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Myelin Basic Protein-Specific TCR/HLA-DRB5*01:01 Transgenic Mice Support the Etiologic Role of DRB5*01:01 in Multiple Sclerosis

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Genetic susceptibility to multiple sclerosis (MS) has been linked to the HLA-DR15 haplotype consisting of DRB1*15:01(DR2b) and DRB5*01:01(DR2a) alleles. Given almost complete linkage disequilibrium of the two alleles, recent studies suggested differential roles in susceptibility (DR2b) or protection from MS (DR2a). Our objective was to assess the potential contribution of DR2a to disease etiology in MS using a humanized model of autoimmunity. To assess the potential contribution of DR2a to disease etiology, we created DR2a humanized transgenic (Tg) mice and subsequently crossed them to Tg mice expressing TL3A6, an MS patient-derived myelin basic protein 83-99–specific TCR. In TL3A6/DR2a Tg mice, CD4 Tg T cells escape thymic and peripheral deletion and initiate spontaneous experimental autoimmune encephalomyelitis (EAE) at low rates, depending on the level of DR2a expression. The ability to induce active EAE was also increased in animals expressing higher levels of DR2a. Inflammatory infiltrates and neuronal damage were present throughout the spinal cord, consistent with a classical ascending EAE phenotype with minor involvement of the cerebellum, brainstem, and peripheral nerve roots in spontaneous, as well as actively induced, disease. These studies emphasize the pathologic contribution of the DR2a allele to the development of autoimmunity when expressed as the sole MHC class II molecule, as well as strongly argue for DR2a as a contributor to the CNS autoimmunity in MS.

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ondary progressive form of MS (28) and the disease-ameliorating effect of DR2a in protecting from severe MBP-specific experimental autoimmune encephalomyelitis (EAE) in DR2b Tg mice (29) were interpreted as evidence that evolutionary pressure to counterbalance the negative influence of DR2b by DR2a underlies the linkage disequilibrium of the two alleles. Therefore, Gregersen et al. (29) argued for an epistatic interaction, such that the risk for developing autoimmune disease that is conferred by one allele (DR2b) is offset by the linked second allele (DR2a).

However, a number of observations, primarily from our studies, argue that the situation may be more complex and that both alleles are indeed jointly involved in MS etiopathogenesis. When characterizing, in detail, the MBP-specific CD4 T cell response in MS patients and healthy controls, T cell lines (TCL) and T cell clones (TCC) restricted by DR2b or DR2a can both easily be established (11, 15, 17, 30). As mentioned above, the immunodominant MBP peptide (83-99) binds strongly to both DR2b and DR2a (17, 20). Perhaps more importantly, DR2b binds only one MBP peptide (83-99), whereas DR2a binds at least three (83-99, 131-145/139-153, 76-91) (11, 12). Furthermore, MBP-specific DR2a- and DR2b-restricted CD4 TCL/TCC show distinct phenotypes. DR2a-restricted TCC are often capable of perforin-mediated and very efficient target cell lysis and more frequently show a Th1 phenotype (15). In contrast, DR2b-restricted TCL/TCC lyse target cells relatively inefficiently by Fas/FasL-mediated mechanisms (15, 31, 32). When examining the relative expression of DR2b versus DR2a in different cells and tissues, the DR2a heterodimers are expressed at higher levels in almost all cells and tissues compared with DR2b heterodimers at the mRNA and protein levels (33).

The above discrepancies led us to address whether DR2a contributes to autoimmune inflammation in the CNS in a humanized Tg EAE model. Toward this end, we chose a well-characterized MBP (83-99)–specific and DR2a-restricted TCR, TL3A6, which we had previously described (40, 41). The HLA-DRB5*01:01 exon 2 gene of HLA-DRB1*04:01-IE and a primer located in the 3′ flanking region of the Jα1.2 PCR products were then subcloned into 96-well plates and vectors (38), respectively. TCR functional identity was confirmed in TCR−/− hybrids prior to microinjection of chimeric TL3A6 TCR α and β gene constructs into fertilized eggs of C57BL/6 mice. Offspring were screened with Southern blotting and PCR and crossed to DR2a/MHC class II knockout (KO) mice to generate TL3A6/DR2a/MHC class II KO mice. These mice were crossed again to DR2b/MBP mice (The Jackson Laboratory) and seem to contribute to the etiopathogenesis of MS.

Proteins, peptides, and positional scanning combinatorial libraries

MBP (83-99): (ENPVVHFFKNIVTPRTP) was synthesized to 95% purity (Stanford University Protein and Nucleic Acid Facility, Stanford, CA) for use in both animal immunizations and cell culture assays. A synthetic N-acetylated, C-amide L-amino acid decapetide synthetic combinatorial library in a positional scanning format (PS-SCL; 200 mixtures in the O3′X to X″O′ format, where O3′ represents 1 of the 20 defined L-amino acids and X represents a randomized position containing all 20 l-amino acids, with the exception of cysteine to avoid generation of secondary structures) was prepared as previously described (39). Each O3′X mixture consists of 3.2 × 1010 (19) different decamer peptides at approximately equimolar concentration. Both combinatorial libraries and individual 10- or 20-mers of MBP and agonists of MBP were synthesized by Torrey Pines Institute for Molecular Studies. Individual peptides were synthesized using solid-phase Fmoc technology, whereas peptide libraries were synthesized using the simultaneous multiple peptide-synthesis approach. Purity and identity were assessed by mass spectrometry. Whole human MBP was prepared as previously described (40, 41).

Flow cytometric analysis and T cell proliferation

Thymi, spleens, or lymph nodes (LN; axillary, brachial, and inguinal) were aseptically removed from mice immediately after euthanasia. To isolate CNS infiltrates, mice were anesthetized and perfused intracardially through the left ventricle with 30 ml ice-cold PBS. CNS cell infiltrates were purified with a 50/70% Percoll-density gradient after digestion of the homogenized brain and spinal cord with a Neutral Tissue Dissociation kit (Miltenyi Biotech, Auburn, CA). Tissues were passed through a 40-μm nylon mesh to create a single-cell suspension for subsequent RBC lysis of spleen cells. For FACS analysis, 1 × 106 cells were washed twice in staining buffer (0.1% NaN3, 2% FCS in PBS), preincubated with Fc block (Pharmingen, San Jose, CA), and stained with biotinylated-, PE-, allophycocyanin-, or FITC-labeled Abs to murine CD4, CD8, CD3, CD69 (BD Pharmingen, Franklin Lakes, NJ), HLA-DR, mouse Foxp3, IFN-γ, IL-17A, GM-CSF (eBiosciences, San Diego, CA), or human Vβ1.1 (Beckman Coulter, Brea, CA). The cells stained with biotinylated mAb were further incubated with Streptavidin-Cyochrome (BD Pharmingen). An intracellular staining kit (eBioscience) was used to fix and permeabilize the cells and staining with anti-human Vβ-mAb. To examine the expression of IL-17A, GM-CSF, and IFN-γ, cells were cultured with MBP (83-99) at 10 µg/ml for 5 h in the presence of breflidin A (10 µg/ml) and then stained with FITC–anti-IL-17A, PE-anti-GM-CSF, or allophycocyanin–IFN-γ mAbs. FACS analysis was performed on a BD FACS calibur using CellQuest software (BD Pharmingen) and a Cytomix FC 500 using CXP Analysis Software (Beckman Coulter). Production of IFN-γ, IL-17, and GM-CSF was measured by ELISA (R&D Systems, Minneapolis, MN).

Spleen and LN populations were tested for proliferative responses at 4 × 106 cells/ml in 96-well round-bottom plates in the presence of dose titrations of Ags. Bare lymphocyte syndrome (BLS) patient cells devoid of MHC class II expression and transfected to express HLA-DR2a were kindly provided by G. Nepom and W. Kwok (University of Washington, Seattle, WA). TL3A6 Tg TCL were prepared by repeated stimulation of TL3A6/DR2a/Rag-1−/− or DR2b/MBP spleen cells (2 × 105/well, 3000 RA). A total of 3 × 106 rested TL3A6 TCL or TL3A6 Tg TCL was cocultured with 9 × 105 irradiated (25,000 rad) DR2a-transfected BLS cells or DR2a Tg spleen cells in serum-free X-
VIVO 15 media (BioWhittaker, Walkersville, MD), respectively. PS-SCL mixtures or peptides were added at various concentrations in 96-well round-bottom plates for 48 h at 37˚C. One microCurie of [3H]thymidine (Perkin Elmer, Waltham, MA) was added to each well for an additional 16 h before harvesting on a Tomtec II IM harvester (Hamden, CT) and measuring the incorporated radioactivity on a Wallac Trilux Microbeta Scintillation Counter (Perkin Elmer).

**Immunization for epitope mapping or the induction of active or passive EAE**

All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals and approved by the National Institute of Neurological Disorders and Stroke and University of Medicine and Dentistry of New Jersey Animal Care and Use Committee. Six- to ten-week-old male and female mice were immunized with 500 µg human MBP or 200, 100, or 50 µg MBP (83-99) or agonist peptide in 1 or 4 mg/ml Mycobacterium H37Ra in IFA (Difco Laboratories, Detroit, MI) for epitope mapping or disease induction, respectively. For active EAE induction, 200 ng pertussis toxin (List Biologicals, St. Louis, MO) was administered i.p. on days 0 and 2 to initiate disease.

Animals were similarly immunized for passive EAE; 10 d later, the inguinal, brachial, and axillary LN were removed. Tissues were dissociated on steel mesh, and single-cell suspensions were seeded at 4 × 10^6 cells/ml in T cell media consisting of RPMI 1640 (Invitrogen, Carlsbad, CA), 10% FCS (Invitrogen) with glutamine, nonessential amino acids, penicillin/

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**FIGURE 1.** TL3A6 Tg T cells are positively selected in the thymus and develop efficiently into the periphery. (A) Comparative expression of HLA-DR in DR2a Tg mice and humans. Peripheral blood cells isolated from two founder DR2a Tg mouse lines, DR2ahi and DR2alo, and healthy donors carrying the DR15 haplotype were stained with anti-HLA-DR and anti-B220 mAb. (B) Greater DR2a expression promoted the development of TL3A6 Tg CD4 T cells in TL3A6/DR2a double-Tg mice. Thymocytes and splenocytes isolated from TL3A6/DR2a double-Tg mice at 6 wk of age were stained with anti-CD4, anti-CD8, and anti-Vβ5.1 Abs. The number of thymocytes and splenocytes are as follows: TL3A6/DR2ahi mice (n = 5), 7.4 ± 2.8 × 10^7 (thymocytes) and 7.9 ± 4.4 × 10^7 (splenocytes). (C) Inefficient positive selection of TL3A6 Tg CD4 T cells in TL3A6/DR2alo mice. Thymocytes isolated from TL3A6/DR2alo and TL3A6/DR2ahi mice were stained with anti-CD4, anti-CD8, and anti-CD69 mAbs. CD4⁺CD8⁺ double-positive (DP) cells are gated for analysis of CD69 expression. Gray line, IgG control; dashed line, TL3A6/DR2alo mouse; black line, TL3A6/DR2ahi mouse. Data shown are representative of three experiments. (D) Development of Vβ5.1 Tg CD4 T cells is less efficient in TL3A6/DR2alo/Rag-1 KO mice compared with TL3A6/DR2ahi/Rag-1 KO mice. TL3A6/DR2alo/Rag-1 KO mice (n = 4), 2.9 ± 1.2 × 10^7 (thymocytes) and 2.3 ± 0.8 × 10^7 (splenocytes); TL3A6/DR2ahi/Rag-1 KO (n = 3); 4.9 ± 0.4 × 10^7 (thymocytes) and 11.1 ± 0.6 × 10^7 (splenocytes).
streptomycin (BioWhittaker), and 5 μg/ml MBP (83-99). Four days later, cell suspensions were washed well in T cell media and resuspended in PBS. A total of 1–2 × 10^5 cells was delivered i.v. to each recipient, and pertussis toxin was administered as for active disease. Animals were weighed and monitored daily; clinical signs were assessed according to the following scale: 0: no disease; 1: limp tail; 2: hindlimb paresis-mild; 2.5: severe paresis; 3: single hindlimb paralysis; 3.5: two limbs paralyzed; 4: hindlimb paralysis, forelimb paresis; and 5: no mobility/moribund.

**Histology**

For histological analysis, deeply anesthetized mice were perfused with 50 ml cold PBS, followed by 50 ml 4% paraformaldehyde in PBS. The brain and spinal column were removed and placed in 10% paraformaldehyde for 5–10 d before systematic processing. Brains were cut into 3-mm slices coronally and embedded in paraffin; after decalcification, spinal cords were cut and divided into upper and lower columns. Each was cut into 5-mm cross sections and 8-mm longitudinal sections; paraffin-embedded tissue was cut at 6 μm, followed by H&E staining or Bielschowsky silver impregnation stain to detect axons (Histoseve, Gaithersburg, MD). To prepare frozen sections, the parafformaldehyde-fixed brain and spinal cord were soaked in 30% sucrose/PBS for 3 d. The frozen sections (thickness: 20 μm) were prepared with a HM505E cryostat (Microm) and stained with anti-CD4, anti-Mac 3, and anti–Ly-6G mAbs (BD Bioscience) and rhodamine- and fluorescein-labeled secondary Abs (Jackson Immunoresearch, West Grove, PA). Nuclei were stained with Hoechst 33258 (Sigma-Aldrich). The stained sections were analyzed with an Axiovert 200 microscope (Carl Zeiss, Thornwood, NY).

**Online supplemental material**

Supplemental Fig. 1 illustrates the thymic and peripheral development of Foxp3 T cells in TL3A6/DR2a Tg mice. Supplemental Fig. 2 illustrates the development of Foxp3 T cells in TL3A6/DR2a/Rag-1 KO mice. Supplemental Fig. 3 illustrates the immunohistopathology of spontaneous EAE in TL3A6/DR2alo Tg mice. Supplemental Table I outlines the localization of histological infiltrates in spontaneous and inducible EAE observed in TL3A6/DR2alo Tg mice.

**Results**

**TL3A6 TCR Tg myelin-reactive T cells are positively selected in HLA-DR2a Tg animals**

HLA-DR plays an essential role in the development of autoreactive CD4 T cells, including myelin-specific CD4 T cells. Thus, we first examined whether HLA-DRB5*01:01 can promote the development of MBP-specific CD4 T cells. Tg mice expressing HLA-DRB5*01:01 genes were created and crossed with MHC class II KO mice (referred to as TL3A6/DR2alo Tg mice). To examine the influence of expression levels of DR2a gene on the development of MBP-specific CD4 T cells, two lines of DR2a Tg mice were selected: one with higher levels (DR2a<sup>hi</sup>) and one with lower levels (DR2a<sup>lo</sup>) of HLA-DRB5*01:01 expression (Fig. 1A). Expression levels of HLA-DR in the DR2a<sup>hi</sup> mice were similar to those of human PBMCs, whereas the transgene expression in the DR2a<sup>lo</sup> mice was roughly 10 times lower compared with DR2a<sup>hi</sup> mice (Fig. 1A). The latter DR2a Tg animals were immunized with whole human MBP, and draining LN cells were tested ex vivo against overlapping 20-mer MBP peptides for myelin reactivity. However, proliferative responses were low, and MBP (83-99)–specific T cells could not be detected (data not shown), suggesting that DR2a-restricted MBP (83-99)–specific type A T cells, which can recognize naturally processed MBP peptides, are largely deleted in DR2a mice, as reported previously (42). In the same study, MBP-specific T cells are also deleted in DR2b Tg mice. In contrast to this observation, DR2a- and DR2b-restricted MBP (83-99)–specific type A T cells, including TL 3A6 Tg, have been isolated from humans expressing DR15. This difference could be due to distinct TCR diversity when comparing mice and humans because, indeed, T cell diversity in humans is 10–20 times higher than that in mice (43, 44). Thus, Tg mice expressing human TCR (TL3A6 specific for MBP (83-99)/DR2alo) were created and crossed with DR2alo<sup>hi</sup> and DR2alo<sup>lo</sup> Tg mice (referred to as TL3A6/DR2a Tg mice) to examine whether MBP-specific human TCR Tg T cells can develop in DR2a Tg mice. To our surprise, CD4<sup>+</sup>TL3A6 TCR Tg T cells were positively selected in the thymus and efficiently developed in the periphery of TL3A6/DR2alo Tg mice (Fig. 1B). Although CD8<sup>+</sup> TL3A6 TCR Tg T cells developed as well as CD4<sup>+</sup> TL3A6 TCR Tg T cells in the thymus of TL3A6/DR2alo Tg mice, increased expression of DR2a in TL3A6/DR2alo Tg mice preferentially promoted the development of CD4<sup>+</sup> TL3A6 TCR Tg T cells without a significant reduction in thymic cellularity (Fig. 1B). Poor development of CD4 T cells in TL3A6/DR2alo Tg mice could be due to inefficient positive selection, because expression of CD69 on CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes was reduced in TL3A6/DR2alo Tg mice (Fig. 1C), as observed in other TCR Tg mice in which inefficient positive selection occurs (45, 46). To eliminate the effect of endogenous mouse TCR on thymic and peripheral development of TL3A6 Tg T cells, TL3A6/DR2alo Tg mice were crossed to Rag-1 KO mice to create TL3A6/DR2alo/mouse MHC class II KO/Rag-1 KO mice (referred to as TL3A6/DR2alo/Rag-1 KO mice). CD4<sup>+</sup>TL3A6 TCR Tg T cells were still positively selected in the thymus and developed efficiently in the periphery of TL3A6/DR2alo/Rag-1 KO mice. In contrast, development of CD4<sup>+</sup>TL3A6 Tg T cells is inefficient in the thymus and spleen of TL3A6/DR2alo/Rag-1 KO mice (Fig. 1D). These data suggest that DR2a-restricted MBP (83-99)–specific CD4<sup>+</sup> T cells can develop in DR2a Tg mice; however, their development is dependent on the
FIGURE 3. PS-SCL comparison of TL3A6 TCC with TL3A6 Tg TCC Ag recognition in the context of DR2a. (A) TL3A6 TCC was tested with BLS-DR2a transfectants against PS-SCL at 100 μg/ml to identify stimulatory amino acids throughout scanning 10-mers to distinguish recognition in the context of DR2a. (B) TL3A6 Tg TCC established from a TL3A6/DR2a/Rag-1 KO animal was tested with DR2a Tg spleen cells. The stimulation index (SI) is the ratio of cpm of duplicate wells/controls without Ag. Data represent the mean SI from two experiments; error bars represent the SD.
expression level of DR2a. We also examined the development of TL3A6 Tg Foxp3+ regulatory T cells; 5.2% of CD4+ TL3A6 Tg T cells express Foxp3 in the spleen of TL3A6/DR2a mice, whereas the percentage of Foxp3+ CD4+ T cells increased to 22.5% in the spleen of TL3A6/DR2a mice (Supplemental Fig. 1). In accordance with other MBP-TCR Tg mice (47), development of Foxp3+ regulatory T cells was not detected in TL3A6/DR2a/Rag-1 KO mice (Supplemental Fig. 2).

Specificity and HLA restriction of the human TCC TL3A6 are preserved in TL3A6 Tg mice

To determine whether TL3A6 Tg T cells can recognize naturally processed MBP peptides, as well as exogenously added MBP (83-99) peptide in the context of DR2a, splenocytes from TL3A6 Tg mice expressing high or low levels of DR2a were cultered with whole MBP or MBP (83-99) peptide. Splenocytes isolated from TL3A6/DR2a and TL3A6/DR2a mice proliferated in response to MBP and MBP (83-99), yet cells from TL3A6/DR2a mice responded more strongly than did those from TL3A6/DR2a mice (Fig. 2). Lower proliferative responses of spleen cells isolated from the TL3A6/DR2a mice correlated with lower expression of DR and less efficient development of CD4+ T cells expressing the TL3A6 V beta chain Vβ5.1+ in TL3A6/DR2a mice (Fig. 1A, 1B). Importantly, MBP (83-99) specificity (type A) was preserved in the TL3A6 Tg T cells that had been selected in TL3A6/DR2a mice.

We next examined whether the fine specificity of TL3A6 TCR is also preserved in this humanized animal. We previously demonstrated systematically, using PS-SCL and bioinformatics approaches, that TL3A6 recognizes a large number of peptides in addition to MBP (83-99) and with higher, similar, or lower affinities (i.e., at lower, similar, or higher Ag concentrations). To assess whether the TL3A6 TCR/HLA-DR2a transgene constructs consisting of human–mouse chimeric TCR and human–mouse chimeric HLA-DR2a maintained this reactivity pattern, we tested human TL3A6 TCC and TL3A6 Tg T cells with PS-SCL. To eliminate any expression of endogenous mouse TCR, the TL3A6 Tg T cells were established from TL3A6/DR2a/Rag-1 KO mice. When comparing the amino acids in each of the 10 positions of PS-SCL that activated the human TL3A6 TCC (Fig. 3A) or a murine TL3A6 Tg TCL (Fig. 3B), the patterns are comparable, confirming that the peptide specificities of the two TCRs (human versus mouse–human chimeric) are nearly identical. Minor differences exist in only a few positions, (only Gly in position 2) and other important residues are recognized by both TCRs (48). These data show that the fine specificity of TL3A6 TCR was preserved in this humanized animal, which was further supported by altered MBP (83-99) peptides. The human TL3A6 TCC has been characterized extensively for recognition of numerous altered peptides derived from MBP (83-99), as well as other molecular mimics (30, 49–51). Superagonists of the native Ag MBP (83-99) for TL3A6 were predicted previously by screening TCC TL3A6 systematically for recognition of amino acid exchanges in the MBP (83-99) peptide (50). These peptides, with up to 6-log lower ECD values, activated the Tg T cells in a similar hierarchy relative to MBP (83-99), confirming that the specificity of the interactions between DR2a and TL3A6 Tg humanized constructs reproduced data obtained with the original TL3A6 TCC (Fig. 4). Notably, mouse TCLs from these mice respond to MBP (81-99 and 83-99) peptides when presented by human APCs expressing DR2a (BLS cells expressing DR2a). However, they do not respond to MBP (83-99) or MBP (81-99) presented by BLS cells transfected with DR2b. The original human TCC demonstrated an ECD of between 0.7 and 1 µg/ml when cultured with either DR2-matched PBMC or DR2a-transfected BLS cells and MBP (83-99). The mouse TL3A6 Tg TCL responded to MBP (83-99) and DR2a-transfected human BLS cells with a similar ECD of 0.8 µg/ml, but it demonstrated

Table I. MBP 83-99 immunization induces EAE in TL3A6/DR2a Tg mice

<table>
<thead>
<tr>
<th>Transgenes</th>
<th>Ag</th>
<th>Incidence of Disease (%)</th>
<th>Average Disease Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Females</td>
<td>Males</td>
<td>Total</td>
</tr>
<tr>
<td>HLA class II (lo)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR2a</td>
<td>MBP83-99/CFA</td>
<td>1/15 (7)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>TL3A6/DR2a</td>
<td>MBP83-99/CFA</td>
<td>8/17 (47)</td>
<td>7/15 (47)</td>
</tr>
<tr>
<td>TL3A6/DR2a</td>
<td>CFA</td>
<td>1/3 (33)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>HLA class II (hi)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR2a</td>
<td>MBP83-99/CFA</td>
<td>1/4 (25)</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>TL3A6/DR2a</td>
<td>MBP83-99/CFA</td>
<td>6/7 (86)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>TL3A6/DR2a</td>
<td>CFA</td>
<td>1/6 (17)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>MHC class II /−−</td>
<td>TL3A6</td>
<td>MBP83-99/CFA</td>
<td>0/2 (0)</td>
</tr>
</tbody>
</table>

Six- to eight-week-old mice were immunized with various combinations of peptide, CFA, and pertussis toxin to induce disease in DR2a-single, TL3A6-single, and double TL3A6/DR2a Tg mice. Mice were weighed and assessed daily for clinical disease.
Table II. Induction of active EAE in TL3A6/DR2ahi Tg mice with superagonist and native ligand MBP (89-98) peptide

<table>
<thead>
<tr>
<th>Peptide EC50 (µg/ml)</th>
<th>Dose (µg)/Mouse</th>
<th>Incidence of Disease (%)</th>
<th>Day of Onset</th>
<th>Peak Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WFKLIPTTKL</td>
<td>200</td>
<td>5/9 (56)</td>
<td>10.8 ± 2.1</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>3.7 × 10⁻⁷</td>
<td>100</td>
<td>5/9 (56)</td>
<td>10.6 ± 6.1</td>
<td>2.1 ± 1.0</td>
</tr>
<tr>
<td>50</td>
<td>5/8 (63)</td>
<td>11.6 ± 5.8</td>
<td>2.3 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1/4 (25)</td>
<td>13.0 ± 2.7</td>
<td>2.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>5/9 (56)</td>
<td>13.0 ± 2.7</td>
<td>2.2 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>FFKNIVTPRT</td>
<td>100</td>
<td>3/9 (33)</td>
<td>12.6 ± 2.9</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>2.1 × 10⁻³</td>
<td>50</td>
<td>0/9 (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0/9 (0)</td>
<td></td>
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TL3A6 Tg T cells are encephalitogenic

We examined whether TL3A6 Tg T cells possess encephalitogenic potential and how DR expression levels affect the development of EAE. We first examined the induction of active EAE in TL3A6/DR2a double-Tg mice. Disease could not be readily induced in DR2a single-Tg animals with MBP (83-99), nor could it be induced in TL3A6 TCR single-Tg mice (Table I). TL3A6/DR2a double-Tg mice rarely exhibited disease when immunized with CFA or pertussis alone, yet immunization with MBP (83-99) in CFA with pertussis elicited disease in 47% and 90% of TL3A6/DR2alo and TL3A6/DR2ahi animals, respectively, suggesting that TL3A6/DR2alo Tg mice are more susceptible to active EAE compared with TL3A6/DR2ahi Tg mice. We next used a stronger stimulus (superagonist peptides) to examine whether it increased the incidence of active EAE in TL3A6/DR2alo Tg mice. However, this was not the case (Table II); the superagonist induced clinical disease at lower doses (50 µg) and with slightly higher clinical scores compared with MBP (89-98) (Table II). In contrast to active EAE, adoptive transfer of TL3A6 Tg TCL established from MBP (83-99)–immunized TL3A6/DR2alo Tg mice induced disease in both lines of DR2a Tg mice at 100% (Fig. 5). Although the severity of disease was not significantly different between these two groups, the DR2alo Tg mouse line showed delayed onset of disease (day 12 versus day 7, p < 0.05) and a tendency to recover with less severe disease than exhibited by DR2ahi animals (Fig. 5). These data suggest that TL3A6 Tg T cells are potentially encephalitogenic and that a high expression of DR2a increases the development of induced EAE.

TL3A6 Tg TCR mice develop spontaneous EAE

We next assessed the occurrence of spontaneous EAE in TL3A6/DR2alo Tg mice. TL3A6/DR2a Tg mice developed spontaneous disease with differing incidences (Table III), ranging from 4.5% in TL3A6/DR2ahi Tg mice to <1% in TL3A6/DR2alo Tg mice. In addition to increased disease incidence, TL3A6/DR2ahi Tg mice showed a trend toward earlier onset of disease (10 versus 21 wk of age), but the numbers were insufficient to reach statistical significance (p = 0.08). To examine the level of DR2a expression required for the development of spontaneous EAE, TL3A6/DR2ahi Tg mice were crossed with TL3A6/DR2alo Tg mice to generate TL3A6/DR2a Tg mice expressing various levels of DR2a (Fig. 6). Expression of HLA-DR in these animals was normalized to that observed in the HLA-high–expressing TL3A6/DR2ahi Tg mice and expressed as the percentage of the maximal mean fluorescence intensity (MFI).

The onset of spontaneous EAE was greatly decreased in mice expressing HLA-DR at low levels (i.e., <50% of that observed in TL3A6/DR2ahi Tg mice) (Fig. 6). Fourteen percent of TL3A6/DR2a Tg mice that express higher levels of DR (>50% of the TL3A6/DR2ahi Tg DR expression level) developed spontaneous EAE.

Because Rag-1 deficiency increases the frequency of spontaneous EAE in MBP-TCR Tg mice as the result of the lack of CD4⁺ CD25⁺ regulatory T cells (52, 53), we also examined this aspect in TL3A6/DR2a/Rag-1 KO mice. As expected, the incidence of spontaneous EAE increased in TL3A6/DR2ahi/Rag-1 KO mice (Table III) but not in TL3A6/DR2ahi/Rag-1 KO mice.

These data suggest that TL3A6 Tg T cells are able to differentiate into pathogenic T cells spontaneously; however, the incidence of spontaneous EAE is dependent on expression levels of DR2a.

Spontaneous development of Th1-, Th17-, and GM-CSF–producing CD4 T cells in TL3A6/DR2ahi mice

We examined the production of IFN-γ, IL-17, and GM-CSF in response to MBP (83-99) in the splenocytes isolated from healthy TL3A6/DR2ahi and TL3A6/DR2alo mice. Production of these cytokines is higher in TL3A6/DR2ahi mice than in TL3A6/DR2alo mice (Fig. 7A), and greater numbers of Th1-, Th17-, and GM-CSF⁺-expressing CD4 T cells exist in TL3A6/DR2ahi mice (Fig. 7B). Intracellular flow cytometric analysis indicated that GM-CSF is predominantly produced by Th1/Th17 cells that coproduce IFN-γ and IL-17 (Fig. 7B).

We next examined the production of IFN-γ, IL-17, and GM-CSF in mice that had spontaneously developed EAE. Although the development of Th1, Th17, and GM-CSF⁺ CD4 T cells in the spleen was comparable in healthy and spontaneous EAE mice (Fig. 7), an increased production of these cytokines was detected in the CNS of spontaneous EAE mice (Fig. 7A). In accordance with the cytokine profile, greater numbers of MBP-specific Th1,
TABLE III. Spontaneous disease in TL3A6/DR2a Tg mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incidence of Disease (%)</th>
<th>Age at Onset (wk)</th>
<th>Peak Severity</th>
<th>Gender</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL3A6/DR2ahi Tg</td>
<td>5/110 (4.5)</td>
<td>10 ± 3</td>
<td>2.7 ± 1.1</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>TL3A6/DR2ahi/Rag-1 KO</td>
<td>3/5 (60.0)</td>
<td>9 ± 1</td>
<td>2.5</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TL3A6/DR2ahi/Rag-1 KO</td>
<td>1/20 (5.0)</td>
<td>12</td>
<td>2.5</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Animals were monitored twice weekly for signs of clinical disease. Animals displaying clinical symptoms were either monitored for disease course or euthanized for histological analysis.

Discussion

Almost 30 y ago, DR2, later termed DR15, and now the DR15 haplotype consisting of DR2a and DR2b, was identified as the DR type most consistently linked to MS (55). In the majority of studies the focus of attention has been on DR2b, and the existence of the second DR15 gene, DR2a, which is in almost absolute linkage disequilibrium with DR2b, has often not been considered. Several lines of evidence seemed to suggest that only DR2b is pathogenic: the above-mentioned genetic data from a small number of African Americans with MS (21), as well as the demonstration that Tg mice expressing the MBP (84-102)-specific and DR2b-restricted TCR Ob.1A12 develop spontaneous EAE and are highly susceptible to actively induced disease (23). The effect of DR2a on the development of spontaneous EAE was examined using a DR2a-restricted EBV peptide-specific TCR (Hy.2E11), which also recognizes MBP (85-99) in the context of DR2b (56). Hy.2E11 TCR/DR2b Tg mice and a second Tg mouse recognizing MBP (85-99) in the context of DR2b, Ob.1A12-TCR/DR2b Tg mice, both develop spontaneous EAE. These observations are the main reason for the currently prevailing assumption that DR2b confers disease risk in MS. However, development of spontaneous EAE is reduced in Hy.2E11-TCR Tg mice that are bred to coexpress DR2b and DR2a genes, and this was attributed to the induction of DR2a-mediated apoptosis in Hy.2E11 Tg T cells in the periphery (29). Based on these data, the investigators concluded that DR2a plays a protective role in the development of spontaneous EAE by deleting autoreactive Tg T cells in the periphery. Because these mice are not infected with EBV, Hy.2E11-TCR Tg T cells may cross-react with an unknown self-Ag associated with DR2a in Hy.2E11-TCR/DR2b Tg mice. In contrast to Hy.2E11-TCR/DR2a/DR2b Tg mice, development of spontaneous EAE is not affected by expression of DR2a in Ob.1A12-TCR/DR2a/DR2b Tg mice (29). Because the investigators did not identify DR2a-restricted peptides recognized by the Ob.1A12-TCR, one may conclude that a DR2a-presented self-peptide mediates suppressive effects or, for example, acts as coinhibitor, only on Hy.2E11-TCR–carrying MBP-specific T cells. However, the pathogenic role of DR2a-restricted MBP-specific T cells has not been addressed. We show in this study that MBP-specific, DR2a-restricted T cells are indeed encephalitogenic and that an increase in DR2a expression is associated with the development of spontaneous EAE in TL3A6/DR2a Tg mice.

Our data and that from other investigators also indicates that it is more likely that both alleles jointly confer risk. The main observations in that direction are that DR2a-restricted, proinflammatory MBP-specific T cells can be readily generated (11, 12), that DR2a is expressed at higher levels in most cells and tissues (33) and binds more MBP peptides (17), and that the two alleles, as the result of similarities of their binding pockets, bind overall similar or overlapping sets of peptides (17–19). The most important example for the last observation is the immunodominant MBP peptide (83-99) that has been described as a target epitope for both...
DR2a- and DR2b-restricted TCC. These observations, combined with our TL3A6/DR2a Tg mouse data, suggest that DR2a-restricted MBP-specific CD4+ T cells are likely to contribute to the pathogenesis of MS.

Thymic and peripheral development of autoreactive T cells are dependent on the avidity of TCR to self-Ag/MHC class II complex. MBP-specific T cells are largely deleted in the thymus and periphery, and only those expressing a low-avidity TCR can escape the tolerance-inducing mechanisms (57). Immunization of DR2a Tg mice with whole MBP and subsequent attempts to map DR2a-restricted MBP epitopes identified very low numbers of responsive cells. As a consequence, disease is difficult to induce by immunization with MBP, suggesting that DR2a-restricted, MBP (83-99)-specific type A T cells, which recognize naturally processed MBP Ags, largely undergo deletion and/or anergy in DR2a Tg mice. Similarly, MBP (83-99)-specific type A T cells also undergo tolerance in DR2b Tg mice (42). The observation that MBP (82-100)-specific type A T cells can be positively selected in DR2a Tg and DR2b Tg mice that are deficient in MBP-expression (42) suggests that MBP (82-100)-specific type A T cells are deleted in the thymus and/or undergo anergy in the periphery as a result of the expression of endogenous MBP. In contrast, MBP (82-100)-

**FIGURE 7.** Development of Th1-, Th17-, and GM-CSF–producing CD4 T cells. (A) Splenocytes isolated from TL3A6/DR2a<sup>lo</sup> mice, TL3A6/DR2a<sup>hi</sup> mice (neither exhibiting clinical disease), and spontaneous EAE mice (TL3A6/DR2a<sup>hi</sup> mice), as well as CNS mononuclear cells isolated from spontaneous EAE mice (TL3A6/DR2a<sup>hi</sup> mice), were cultured with MBP (83-99) at 10 µg/ml for 3 d. Production of IFN-γ, IL-17, and GM-CSF was measured by ELISA. (B) Flow cytometric analysis of the production of IFN-γ, IL-17, and GM-CSF in CD4<sup>+</sup> T cells by stimulation with MBP (83-99) at 10 µg/ml for 5 h. CD4<sup>+</sup> cells are gated for production of IFN-γ and IL-17. IFN-γ<sup>+</sup> (Th1), IL-17<sup>+</sup> (Th17), and IFNγ<sup>+</sup>IL-17<sup>+</sup> (Th1-Th17) cells are further gated for analysis of GM-CSF production. Data are from one representative of more than three independent experiments. *p < 0.05, **p < 0.0001.
specific type B T cells that recognize a cryptic MBP (82-100) epitope can escape immune tolerance in DR2a Tg mice and possess encephalitogenic activity (42). Although thymic negative selection and peripheral tolerance may occur more frequently in MBP-specific type A T cells than in type B T cells, studies showed that MBP-specific type A T cells that escape thymic and peripheral tolerance are more encephalitogenic than type B T cells (57). Despite the tolerance induction in MBP (83-99)–specific type A T cells in DR2a and DR2b Tg mice, DR2a- and DR2b-restricted MBP-specific type A T cells are frequently isolated in humans. This difference may be due to higher TCR diversity in DR15+ humans compared with DR2a Tg mice. The total TCR repertoire in human blood and mouse spleen is estimated as 2.5 × 10^7 and 1–2 × 10^8, respectively (43, 44). Thus, T cell diversity in humans is 10–20 times greater than in mice. Our study examines such a DR2a-restricted MBP (83-99)–specific type A human TCR (TL3A6) that escaped tolerance in a patient and is capable of driving disease. Tg mice that express DR2a positively select the TL3A6 Tg CD4+ type A T cells, and these cells can escape thymic and peripheral deletion in TL3A6/DR2a Tg mice and go on to initiate spontaneous disease.

The escape from thymic and peripheral tolerance could be due to a low avidity of TL3A6TCR for MBP (83-99)/DR2a. Indeed, the crystal structure of the TL3A6/MBP (83-99)/HLA-DR2a complex shows relatively few TCR contact residues available for recognition and an overall low affinity for the peptide–MHC complex, with no high-affinity bonds (salt bridges, hydrogen bonds) and only van der Waals bonds contributing to TCR-MHC/peptide interaction (34). In addition, the crystal structure analysis revealed a nontraditional nesting of the peptide within the DR2a molecule with a slight shift to the N terminus (34). The bound peptide sitting slightly off center in the MHC likely limits optimal availability to the TCR. Such unconventional recognition of the N-terminal portion of the peptide was demonstrated in another autoreactive TCR, and it may contribute to escaping thymic deletion (58).

Spontaneous EAE develops in TL3A6/DR2a Tg mice in ∼4.5% of animals (i.e., roughly 45 times more frequently than the prevalence observed in MS in Northern European and North American populations and similar to the prevalence in first-degree relatives of MS patients) (59), whereas spontaneous autoimmunity was detected in TL3A6/DR2alo Tg mice at considerably reduced levels. Development of spontaneous EAE was significantly reduced in TL3A6/DR2alo Tg mice when DR2a expression decreased to 50% of that in DR2a hi mice. This higher incidence of spontaneous EAE is due to the presence of DR2a molecules in DR2a hi mice, whereas DR2alo Tg mice possess DR2b molecules instead (43, 44). HLA-DR2a may affect the development of spontaneous EAE in several ways. Interestingly, the incidence and disease severity were not significantly affected by the level of DR2a expression in adoptive-transfer EAE (Fig. 5), suggesting that a high expression of DR2a is not required during the effector stages of the disease.

FIGURE 8. Histopathological analysis of induced and spontaneous disease. Inflammatory infiltrates and axonal degeneration were observed primarily in the spinal cord of MBP (83-99)–immunized TL3A6/DR2a Tg mice with relative sparing of the cerebrum or cerebellum. (A) Longitudinal section of the spinal cord reveals both granulocytic (arrows) and lymphocytic infiltrates, similar to those shown in (B) at the margin of the spinal cord adjacent to the meninges. (C) Cross-section of the spinal cord and spinal nerves of the cauda equina depict a swollen axon (arrowhead) and a degenerated axon (arrow) ([(A)–(C), H&E, original magnification ×400]). (D–H) Animals exhibiting spontaneous disease (in the absence of immunization or adjuvant) showed a similar pattern of primarily spinal cord involvement. (D) Longitudinal section of the cord reveals severe white matter infiltration with relative gray matter sparing (H&E, original magnification ×200). (E) Bielschowsky silver-stained specimens indicate axonal injury/loss in spinal cord with several interrupted longitudinal fibers/loss of axonal staining (arrowheads) (original magnification ×200). (F) Spontaneous meningomyelitis in the lumbar spinal cord with numerous infiltrates and swollen/fragmented axons (H&E, original magnification ×100, inset ×200). (G) Spontaneous EAE animals also showed evidence of neuritis where lymphocytic infiltration is associated with cranial nerves (H&E, original magnification ×100). (H) Higher magnification of (G) (original magnification ×300).
Because spontaneous development of Th17 cells depends on the strength of the interaction between the TCR and self-Ag/MHC class II complex (61), low expression of DR2a may not be sufficient for the development of Th17 T cells in TL3A6/DR2aTg mice. Indeed, development of MBP-specific Th1 and Th17 cells is low in TL3A6/DR2aTg Tg mice, and their development increases in TL3A6/DR2aTg Tg mice (Fig. 7). In addition, development of GM-CSF–producing Th1/Th17 cells, which play an essential role in the development of EAE (62, 63), was increased in TL3A6/DR2aTg Tg mice (Fig. 7). Thus, expression levels of DR is one of the factors involved in the differentiation of ependophagetic T cells. In addition, a decrease in the development of MBP-specific Foxp3 regulatory T cells in TL3A6/DR2aTg mice (Supplemental Fig. 1) may contribute to the spontaneous development of ependophagetic T cells. Nevertheless, only a small percentage (4.5–14%) of TL3A6/DR2aTg Tg mice develop spontaneous EAE, although MBP-specific Th1, Th17, and GM-CSF+CD4 T cells develop in the mice. Thus, further experiments are required to investigate other factors influencing the development of spontaneous EAE.

In summary, data from this novel Tg mouse model document that a DR2a-restricted, MBP (83-99)–specific TCR causes EAE spontaneously, upon active induction with MBP and following adoptive transfer. Therefore, we conclude that DR2a, independently or together with the other HLA-DR15 risk allele DR2b, serves as an etiologic risk factor for MS rather than as an epistatic modulator of the other HLA-DR types and for its recognition by human T cell clones. J. Exp. Med. 179: 279–290.


Ramagopalan, S. V., N. J. Department of Neuroimmunology and Multiple Sclerosis Research, Neurology Clinic, University Hospital Zurich, University of Zurich, Zurich, Switzerland. For helpful suggestions and critical reading of the manuscript. We dedicate this manuscript to the memory of Dr. Marco Vergelli, who first isolated and characterized the TL3A6 TCC.

Disclosures
The authors have no financial conflicts of interest.

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