpm, which is equal to the mean cpm cultures with Ag subtracted by the mean cpm of culture medium alone. The background proliferation levels of the unstimulated T cell cultures ranged from 900-3900 cpm. SDs were $<$10%.

*TCR recognition of MBP peptide in two distinct registers on DRB1*1501-positive APC*

Analysis of a panel of single amino acid analog peptides of $MBP_{85–99}$ demonstrated that substitution at positions 90, 91, and 93 resulted in a loss of T cell recognition, consistent with the binding frame identified in the crystal structure of the DR2/MBP peptide complex, where 90H, 91F, and 93K were solvent-exposed and thus available for TCR recognition (28). Very similar data were obtained with these analog peptides using APC that expressed DRB1*1501 and DRB1*0404 (Figs. 2*A* and 4*C*), indicating that the peptide is predominantly recognized in the same register by Ob1A12.TCR on these DR molecules.

However, we also observed that a MBP analog peptide in which the P1 anchor residue (89V) was substituted by lysine (89K) was stimulatory when presented by either DRB1*1501- or DRB1*0404-positive APC. Because the lysine side chain cannot be accommodated in the P1 pocket, this finding raised the question of whether the MBP peptide can be recognized in a second binding frame by Ob1A12.TCR. Indeed, two peptides with double substitutions at positions 88 and 89 (88K89S and 88K89Y) activated the Ob1A12 T cell clone when presented by DRB1*1501-positive APC (Fig. 9). Both substitutions at position 89 (serine in 88K89S and tyrosine in 88K89Y) interfere with binding in the P1 pocket of DRB1*1501, thus documenting TCR recognition of these peptides in an alternative binding frame. The peptide concentrations required for stimulation with these analogues were ~2 log higher than for $MBP_{85–99}$, which may be due in part to the lower affinity binding of the peptides in this frame. The 88K89Y peptide was also recognized by the Ob.1A12 T cell clone when presented by DRB1*0401-positive APC, whereas stimulation by the 88K89S peptide was weak (data not shown). Taken together, these data demonstrate a surprising degree of cross-reactivity for this TCR: recognition in more than one binding frame, as well as recognition in the context of different DR molecules (DRB1*1501-, DRB1*0401-, and DRB1*0404-positive APC). In addition, a number of microbial peptides that are quite distinct in their primary sequence from the MBP peptide are recognized by this TCR in the context of DRB1*1501 (27).

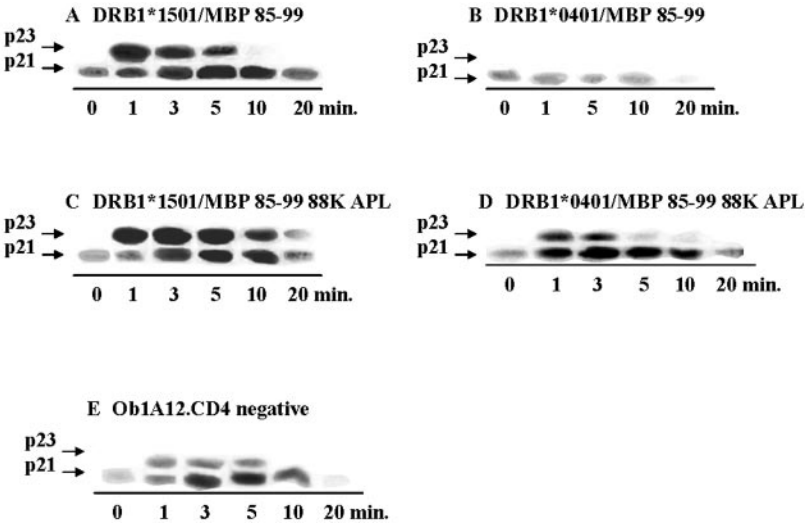

FIGURE 8. CD3ζ phosphorylation kinetics after the stimulation of Ob1A12.TCR. Stimulation by MBP peptide 85–99 in the context of either DRA/DRB1*1501 (*A*) or DRA/DRB1*0401 (*B*) and that of MBP peptide 85–99 88K APL in the context of either DRA/DRB1*1501 (*C*) or DRA/DRB1*0401 (*D*) are shown. CD3ζ phosphorylation kinetics after stimulation of Ob1A12.TCR by the MBP peptide 85–99 88K APL in the context of DRA/DR B1*1501 in the absence (*E*) of CD4 costimulation.

Discussion

MHC restriction of T cells in response to peptide stimulation is a fundamental concept in immunology. Recently, it has become clear that whereas TCRs can be highly specific, at the same time they are also degenerate in their responses to different Ags. In this study we reveal an expected degree of TCR degeneracy related to T cell recognition of peptide presented by multiple HLA-DR molecules. Surprisingly, APL-induced cross-reactivity revealed a further degree of TCR degeneracy among different DR molecules. The degree of cross-reactivity with different DR molecules depended upon the presence of signals provided by CD4 coreceptor engagement of MHC. Furthermore, recruitment of CD4 into the signaling complex may lead to modulation of the initial signal changing the T cell response. These data have important and unexpected consequences for the therapeutic use of altered peptide ligands in the therapy of human autoimmune disease where there is common heterozygosity at the MHC locus.

The phenomenon of TCR degeneracy has been extensively investigated (5). Several reports linked the degeneracy of the TCR with the possible occurrence of T cell recognition of autoantigen (32, 33). $CD4^+$ T cell cross-reactivity between OspA and the autoantigen LFA-1 was demonstrated as a potential mechanism of the induction of autoimmune arthritis after persistent *Borrelia burgdorferai* infection (34). In contrast, constant TCR engagement by MHC was shown to be critical for naive T cells homeostasis (7, 35). Thus, cross-reactivity, although increasing the risk for the initiation of the autoimmune reactions, may represent a fundamental mechanism required for T cell survival and maintenance of T cell diversity. To date, the best-studied model of T cell degeneracy relates to the recognition of APLs. Identification of critical peptide TCR and MHC contact residues, followed by extensive database searches, enabled identification of naturally occurring microbial Ags as candidates for peptides capable of triggering autoreactive T cells (27, 33). In this study, using a classic single amino acid peptide scan approach, we have identified ligands that can activate the same autoreactive TCR in the context of different DR molecules. Due to the vast number of DR alleles, the majority of individuals are heterozygous for the DR allele. Thus, with heterozygosity of MHC class II alleles, the chances of potential TCR cross-reactivity with autoantigens appear to be significantly increased.

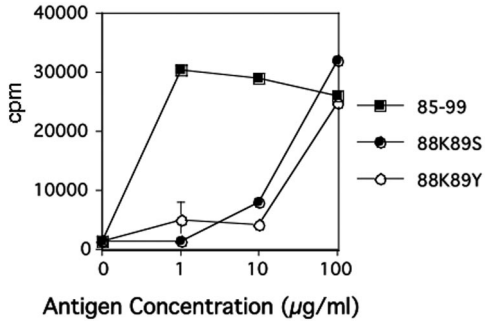

FIGURE 9. Recognition of peptides in more than one binding register by the Ob1A12 T cell clone. Peptides with double substitutions at positions 88 and 89 (88K89S and 88K89Y) were tested at concentrations ranging from 1–100 μg/ml in a [^{3}H]thymidine incorporation assay using DRB1*1501-positive APC. Substitution at position 89 by serine or tyrosine abrogates binding in the previously defined register, because these side chains cannot be accommodated in the P1 pocket of DRB1*1501.

Structural differences in MHC class II molecules provide an environment for escaping negative selection in the thymus while allowing further stimulation of the TCR in the periphery. The Ob1A12 T cell clone has been derived from the peripheral blood of a DR heterozygous patient: DRA/DRB1*0401, DRA/DRB1*1501 (8, 9). Therefore, reactivity of the Ob1A12.TCR toward both DR molecules may represent a naturally occurring phenomenon of biologic significance. Although MBP peptide 85–99 has been described as a primary ligand for that clone, the self ligand(s) in the thymus selecting for this TCR is not known. Interestingly, both DRA/DRB1*1501 and DRA/DRB1*0401 loci were shown to be linked with genetic susceptibility to MS (36, 37). It remains unresolved whether the combination of those two DR alleles may create a natural environment, allowing the development of autoreactivity toward myelin Ags.

The details of the three-dimensional structure of the DRA/DRB1*1501 complexed with the $MBP_{85–99}$ peptide were revealed by direct crystallographic analysis of this complex (28). The crystal structure confirmed previous findings describing P1 and P4 pockets of the MBP peptide 85–99 as the residues determining the HLA binding of this peptide (23, 26). P2, P3, and P5 were shown in the crystal structure analysis as solvent exposed, confirming their role as major TCR contact sites (23, 26, 28). P1 Val and P4 Phe of $MBP_{85–99}$ are positioned against Val at β86 and Ala at β71, respectively, of the β1-chain of DRB1*1501. HLA-DRB1*1501 differs from HLA-DR*0401 by 10 aa within the β1 domain,

including those at positions 71 and 86 (38). Nevertheless, the recognition of MBP peptide 85–99 88V→K APL in the context of HLA-DRA/DRB1*0401 and HLA-DRA/DRB1*0404 (which shares the same amino acids in position β86 as DRB1*1501) by Ob1A12.TCR shows that this peptide could be presented by HLA class II structurally different from DRB1*1501. Furthermore, detailed analysis of MBP peptides using single amino acid APLs presented by HLA-DRA/DRB1*0404 as well as single amino acid mutants of DRB1*0404 at position β71 or β86 suggested that the positioning of MBP peptide 85–99 or MBP peptide 85–99 88K APL in the binding groove of either HLA-DRA/DRB1*0401 or HLA-DRA/DRB1*0404 may not be different from its positioning in HLA-DRA/DRB1*1501. Therefore, the major reason for the lack of recognition of MBP peptide 85–99 in the context of HLA-DRA/DRB1*0401 by the Ob1A12.TCR may be associated with the lack of proper TCR stimulation by this MBP/HLA complex. Interestingly, modification of MBP peptide 85–99 at the P-1 position by mutating a valine to lysine permitted recognition of the MBP peptide by Ob1A12.TCR in the context of DRA/DRB1*0401. The P-1 side chain is solvent-exposed, and the effect of this substitution may therefore be due to changes in the MHC/peptide surface accessible to TCR binding (28). It is also possible that the $MBP_{85–99}$ 88V→K peptide binds the different HLA-DR molecules in a different register, allowing for the observed cross-reactivity, although this is unlikely because the 89V→K did not stimulate a proliferative response when presented by DRB1*0401.

The activation of Ob1A12.TCR by MBP peptide 85–99 88V→K APL in the context of HLA-DRA/DRB1*0401 was critically dependant on the presence of the CD4 coreceptor. During MHC class II recognition, CD4 and TCR colocalize to interact with the same MHC class II. During T cell membrane compartmentalization, CD4 aids recruitment of the TCR to lipid rafts (39, 40). Specifically, CD4 is thought to be important during the early stages of immune synapse formation in which TCR engagement of peptide/MHC occurs in the outer portion of the synapse before the movement of complexes into the central cluster (41). Furthermore, a cysteine motif present in the CD4 tail binds the Src family kinase $p56^{lck}$ (42), which appears to be responsible for phosphorylating CD3ζ, the earliest known event in T cell signaling (43). Thus, CD4, by promoting the formation of rafts and stabilization of the MHC-TCR complex, plays an important role in promoting TCR complex formation and sustaining TCR signal transduction (40, 44, 45). Our data demonstrate a critical role for CD4 in the activation of Ob1A12.TCR by MBP peptide 85–99 88V→K APL in the context of HLA-DRA/DRB1*0401. A recently published analysis of the CD4 crystal structure complexed to MHC class II did not demonstrate direct contact between CD4 and the DR peptide binding groove (46). Therefore, it is unlikely that modification of MBP peptide 85–99 88V→K results in the direct engagement of CD4. Interestingly, mouse CD4 provided better costimulation to the Ob1A12.TCR then human CD4 (our unpublished results) (47). This suggests an important role for CD4 in support of Ob1A12.TCR by MBP peptide 85–99 88V→K APL as related to the direct strengthening of the TCR signal in the transmembrane/cytoplasmal parts of the TCR-CD3 complex. This is in concert with our observation of extended CD3ζ phosphorylation kinetics after stimulation with MBP peptide 85–99 88K APL in the Ob1A12.TCR hybridomas cotransfected with CD4.

The encephalitogenic potential of Ob1A12.TCR has been shown by the generation of an Ob1A12.TCR/HLA-DRA/DRB1*1501 transgenic mouse (47) (S. Ellmerich, M. Mycko, D. A. Hafler, V. Kuchroo, and D. Altmann, unpublished observations). These mice have shown high susceptibility to spontaneous EAE, an animal model of MS. These data indicate that stimulation of the Ob1A12.TCR in the context of HLA-DRA/DRB1*1501 by the relevant MBP peptide is sufficient for the induction of autoimmune demyelination. Nevertheless, it is still unknown whether the residual murine MHC molecules in transgenic mice contributed to the selection and maintenance of Ob1A12.TCR-bearing T cells. HLA-DRA/DR1501 transgenic mice as well as HLA-DRA/DR1501/human CD4 transgenic mice were shown to be completely resistant to the active induction of EAE (47, 48). Therefore, studies involving the heterozygous HLA-DRA/DRB1*0401/HLA-DRA/DRB1*1501 environment may contribute to understanding the homeostasis of MBP-autoreactive T cells.

It was of interest to search for microbial or self-peptides sharing the core sequence of MBP peptide 85–99 88V→K APL that might elucidate new candidate Ags triggering an autoimmune response in MS. However, this search yielded no obvious candidate (data not shown). This TCR is nevertheless highly cross-reactive, because five microbial peptides were previously shown to activate the Ob1A12 human T cell clone (27). Although the in vivo role of the cross-reactivity of the Ob1A12 T cell clone to $MBP_{85–99}$ 88K in the context of both HLA-DRA/DRB1*1501 and HLA-DRA/DRB1*0401APL remains unknown, it was recently shown, using TCR transfectants and TCR transgenic mice, that a TCR from a different MS patient recognized both MBP peptide in the context of DRB1*1501 and EBV peptide in the context of DRB5*0101 (49). These and our data strongly suggest the importance of heterozygous MHC class II cross-reactivity for the regulation of autoreactive CD4 T cell clones, when MHC class II heterozygosity can contribute to the process of both T cell selection and homeostasis. Therefore, elucidation of all cross-reactive peptides should require analysis of wide panel of the ligands, such as combinatorial peptide libraries (50, 51), considering all the self MHC class II molecules as potential restriction elements. Furthermore, our data indicate that APLs used in the therapy of patients with autoimmune diseases where heterozygosity is common may lead to unexpected activation of autoreactive T cells.

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