Activation of $\text{V}_38$ T cells affects spontaneous EAE in MBP TCR transgenic mice

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Abstract

Two strains of transgenic (Tg) mice ($\text{V}_3.2/\text{V}_8.2$ and $\text{V}_4/\text{V}_8.2$) have T cell receptors (TCR) that recognize the NAc1-11 immunodominant epitope of the myelin basic protein (MBP). Spontaneous experimental autoimmune encephalomyelitis (sEAE) readily develops in $\text{V}_3.2/\text{V}_8.2$ mice. T cells in $\text{V}_3.2/\text{V}_8.2$ mice demonstrate increased levels of CD69, CD44$^{\text{high}}$ and decreased CD45RB relative to $\text{V}_4/\text{V}_8.2$ mice. Increased proliferative responses to MBP and high levels of TNF-$\alpha$ are seen in $\text{V}_3.2/\text{V}_8.2$ mice. High IL-4 and TGF-$\beta$ production is observed in $\text{V}_4/\text{V}_8.2$ mice. CC chemokines (macrophage inflammatory protein-1 $\alpha$ (MIP-1$\alpha$), RANTES and monocyte chemotactic protein 1 (MCP-1)) are increased in the central nervous system (CNS) of $\text{V}_3.2/\text{V}_8.2$ mice. Thus, activated Th1 cells in the periphery of $\text{V}_3.2/\text{V}_8.2$ mice may traffic to the CNS in response to CC chemokines, influencing sEAE. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Autoimmunity; EAE/MS; T cell receptors; Cytokine; Chemokine

1. Introduction

Experimental autoimmune encephalomyelitis (EAE) serves as a useful animal model for studying the human demyelinating disease, multiple sclerosis (MS). EAE, a T cell-mediated autoimmune disease of the central nervous system (CNS), is induced by immunization with myelin components including myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) or peptides (Martin and McFarland, 1995). Immunization of SJL (H-2$d$) or B10.PL (H-2$^u$) mice with MBP or MBP-derived peptides in adjuvant induces a relapsing–remitting chronic form of EAE (Zamvil and Steinman, 1990). The disease is also inducible in naive susceptible mice by the passive transfer of activated MBP-specific CD4$^+$ T cells or T cell lines (Zamvil and Steinman, 1990; Lafaille et al., 1997). Two strains of T cell receptor (TCR) transgenic (Tg) mice ($\text{V}_3.2/\text{V}_8.2$ and $\text{V}_4/\text{V}_8.2$) have been generated by Goverman et al. (1993) and Lafaille et al. (1994), respectively, and recognize the NAc1-11 immunodominant epitope of MBP. Both Tg lines have been bred onto the B10.PL background and are highly susceptible to EAE. Spontaneous EAE (sEAE) was observed in $\text{V}_3.2/\text{V}_8.2$ Tg mice housed in a conventional animal facility but not in those maintained in a pathogen-free facility (Goverman et al., 1993). In contrast, very few $\text{V}_4/\text{V}_8.2$ Tg mice developed spontaneous EAE during the first 12 months of life (Lafaille et al., 1994).

In the normal mammalian CNS, T lymphocytes are very rare (Wekerle et al., 1986; Hickey, 1991). However, T cells extravasate into the CNS during conditions when an immunological stimulus is sufficient to produce inflammation. Such conditions include viral encephalitis, MS and experimentally induced diseases such as EAE. When EAE-inducing T cell lines and clones are introduced, which are specific for a single CNS antigen, activated cells are observed to produce disease, whereas non-activated cells do not (Wekerle et al., 1986; Hickey, 1991). It has been reported that activated T cells are able to cross the blood–brain barrier and perform immune surveillance of the CNS (Williams and Hickey, 1995; Hickey et al., 1991). Entry into the CNS was not dependent on cell phenotype, antigen specificity, MHC restriction element or organ location of the antigen recognized by the T cell. The activation state seemed to be the critical requirement for T cell

Abbreviations: CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; MS, multiple sclerosis; sEAE, spontaneous EAE; Tg, transgenic.

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entry. However, this notion has recently been challenged since it has been reported that naive MBP-specific T cells can also traffic to the CNS but do not trigger autoimmune reactions because they undergo tolerance induction in situ (Brabb et al., 2000).

There are several possible mechanisms by which Ag-specific T cells might direct inflammatory cell recruitment to the CNS. The first is that neuroantigen-specific T cells enter the intrathecal perivascular space and recruit additional CNS Ag-specific and Ag-nonspecific T cells and Mφ by secreting chemokines within the CNS target organ. A second more indirect mechanism for encephalitogenic T cells to induce leukocyte accumulation in the CNS entails induction of chemokine expression by cellular elements associated with the cerebral vasculature and blood–brain barrier (Karpus and Ransohoff, 1998). CC chemokines have been shown to play a role in the pathogenesis of EAE as well as in the induction of tolerance (Karpus et al., 1995, 1997, 1998; Ransohoff et al., 1993; Hayashi et al., 1995). It has been reported that macrophage inflammatory protein-1 α (MIP-1α) plays an important role in the chemotraction of mononuclear inflammatory cells to the CNS in T cell-mediated autoimmune diseases such as EAE (Karpus et al., 1995). Monocyte chemotactic protein 1 (MCP-1) has been shown to regulate tolerance by induction of Th2 cytokine production in vitro (Karpus et al., 1998) and to play a biologically relevant role in relapsing EAE disease in vivo (Kennedy et al., 1998).

In this study, we evaluated the peripheral lymphoid tissue compartment of two strains of MBP TCR Tg mice, which differ in their susceptibility to spontaneous EAE. We found that peripheral T cells from Vα2.3/Vβ8.2 Tg mice are more activated, correlating with the appearance of sEAE and secrete higher levels of TNF-α than cells from Vα4/Vβ3.2 Tg animals. In addition, we observed high levels of CC chemokines in the CNS of Vα2.3/Vβ8.2 Tg mice with sEAE. These results suggest that the activated T cells in the periphery of Vα2.3/Vβ8.2 Tg mice are attracted to the CNS, which has important implications for sEAE.

2. Materials and methods

2.1. Tg mice

2.1.1. Vα2.3/Vβ8.2 Tg mice

Vα2.3/Vβ3.2 Tg mice, which overexpress the TCR specific for MBP (NAc1-11), were generated as previously reported (Goverman et al., 1993). Separate TCR α and β chain transgenic lines were obtained from Dr. Joan Goverman at the University of Washington and backcrossed onto the B10.PL background. Mouse lines homozygous for the transgenic TCRα and for the TCRβ chains were bred together to generate Vα2.3/Vβ3.2 double transgenic mice. Vα2.3/Vβ3.2 mice are therefore homozygous for each of the TCR transgenes. We were discouraged from maintaining homozygous parental strains due to the appearance of spontaneous lymphoma in TCRα2 homozygous mice (Brabb et al., 1997a). Mouse breeding was performed at the Ohio State University (OSU) in a clean specific pathogen-free facility (SPF) with autoclaved cages, autoclaved bedding, food, and water. To detect the expression of the transgene, peripheral blood leukocytes were labeled with monoclonal antibodies directed against Vα2 and Vβ3.2 and assessed by flow cytometry. We compared transgenic TCR expression using labeling with Vα2 and Vβ3.2 antibodies versus Vβ3.2 alone. The results showed similar values differing by less than 5%.

2.1.2. Vα4/Vβ3.2 Tg mice

Mice overexpressing the Vα4/Vβ3.2 Tg TCR, which also recognizes the NAc1-11 peptide of MBP, were obtained from Dr. Charles Janeway, Yale University (Laflaile et al., 1994). The Vα4/Vβ3.2 Tg mice had been extensively backcrossed onto the B10.PL background and were housed and bred at OSU in a clean SPF facility as outlined above. For the generation of Tg mice for this study, Vα4/Vβ3.2 mice were bred with B10.PL mice to produce offspring heterozygous for the transgenic TCR. Mice were screened by flow cytometry using peripheral blood leukocytes labeled with monoclonal antibodies directed against Vβ3 and CD4 (or anti-clonotypic antibody G19, Vβ3 and CD4). We compared transgenic TCR expression using labeling with G19 and Vβ3.2 antibodies versus Vβ3.2 alone. The results showed similar values differing by less than 5%.

For analyses of immune function, Tg mice of both strains were housed under SPF conditions and sacrificed at 2–4.5 months of age. At this point in time, mice were clinically healthy, had no observed sEAE and were age- and gender-matched. To compare the incidence and severity of sEAE between the two strains of Tg mice, specific mice were removed from the SPF facility and placed in conventional animal housing conditions. Both the SPF facility and the conventional mouse rooms were shown to be free of typical mouse pathogens (including MHV and GD VII viruses).

2.2. Evaluation of spontaneous EAE

Individual animals from the two strains of MBP TCR Tg mice were removed from the pathogen-free facility and housed under conventional conditions after 6 weeks of age for observation of sEAE. Mice were observed for clinical signs of sEAE and scored as follows: +1, limp tail or waddling gait with tail tonicity; +2, ataxia or waddling gait with tail limppness; +3, partial hindlimb paralysis; +4, total hindlimb paralysis; and +5, death. Mice were observed for 12 months.

2.3. Lymphocyte proliferation

Single cell suspensions were prepared from peripheral (inguinal, axillary, brachial, cervical, deep cervical, popliteal
and periaortic) lymph nodes and mesenteric lymph nodes of individual animals from the two transgenic strains. Lymphoid cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 25 mM HEPES, 2 mM l-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 5 × 10⁻⁵ M 2-ME in round-bottom 96-well plates (4 × 10⁵ cells/well). Cells were cultured with MBP (0.1–1000 μg/ml) in triplicate for 72 h, including a final 18-h pulse with ³H-thymidine. Cultures were harvested onto glass-fiber filter mats using a Skatron harvester (Skatron, Sterling, VA) and were counted by liquid scintillation on a Wallac beta-plate (LKB, Wallac, MD). For each individual mouse, the percentage of Vβ₈⁺ cells as determined by flow cytometry (Vβ₈⁺ T cells) was multiplied by the number of cells per well (4 × 10⁵) to give the number of Vβ₈⁺ cells per well, approximating the number of Tg⁺ cells. The MBP-specific ³H-thymidine incorporation (average of triplicate wells) was divided by the number of Vβ₈⁺ cells per well to give the cpm per Vβ₈⁺ cell for each animal:

\[
\text{Δcpm} = \frac{\% \text{Vβ₈⁺}}{100} \times 4 \times 10^5 = \text{cpm/Vβ₈⁺ cell.}
\]

2.4. ELISPOT assay for the frequency of cytokine-secreting cells

Microtiter plates with nitrocellulose bottoms (PolyFiltronics, Rockland, MA) were coated overnight with IFN-γ (R46A2), IL-2 (JES6-1A12) or IL-4 (11B11)-specific capture antibodies (Pharmingen, San Diego, CA) at 2–4 μg/ml (Forsthuber et al., 1996) or chicken anti-TGF-β (R&D Systems, Minneapolis, MN) at 4 μg/ml. After the plates were washed, they were blocked with 1% BSA (enzyme grade) (Sigma) in DMEM for 1 h at room temperature and washed again. Cells (4 × 10⁵/well) were cultured alone or together with OVA (40 μg/ml), MBP (40 μg/ml) or anti-CD3 mAb (2 μg/ml, Pharmingen) in HL-1 medium (Biowhittaker, Walkersville, MD) for 24 h (IL-2 and IFN-γ) or 48 h (IL-4 and TGF-β). Subsequently, the cells were removed by washing. The appropriate biotinylated detection antibodies, IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-4 (BVD6-24G2) (Pharmingen) or TGF-β (Genzyme, Cambridge, MA) were added, and the plates were incubated overnight at 4 °C. Goat anti-biotin/alkaline phosphatase conjugate (IL-2, IFN-γ and IL-4) (Vector Laboratories, Burlingame, CA) or alkaline

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Incidence of clinical EAE (%)</th>
<th>Mean weeks of onset ± S.D.ᵃ</th>
<th>Cumulative clinical scoreᵇ</th>
<th>Mean maximum severity ± S.D.ᶜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vα2.3/Vβ₈.2 Tg</td>
<td>8/10 (80%)</td>
<td>13.6 ± 4.17 **</td>
<td>12.9 ± 3.46 *</td>
<td>3.0 ± 0.93</td>
</tr>
<tr>
<td>Vα4/Vβ₈.2 Tg</td>
<td>3/10 (30%)</td>
<td>32.3 ± 8.08</td>
<td>2.3 ± 1.40</td>
<td>3.0 ± 0.87</td>
</tr>
</tbody>
</table>

ᵃ The two strains of Tg mice were housed under conventional conditions from 6–50 weeks of age.
ᵇ Mean of the sum of scores for each animal over the entire observation period. Animals who died during the observation period received only a single score of 5 on the day of death (5 = death).
ᶜ Mean of the highest clinical score exhibited by individual animals within a group during the entire course of sEAE.
* p < 0.05.
** p < 0.001 by t-test.

Fig. 1. Spontaneous EAE in Vα2.3/Vβ₈.2 and Vα4/Vβ₈.2 Tg mice. Two strains (Vα2.3/Vβ₈.2, six males and four females, n = 10; Vα4/Vβ₈.2, four males and six females, n = 10) of MBP TCR Tg mice were housed under SPF conditions for 6 weeks. They were placed in conventional conditions after 6 weeks of age. The mice were observed for the onset of disease, and the clinical signs were scored for 12 months.
phosphatase conjugated horse anti-mouse IgG (TGF-\(\beta\)) (Vector Laboratories) was then added, and the reaction was visualized by adding BCIP/NBT phosphatase substrate (Kirkegaard and Perry, Gaithersburg, MD). The number of spots was determined by computerized image analysis (Zeiss, Thornwood, NY):

\[
\text{Spots} = \frac{\%V_{\beta8}^+}{100} \times 4 \times 10^5 = \text{spots/V}_{38}^+ \text{cell.}
\]

2.5. Analysis of TNF-\(\alpha\) by ELISA

Lymphocytes (\(4 \times 10^5\) cells/well) from LN and MLN of individual mice were cultured with the medium alone, OVA (40 \(\mu\)g/ml), MBP (40 \(\mu\)g/ml) or anti-CD3 mAb (2 \(\mu\)g/ml, Pharmingen) for 24 h. Supernatants were collected and analyzed for TNF-\(\alpha\) production by ELISA using OptEIA Set (Pharmingen). The levels of TNF-\(\alpha\) secreted in response to MBP stimulation were divided by the number of V\(\beta8^+\) cells per well to give the amount of TNF-\(\alpha\) (pg/ml) per 10^4 V\(\beta8^+\) cell for each animal:

\[
\frac{\text{TNF-}\alpha}{V_{38}^+} = \frac{\%V_{\beta8}^+}{100} \times 4 \times 10^5 = \text{TNF-}\alpha/V_{38}^+ \text{ cell.}
\]

2.6. Analysis of chemokines by ELISA

For chemokine protein determination, individual Vo2.3/V\(\beta8.2\) or Vo4/V\(\beta8.2\) Tg mice were anesthetized with a mixture of ketamine (82.5 mg/kg) and xylazine (7.5 mg/kg) diluted in PBS and administered by intraperitoneal injection. Deeply anesthetized mice were perfused with 50 ml of 0.2% D-glucose in PBS (pH 7.4) via the left ventricle. The brain was removed and homogenized in 2 ml of PBS (pH 7.4). Supernatants obtained by centrifugation at 10,000 \(\times\) g for 10 min were frozen at \(-70^\circ\)C until assay (Song et al., 1999). MIP-1\(\alpha\), MCP-1 and RANTES levels were quantitated using a commercially available ELISA (R&D Systems) and carried

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**Table 2**

Subsets of lymphocytes in Vo2.3/V\(\beta8.2\) and Vo4/V\(\beta8.2\) Tg mice

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Strain of Tg animals</th>
<th>V(\beta8^+) ((\times 10^6))</th>
<th>CD4 ((\times 10^6))</th>
<th>CD8 ((\times 10^6))</th>
<th>Ratio for CD4/CD8</th>
<th>CD3 ((\times 10^6))</th>
<th>CD19 ((\times 10^6))</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNC</td>
<td>Vo2.3/V(\beta8.2)</td>
<td>13.30 ± 1.78</td>
<td>14.19 ± 6.95</td>
<td>3.38 ± 2.67</td>
<td>4.20</td>
<td>23.54 ± 6.36</td>
<td>32.93 ± 6.99</td>
</tr>
<tr>
<td></td>
<td>Vo4/V(\beta8.2)</td>
<td>27.30 ± 2.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLN</td>
<td>Vo2.3/V(\beta8.2)</td>
<td>18.35 ± 1.87</td>
<td>11.77 ± 1.88</td>
<td>3.76 ± 1.56</td>
<td>3.13</td>
<td>21.32 ± 3.52</td>
<td>25.04 ± 3.52</td>
</tr>
<tr>
<td></td>
<td>Vo4/V(\beta8.2)</td>
<td>21.46 ± 0.71</td>
<td>21.73 ± 0.54</td>
<td>11.8 ± 1.22</td>
<td>18.40</td>
<td>24.40 ± 0.85</td>
<td>10.05 ± 0.57</td>
</tr>
</tbody>
</table>

* Lymphocytes from LN and MLN were stained with FITC-conjugated V\(\beta8.2\) or CD3 followed by PE-conjugated CD4, CD8 or CD19. Analysis gates were set on the lymphocyte population, and the expression of the TCRV\(\beta8\), CD4, CD8, CD3 and CD19 on these cell populations is shown.

* Different between the two strains of Tg mice (\(n = 3\) mice/strain) at \(p < 0.05\) by \(t\)-test.

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*Fig. 2.* Increased CD69, CD44<sup>high</sup> and decreased CD45RB expression on V\(\beta8.2\) T cells from Vo2.3/V\(\beta8.2\) Tg mice. MLN and LN from individual mice were collected from Vo2.3/V\(\beta8.2\) (\(n = 2\), male, 3 months of age) and Vo4/V\(\beta8.2\) Tg mice (\(n = 2\), male, 3.5 months of age). Cells from individual mice were stained with FITC-conjugated anti-V\(\beta8.1/8.2\) and PE-conjugated anti-CD69, CD44 or CD45RB. Analysis gates were set on the V\(\beta8.1/8.2^+\) population, and the expression of activation markers on these cells is shown. The data are shown from one of two representative experiments.
out according to the manufacturer’s instructions. The reaction was terminated by adding 100 µl of stop solution to each well within 30 min, and the absorbance was measured at a wavelength of 450 nm. Tissue chemokine levels were expressed relative to the total protein measured using a protein quantification kit (Pierce, Rockford, IL).

2.7. Flow cytometric analysis

Single cell suspensions of lymphoid cells derived from lymphoid tissue (peripheral lymph nodes and mesenteric lymph nodes) were stained for Vβ8.2 or CD3ε with FITC-conjugated mAb and CD4, CD8α, CD44, CD45RB or CD69 with PE-conjugated mAb, respectively, using twocolor flow cytometry. Isotype control mAbs (Pharmingen) were matched for fluorochrome and used for cursor placement. Lymphocytes were gated based on forward versus side scatter and a total of 10,000 events were analyzed on an EPICS XL flow cytometer (Beckman Coulter, Miami, FL).

2.8. Statistical analysis

The data for sEAE, lymphocyte subsets and chemokine levels were analyzed by Student’s t-test and considered significant at \( p < 0.05 \). For cytokine ELISPOT and ELISA data and proliferation analyses, a non-parametric ANOVA with Tukey’s post-hoc test was performed to determine the differences between the groups. Groups were considered significantly different at \( p < 0.05 \).

3. Results

3.1. Spontaneous EAE in MBP TCR Tg mice

Goverman et al. (1993) previously reported that sEAE is observed in Vα2.3/Vβ8.2 TCR Tg mice housed in a conventional animal facility with less sEAE in a pathogen-free facility. We compared the incidence of spontaneous disease in the two strains of Tg mice while they were simultaneously housed in two different environments.

![Graph showing increased proliferative response to MBP in T cells from Vα2.3/Vβ8.2 mice.](image)

Fig. 3. Increased proliferative response to MBP in T cells from Vα2.3/Vβ8.2 mice. Cells from individual mice (Vα2.3/Vβ8.2, \( n = 3 \), male, 2–3.5 months of age; Vα4/Vβ8, \( n = 3 \), male, 4.5 months of age) were cultured at \( 4 \times 10^6 \) cells per well with MBP (0.1–1000 µg/ml) for 72 h, and \(^3\)H-thymidine was added for the final 18 h of the culture. The data are shown from one of two representative experiments. *: Different between the two strains of Tg mice at \( p < 0.05 \) by ANOVA.
housed under identical SPF conditions as well as conventional animal housing conditions. We observed a similar incidence of sEAE between the two strains of mice housed in the SPF facility for 5–6 months: 14–15% for Vα2.3/Vβ8.2 Tg mice and 11% for Vα4/Vβ8.2 Tg mice (Brabb et al., 1997b; Governor, 1999; Lafaille et al., 1994; and our own observation). However, under conventional conditions, Fig. 1 and Table 1 show that 80% of the Vα2.3/Vβ8.2 Tg mice developed sEAE over an observation period of 30 weeks, with disease incidence and severity increasing over time. We observed that the majority of the Vα2.3/Vβ8.2 mice died by 30 weeks of age when housed in a conventional animal facility. Clinical signs were noted starting at 6 weeks of age in Vα2.3/Vβ8.2 mice, but none of the Vα4/Vβ8.2 Tg mice developed sEAE until 25 weeks. We have noted a similar incidence of sEAE in Vα4/Vβ8.2 Tg mice housed under SPF (11%) (Lafaille et al., 1994) versus conventional conditions (10%) (Fig. 1) until 30 weeks of age. Over a 12-month observation period, however, only 30% of the Vα4/Vβ8.2 Tg mice developed sEAE, with most disease occurring after 40 weeks of age. The data shows that the incidence of sEAE in Vα4/Vβ8.2 Tg mice is markedly less than that observed for Vα2.3/Vβ8.2 Tg mice when these mice are housed under conventional conditions.

3.2. Tg cells from Vα2.3/Vβ8.2 Tg mice are more activated than cells from Vα4/Vβ8.2 mice

To explore the greater degree of sEAE in Vα2.3/Vβ8.2 Tg mice, we undertook an examination of lymphoid cell composition and activation between the two Tg strains. Table 2 shows that there are fewer Vβ+ T cells in the LN and fewer CD4+ T cells in the mesenteric LN of Vα2.3/Vβ8.2 Tg mice, while no differences were observed in CD3 or CD8 populations. In contrast, a greater number of CD19+ B cells was observed in Vα2.3/Vβ8.2 Tg mice. The difference in CD4 levels between the strains produces a difference in the CD4/CD8 ratio, with a lower ratio in both

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**Fig. 4.** Increase in the frequency of Th1-producing cells in Vα2.3/Vβ8.2 Tg mice and increase in Th2-producing cells in Vα4/Vβ8.2 Tg mice. Lymphocytes (4 × 10^5 cells/well) from individual mice (Vα2.3/Vβ8.2, n = 3, male, 2 months of age; Vα4/Vβ8.2, n = 3, male, 2.5 months of age) were cultured with the medium alone or MBP (40 μg/ml) for 24 h (IL-2 and IFN-γ) or 48 h (IL-4 and TGF-β). Wells were then processed for the detection of IL-2, IFN-γ, IL-4 and TGF-β by ELISPOT and TNF-α by ELISA. The data are shown from one of two representative experiments. #: Different between the two strains of Tg mice at p < 0.02–0.001 by ANOVA; @: different from the unstimulated culture (Med) in the same Tg strain at p < 0.02–0.001 by ANOVA; ND: none detectable.
the LN and mesenteric LN for the Vα2.3/Vβ8.2 strain. Interestingly, the larger number of CD4⁺Vβ8.2⁺ cells in Vα4/Vβ8.2 Tg mice does not correlate with the development of sEAE in these mice.

To determine the activation state of the MBP-specific T cells, we analyzed the expression of the early activation antigen CD69 expressed on the surface of recently activated T lymphocytes but not on the resting mature T cells. Fig. 2 demonstrates that when compared to the cells from Vα4/Vβ8.2 Tg mice, T cells from Vα2.3/Vβ8.2 Tg mice have higher levels of CD69 and CD44high expression on Vβ8.2⁺ cells in the MLN. In addition, a decrease in CD45RB expression on Vβ8.2⁺ cells in the MLN and LN of Vα2.3/Vβ8.2 Tg mice (Fig. 2) was also observed, indicative of prior activation.

The proliferative response of lymphocytes cultured in the presence of a wide range of concentrations of MBP is significantly increased in the MLN and LN of Vα2.3/Vβ8.2 Tg mice calculated as cpm per Vβ8⁺ T cell (Fig. 3). These results, taken together, suggest that Vβ8⁺ cells from peripheral lymphoid tissue in Vα2.3/Vβ8.2 Tg mice exhibit a higher level of T cell activation than cells from Vα4/Vβ8.2 Tg mice.

3.3. Cytokine production in TCR Tg mice

Previous studies have shown that Th1 cytokine production is critically linked with the development of EAE (Weinberg et al., 1994; Renno et al., 1994; Yoskuhl et al., 1993; Ando et al., 1989). We therefore compared the

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**Fig. 5.** Increase in chemokine MIP-1α, RANTES and MCP-1 production in the CNS of Vα2.3/Vβ8.2 Tg mice. Brains were collected from individual Tg mice (Vα2.3/Vβ8.2, n = 4, male, 2 months of age; Vα4/Vβ8.2, n = 3, male, 3–4.5 months of age; Vα2 mouse with sEAE, n = 1, male, 6 months of age) solubilized as described in Materials and methods, and the supernatants from individual mice were assayed by ELISA. Values are expressed as the concentration of chemokine/mg protein. The data are shown from one of two representative experiments. * * p < 0.01 by t-test.
frequency of cytokine-producing cells from lymphoid organs of V\textsubscript{a}2.3/V\textsubscript{38.2} and V\textsubscript{a}4/V\textsubscript{38.2} Tg mice. As shown in Fig. 4, TNF-\(\alpha\) levels are dramatically increased in both the LN and MLN of V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice relative to quite low levels of this cytokine in V\textsubscript{a}4/V\textsubscript{38.2} Tg mice. It is notable that V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice also constitutively produce IFN-\(\gamma\) in the MLN but not in the LN. The V\textsubscript{a}4/V\textsubscript{38.2} Tg mice produce quite low levels of IFN-\(\gamma\). In contrast, both strains produce IL-2 in response to MBP stimulation in the LN but only V\textsubscript{a}2.3/V\textsubscript{38.2} Tg produce IL-2 in response to MBP in the MLN. There are clear strain differences in IL-4 and TGF-\(\beta\) levels, with the V\textsubscript{a}4/V\textsubscript{38.2} strain showing higher production of both cytokines in both the LN and MLN than the V\textsubscript{a}2.3/V\textsubscript{38.2} strain. Taken together, these results show an increased level of Th1 cytokines (TNF-\(\alpha\) and IFN-\(\gamma\)) in the V\textsubscript{a}2.3/V\textsubscript{38.2} Tg cells and increased Th2 cytokines (IL-4) and TGF-\(\beta\) levels in V\textsubscript{a}4/V\textsubscript{38.2} Tg cells following antigen stimulation.

3.4. Chemokines produced in the CNS of V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice

It has been reported that the administration of anti-MIP-1\(\alpha\) prevented the development of EAE clinical signs as well as the infiltration of mononuclear cells into the CNS following the transfer of neuroantigen peptide-activated T cells (Karpus et al., 1995). MIP-1\(\alpha\) has also been shown to enhance Th1 cytokine production (Karpus et al., 1997). We hypothesized that the greater degree of sEAE in V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice might be accompanied by increased CNS chemokines. Therefore, we measured CC chemokine (MIP-1\(\alpha\), RANTES and MCP-1) production in the CNS of the two strains of Tg mice. Fig. 5 shows that these three chemokines were significantly increased in the CNS from V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice relative to V\textsubscript{a}4/V\textsubscript{38.2} Tg mice. A comparison of V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice with and without sEAE showed high levels of MIP-1\(\alpha\) under both conditions and elevated RANTES with spontaneous disease. Interestingly, disease-free V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice showed high levels of MCP-1, while mice with spontaneous disease showed none. It is notable that MCP-1 has been reported to control relapsing EAE but not acute EAE (Kennedy et al., 1998).

4. Discussion

Spontaneous initiation of autoimmune disease is thought to be the result of a combination of genetic and environmental factors (Goverman et al., 1993, 1997; Goverman, 1999; Lafaille et al., 1994; Olivares-Villagomez et al., 1998; Van de Keere and Tonegawa, 1998). We report that sEAE occurs in both the V\textsubscript{a}2.3/V\textsubscript{38.2} and the V\textsubscript{a}4/V\textsubscript{38.2} strains of MBP TCR Tg mice housed under conventional conditions. The incidence and the age of onset of sEAE are very different between the two strains of Tg mice. V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice develop sEAE beginning at 6 weeks of age, and over the course of 50 weeks exhibit a high incidence of disease (80%). In contrast, during the same period of observation and the same housing conditions, only 30% of V\textsubscript{a}4/V\textsubscript{38.2} Tg animals showed clinical signs at 6 months of age or older (Fig. 1 and Table 1). We have noted that the incidence of sEAE increases with increasing age in both strains (Fig. 1). We noted a higher incidence of sEAE in V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice than previously reported (Goverman, 1999). An interim analysis of sEAE in V\textsubscript{a}2.3/V\textsubscript{38.2} mice conducted at 5 months of age revealed that 40% exhibited sEAE. This value is consistent with the incidence of sEAE in V\textsubscript{a}2.3/V\textsubscript{38.2} mice of similar age housed at the University of Washington (Goverman, 1999). In consideration of the environmental factors, we also analyzed the sera of these animals for evidence of MHV and GD VII virus infection. All titers were negative in V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice and in the OSU animal facility. Thus, these specific agents are not responsible for the delay in the disease noted in the V\textsubscript{a}4/V\textsubscript{38.2} mice. Taken together, these results suggest that V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice may be more sensitive to age-related, microbial or environmental triggers of autoimmunity disease.

The two strains of MBP TCR Tg mice differ in the usage of the V\textsubscript{a} chain of the predominant TCR. Olivares-Villagomez et al. (1998) reported that V\textsubscript{a}4/V\textsubscript{38.2}/RAG-1\(^{-}\) mice crossed with TCR-\(\alpha\) or -\(\beta\) knockout mice developed sEAE, similar in incidence and severity to V\textsubscript{a}4/V\textsubscript{38.2}/RAG-1\(^{-}\) mice (Olivares-Villagomez et al., 1998). Moreover, it has been reported that a lower incidence of sEAE is observed in mice heterozygous for the V\textsubscript{a}4/V\textsubscript{38.2} transgene relative to homozygous mice (Olivares-Villagomez et al., 1998). Similar studies have not been done in the V\textsubscript{a}2/V\textsubscript{38.2} Tg mice. Therefore, it would be most interesting to compare the development of sEAE in both strains heterozygous for the transgenic TCR under SPF or conventional conditions and/or to compare the incidence of sEAE in the two strains bred onto a RAG (–/–) background.

Our data suggest that the high rate of spontaneous disease noted in V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice is accompanied by the activation of Tg cells in peripheral lymphoid tissues (Figs. 1–3). Tg cells from V\textsubscript{a}2.3/V\textsubscript{38.2} mice demonstrate higher levels of CD69 and CD44 expression and lower amounts of CD45RB than V\textsubscript{a}4/V\textsubscript{38.2} Tg mice (Fig. 2). Furthermore, Tg cells from the V\textsubscript{a}2.3/V\textsubscript{38.2} mice show significantly higher proliferative responses to MBP than cells from the V\textsubscript{a}4/V\textsubscript{38.2} mice (Fig. 3). The high incidence of spontaneous EAE in the V\textsubscript{a}2.3/V\textsubscript{38.2} mice may well be explained by the presence of activated T lymphocytes in the periphery of this mouse. It is unclear why there is an increase in Tg T cell activation in V\textsubscript{a}2.3/V\textsubscript{38.2} Tg mice since both strains of Tg mice were housed under the same conditions. Possibilities to explain this T cell activation state include endogenous retroviral infection, molecular mimicry between MBP NAc1-11 and epitopes on microbial agents or superantigen activation. We are currently exploring these avenues along with the contributions by the V\textsubscript{a}2 chain of the TCR.
CD28 costimulation has been shown to be important in MBP-specific T cell activation in vivo and the initiation of spontaneous EAE (Oliveira-dos-Santos et al., 1999). However, we found no significant differences in the expression of CD28 on Tg cells in the periphery between the two strains of Tg mice (data not shown). Expression of the early activation marker, CD69, is correlated with development of sEAE (Figs. 1 and 2). It may be that CD69 is a more sensitive activation marker, or it may be more important for developing sEAE in the two strains of Tg mice. In humans, it has been reported that anti-CD3+ anti-CD28 synergize in inducing maximal expression of the CD69 activation marker (Secchiero et al., 2000).

Our previous studies have shown that the kinetics of oral tolerance induction also differ significantly between the two strains of Tg mice (Benson et al., 2000; Meyer et al., 2001). MBP administered orally 10 days before immunization protected V\textsubscript{4}/V\textsubscript{8.2} Tg mice from EAE and this was the only time at which protection was observed (Meyer et al., 2001). In contrast, oral MBP protected the V\textsubscript{4}/V\textsubscript{8.2} Tg mice from EAE only when administered 1 day prior to immunization and not at other times (including 10 days) (Benson et al., 2000). In order to provide a potential explanation for these discrepancies, we compared the immune cell composition and responsiveness of the lymphoid tissue in the V\textsubscript{2.3}/V\textsubscript{8.2} and V\textsubscript{4}/V\textsubscript{8.2} mice. As shown here, the Tg T cell activation state and cytokine secretion profiles are quite different between these two mouse strains. It is possible that a longer time interval is required in the V\textsubscript{2.3} mouse to adequately tolerize or delete the strong Th1-prone response in this mouse. On the other hand, the Th2-prone response in the V\textsubscript{4}/V\textsubscript{8.2} mouse may need only a brief restimulation to exhibit tolerance.

Recently, it has been reported that CD4\textsuperscript{+} regulatory T cells play a critical role in controlling the self-reactive T cell in V\textsubscript{4}/V\textsubscript{8.2} Tg mice (Van de Keere and Tonegawa, 1998; Olivares-Villagomez et al., 1998, 2000). Our data show a high frequency of IL-4- and TGF-\beta-producing cells in V\textsubscript{4}/V\textsubscript{8.2} Tg mice. The regulatory T cells may be the source of these cytokines, which could serve to protect the mice from sEAE.

We utilized V\textsubscript{8.2} Tg cells as representative of Tg \textsuperscript{+} cells for the two strains of mice since experimental analyses showed similar values whether the cells were stained with V\textsubscript{38}-specific antibodies or reagents identifying both chains of the TCR. In the proliferative and the cytokine assays, cpm and cytokine levels were expressed per V\textsubscript{8.2} cell. Using V\textsubscript{8.2} as representative of Tg \textsuperscript{+} cells should not affect the overall trends in the data since the value for double positive Tg cells is almost identical to that for V\textsubscript{8.2} cells in both V\textsubscript{2.3}/V\textsubscript{38} and V\textsubscript{4}/V\textsubscript{38} Tg mice.

Both myelin-reactive and non-reactive T cells can enter the CNS (Hickey et al., 1991; Owens et al., 1994; Lannes-Vieira et al., 1994). The prevailing view is that activated T cells have a greater propensity to extravasate and enter the CNS due to the up-regulation of critical adhesion molecules and chemokines (Owens et al., 1994). In EAE, cytokines play a critical role in defining the Th1 or Th2 nature of the autoreactive immune response and in propagating and regulating inflammation within the CNS (Bettelli and Nicholson, 2000). Our data suggest that the MBP-specific Th1 cells are activated and proliferate in the periphery of V\textsubscript{2.3}/V\textsubscript{8.2} Tg mice, and then are able to traffic to the CNS in response to the high levels of chemokine produced. These cells likely still continue to produce TNF-\alpha and induce inflammation in the CNS, which leads to the development of sEAE. In contrast, T cells producing IL-4 and TGF-\beta may act as regulatory cells in V\textsubscript{4}/V\textsubscript{8.2} mice. Such cells could protect the mice from sEAE and have been shown to be capable of trafficking into the CNS (Lafaille et al., 1997). Naive MBP-specific T cells can also traffic to the CNS but do not trigger autoimmune disease (Brabb et al., 2000), possibly because they are not expressing the appropriate costimulatory and activation-enhancing molecules (e.g. CD40L).

CC chemokine family members have been implicated as functional mediators of immunopathology in EAE (Karpus and Ransohoff, 1998). For example, MIP-1\alpha controls mononuclear cell accumulation during acute EAE, while MCP-1 controls mononuclear cell infiltration during relapsing EAE in SJL mice (Kennedy et al., 1998). sEAE in MBP TCR Tg mice differs from relapsing EAE in B10.PL mice, which tends to have a greater degree of relapse and remission (Meyer et al., 1996). We observed that MIP-1\alpha, RANTES and MCP-1 are significantly increased in the CNS of V\textsubscript{2.3}/V\textsubscript{8.2} Tg mice. The activated Th1 cells in the periphery may traffic to the CNS in response to CC chemokines, influencing the development of sEAE in V\textsubscript{2.3}/V\textsubscript{8.2} Tg mice. We examined the CNS of the two Tg strains for V\textsubscript{38}\textsuperscript{+} T cells. These cells were not detected in the CNS from either strain of Tg mouse housed under SPF conditions without immunization or sEAE (data not shown).

In summary, there are definite differences in the lymphoid tissue between V\textsubscript{2.3}/V\textsubscript{8.2} and V\textsubscript{4}/V\textsubscript{8.2} Tg mice. When compared to V\textsubscript{4}/V\textsubscript{8.2} Tg mice, V\textsubscript{2.3}/V\textsubscript{8.2} Tg mice exhibit much more sEAE and require a longer interval of time after oral antigen exposure to demonstrate tolerance (Benson et al., 2000; Meyer et al., 2001). Activated T cells from V\textsubscript{2.3}/V\textsubscript{8.2} Tg mice produce high levels of TNF-\alpha. These activated T cells appear to traffic to the CNS since CNS tissues from V\textsubscript{2.3}/V\textsubscript{8.2} mice produce high levels of the chemokines MIP-1\alpha, RANTES and MCP-1. Therefore, a greater degree of T cell activation and an enhancement of inflammatory signals are observed to accompany the development of sEAE in the V\textsubscript{2.3}/V\textsubscript{8.2} Tg mice.

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References


