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Differences Between Two Strains of Myelin Basic Protein (MBP) TCR Transgenic Mice: Implications for Tolerance Induction

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Experimental autoimmune encephalomyelitis (EAE) is mediated by $CD4^+$ T cells which preferentially use the Vβ8.2 TCR in response to myelin basic protein (MBP). Two strains of Tg mice (Vα2.3/Vβ8.2 and Vα4/Vβ8.2) have T cell receptors that recognize the NAc1-11 immunodominant epitope of MBP. We previously reported that oral administration of MBP protects both Vα2.3/Vβ8.2 and Vα4/Vβ8.2 mice from EAE; however, tolerance induction differs between strains and is dependent on the timing of oral antigen. Here we analyze the peripheral and gut-associated lymphoid tissue (GALT) environments of the two strains of Tg mice. Tg cells in the Peyer's patch (PP) but not the spleen of Vα2.3/Vβ8.2 mice demonstrate increased CD69 and decreased CD45RB relative to Vα4/Vβ8.2 mice. High levels of Th1 and Th2 cytokines, proliferative activity and CC chemokines (MCP-1) are observed in the periphery and GALT of Vα2.3/Vβ8.2 Tg mice. In contrast, more non-Tg $CD4^+$ cells are seen in the PP of Vα4/Vβ8.2 mice. These studies suggest that activated Tg T cells and fewer potential regulatory cells in the PP of Vα2.3/Vβ8.2 Tg mice may influence oral tolerance.   © 2002 Elsevier Science Ltd.

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 [1]. Immunization of H-2^s (SJL) or H-2^u (B10.PL or PL) mice with MBP or MBP-derived peptides in adjuvant induces a relapsing-remitting chronic form of EAE [2]. The disease is also inducible in naïve susceptible mice by passive transfer of activated MBP-specific $CD4^+$ T cells or T cell lines [2, 3]. Two strains of T cell receptor (TCR) transgenic (Tg) mice (Vα2.3/Vβ8.2 and Vα4/Vβ8.2) have been generated by Goverman *et al*. [4] and Lafaille *et al*. [5], 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 was observed in Vα2.3/Vβ8.2 Tg mice housed in a conventional animal facility, but not in those maintained in a pathogen-free facility [4]. In contrast, very few Vα4/Vβ8.2 Tg mice developed spontaneous EAE during their first 12 months of life [5].

Antigen-specific tolerance can be induced by the oral administration of myelin proteins. We and others

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have shown that the oral administration of MBP prior to encephalitogenic challenge results in protection from clinical signs and histopathologic changes of EAE in Lewis rats [6–9], B10.PL (H-2^u) mice [10] and MBP TCR Tg mice [11, 12]. The primary mechanisms by which oral tolerance is mediated include anergy, deletion, and active suppression. Feeding low doses of autoantigen produces active suppression mediated by the secretion of TGFβ, which is reported to suppress autoreactive T cells in lymphoid tissue and the target organ [13, 14]. In contrast, feeding high doses of autoantigen induces anergy and deletion of autoreactive T cells [6, 11, 14–16].

Orally administered antigen first encounters the gut-associated lymphoid tissue (GALT), which is a well-developed immune network that evolved not only to protect the host from ingested pathogens, but also to prevent the host from reacting to dietary proteins. The GALT is composed of villi that contain epithelial cells, intraepithelial lymphocytes (IEL), lamina propria lymphocytes (LPL), and Peyer's patches (PP). It has been reported that feeding low doses of antigen upregulates the number of TGFβ-secreting regulatory cells in the GALT (e.g. in the Peyer's patch) [17], which are released into the periphery. We have observed that oral administration of a large dose of MBP to MBP TCR Tg mice decreases the number of Tg lymphocytes in the peripheral lymphoid organs, with an increase in Tg lymphocytes in the intestine [12]. Recently, Veazey *et al.* [18] reported that the gastrointestinal tract is a major site for CD4$^+$ T cell deletion and viral replication in simian immunodeficiency virus (SIV) infection. These findings suggest that the intestine represents an important site for priming/differentiation of activated T cells [19–21].

CC chemokines have been shown to play a role both in the pathogenesis of EAE and in the induction of oral tolerance [22–26]. It has been reported that administration of anti-MIP-α prevented the development of EAE [22]. Monocyte chemotactic protein 1 (MCP-1) has been shown to regulate tolerance by induction of Th2 cytokine production *in vitro* [26], and to play a biologically relevant role in relapsing EAE disease *in vivo* [27]. Chemokine production within the gastrointestinal compartment and mucosal lymphoid tissue is thought to direct leukocyte trafficking and/or differentiation of T helper lymphocytes, which subsequently influences peripheral immune responses.

In this study, we evaluated the immune environment in the periphery and gut of two strains of MBP TCR Tg mice, which differ in their induction of oral tolerance. We found that PP Tg cells from Vα2.3/Vβ8.2 Tg mice express greater activation markers than Tg cells from the spleen. Both spleen and GALT T cells proliferate more, secrete higher levels of Th1 and Th2 cytokines and chemokines (MCP-1 and MIP-1a) than cells from Vα4/Vβ8.2 Tg animals. These results suggest that the two lines differ markedly with respect to lymphoid cell function in the periphery and GALT, which has important implications for oral tolerance induction.

Materials and methods

Tg mice

Vα2.3/Vβ8.2 Tg mice

Vα2.3/Vβ8.2 Tg animals which overexpress the TCR specific for MBP (NAc1-11) were generated as previously reported [4]. Separate TCR α and β chain transgenic lines were obtained from the University of Washington and backcrossed onto the B10.PL background. α chain and β chain expressing mice were bred together to generate mice expressing complete transgenic TcRs. Vα2.3/Vβ8.2 Tg mice were bred at the Ohio State University (OSU) in a clean specific pathogen-free facility with autoclaved cages, autoclaved bedding, food, and water. To detect expression of the transgene, mice were screened by flow cytometry and peripheral blood leukocytes labeled with monoclonal antibodies directed against Vα2.3 and Vβ8.2. We compared transgenic TCR expression using labelling with Vα2 and Vβ8.2 antibodies versus Vβ8.2 alone. The results showed similar values differing by less than 5%.

Vα4/Vβ8.2 Tg mice

Mice overexpressing the Vα4/Vβ8.2 Tg TCR, which also recognizes the NAc1-11 peptide of MBP, were obtained from Dr Charles Janeway, Yale University [5]. The Vα4/Vβ8.2 Tg mice had been extensively backcrossed onto the B10.PL background and were housed at OSU in a clean specific pathogen-free facility as outlined above. Mice were screened by flow cytometry using peripheral blood leukocytes labeled with monoclonal antibodies directed against Vβ8 and CD4 (or a clonotypic antibody G19 [5], Vβ8.2 and CD4). We compared transgenic TCR expression using labelling with G19 and Vβ8.2 antibodies versus Vβ8.2 alone. The results showed similar values differing by less than 5%.

Isolation of lymphocytes from peripheral lymphoid tissues, and GALT (PP, IEL and LPL)

Peripheral lymphocytes

Single-cell suspensions were prepared from the spleens of the two Tg mouse strains for flow cytometry, ELISPOT, and proliferation.

PP

PP were excised from the wall of the small intestine. PP were slit using a surgical blade and then teased gently in RPMI 1640 medium containing 10% fetal bovine serum (FBS). The cell suspension was passed through a stainless steel screen to remove cell debris and washed twice.

IEL

IEL were obtained as originally described by Cerf-Benussan *et al.* [28] but with minor modifications [29, 30]. Briefly, the small intestine was flushed with PBS followed by excision of PP. The intestine was then opened longitudinally and cut into 5–10 mm pieces. The intestinal pieces were placed into Medium 199 (GIBCO, Grand Island, NY) supplemented with 1 mM dithiothreitol followed by shaking at 37°C for 30 min. The suspensions contained epithelial cells and IEL. The IEL were purified by centrifugation using a 44–67.5% Percoll (Pharmacia Biotech, Santa Ana, CA) gradient. The cells at the interface were harvested and washed twice. The IEL were resuspended in RPMI 1640 containing 10% FBS.

LPL

The remaining gut pieces after removal of IEL were used to isolate LPL as described [31]. The tissue was floated in medium and digested using collagenase type VIII (Sigma, St Louis, MO) with constant shaking for 60 min at 37°C. Cells in the supernatant were harvested, washed, filtered through a 70 µm cell strainer (Becton Dickinson, Franklin Lake, NJ), washed, and placed on a discontinuous 40–100% Percoll gradient. After centrifugation for 20 min at $600 \times g$, lymphoid cells were collected from the interface, washed, and resuspended in medium containing 10% FBS.

Flow cytometric analysis

Single cell suspensions of systemic lymphoid cells (spleen) and mucosal lymphoid tissues (PP, isolated IEL and LPL) were stained for Vβ8.2 with FITC-conjugated mAb and CD4, CD8α, CD69, or CD45RB with PE-conjugated mAb respectively using two-colour flow cytometry. Alternatively, cells were stained for CD3-ε with PE-conjugated mAb and TCRγδ with FITC-conjugated mAb, respectively. Isotype control mAbs (Pharmingen, San Diego, CA) were matched for fluorochrome and used to set gate cursors. Lymphocytes were gated based on forward versus side scatter and a total of 10,000 events were analyzed on an EPICS XL flow cytometer (Coulter, Miami Lakes, FL).

ELISPOT assay for frequency of cytokine secreting cells

Microtiter plates with nitrocellulose bottoms (Poly-Filtronics, Rockland, MA) were coated overnight with IFN-γ (R46A2), IL-2 (JES6-1A12), IL-4 (11B11), or IL-5 (TRFK5)-specific capture antibodies (Pharmingen, San Diego, CA) at 2–4 µg/ml [32], or 4 µg/ml chicken anti-TGF-β (R&D Systems, Minneapolis, MN). 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. Spleen cells were plated at 4×10^5 cells/well, PP cells at 1×10^6 cells/well, and IEL and LPL were plated at 5×10^5 cells/well in the presence of PEC (1×10^5 cells/ml) as APC. Cells were cultured alone, or together with OVA (40 µg/ml), MBP (40 µg/ml), or anti-CD3 mAb (2 µg/ml, Pharmingen, San Diego, CA) in HL-1 medium (Bio-whittaker, Walkersville, MD) for 24 h (IL-2 and IFN-γ) or 48 h (IL-4, IL-5 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), IL-5 (TRFK4) (Pharmingen, San Diego, CA) 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, IL-4, and IL-5) (Vector Lab., Burlingame, CA) or alkaline phosphatase conjugated horse anti-mouse IgG (TGF-β) (Vector Lab.) was then added and the reaction visualized by adding BCIP/NBT phosphatase substrate (Kirkegaard and Perry, Gaithersburg, MD). The number of spots were counted by computerized image analysis (Zeiss, Thornwood, NY).

Analysis of chemokine by ELISA

For chemokine protein determination, small intestine tissue from Vα2.3/Vβ8.2 or Vα4/Vβ8.2 Tg mice was homogenized in 2.0 ml of PBS (pH 7.4) (for <3 min in homogenizer). The supernatants obtained by centrifugation ($10,000 \times g$ for 10 min) were frozen at −70°C in polypropylene tubes until assay [33]. Supernatants were collected from the culture of spleen cells (4×10^5 cells/well) with medium, OVA (40 µg/ml), MBP (40 µg/ml), or LPS (10 µg/ml) for 24 h. MCP-1 and MIP-1α were tested using a commercially available ELISA kit (R&D Systems, Minneapolis, MN) carried out according to manufacturer's instructions. The reaction was terminated by adding 100 µl of stop solution to each well after 30 min, and absorbance was measured at a wavelength of 450 nm. Tissue chemokine levels were expressed relative to total protein, measured using a protein quantification kit (Pierce, Rockford, IL).

Proliferation analysis

Cells from spleen and PP were prepared, and cultured in RPMI 1640 containing 10% 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. Cells (4×10^5/well for spleen, 1×10^6/well for PP) were cultured with NAc1-11 (0.01–200 µg/ml), anti-CD3 mAb (2 µg/ml), or medium alone in triplicate for 72 h, including a final 18 h pulse with ^{3}H-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 betaplate (LKB, Wallac, MD). The percentage of Vβ8$^+$ cells from each individual mouse, as determined by flow cytometry (Vβ8$^+$ T cells) was multiplied by the number of cells per well

Table 1. *Subsets of lymphocytes in spleen and GALT from Vα2.3/Vβ8.2 and Vα4/Vβ8.2 Tg mice*

Cells	Mouse strains	CD3	CD4	CD8	Ratio for CD4/CD8	Vβ8	TCRδγ	CD19
Spleen	Vα2.3/Vβ8.2	12.40±2.12	9.43±1.79	12.4±2.05	0.76	11.33±4.37	ND	23.4±8.63
	Vα4/Vβ8.2	22.4±3.25*	22.05±3.56**	1.93±1.44**	11.42	18.28±4.16*	ND	29.6±2.12
PP	Vα2.3/Vβ8.2	17.30±0.71	10.95±2.33	3.29±3.33	3.33	10.26±3.50	4.81±6.36	73.80±8.91
	Vα4/Vβ8.2	13.70±6.08	17.40±2.40	2.28±1.66	7.63	14.53±5.42	3.78±3.29	65.10±14.57
IEL	Vα2.3/Vβ8.2	71.65±3.46*	13.90±2.26	55.80±8.95*	0.25	71.43±3.71**	8.25±8.70	2.88±1.87
	Vα4/Vβ8.2	86.55±1.48	24.55±6.86	83.15±6.29	0.30	22.45±2.40	9.55±1.91	2.01±1.27
LPL	Vα2.3/Vβ8.2	47.50±5.41	14.60±4.10	10.75±6.72	1.36	40.76±2.02**	3.65±3.75	5.54±4.19
	Vα4/Vβ8.2	48.85±8.75	11.30±6.12	28.80±9.94	0.39	19.10±5.30	3.80±4.58	5.30±5.27

Spleen, PP cells and isolated IEL, LPL were stained with FITC-conjugated Vβ8.2, TCRγδ, CD3 followed by PE-conjugated CD4, CD8 or CD19. Analysis gates were set on the lymphocyte population, and expression of the TCRVβ8, TCRγδ, CD3, CD4, CD8 and CD19 on these cell populations are shown. *, Difference between the two strains of Tg mice at $P<0.05$, and **, $P<0.001$ by *t*-test.

(4×10^5) 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 [34]:

$$\text{cpm/Vβ8}^+ \text{ cell} = \frac{\Delta \text{ cpm}}{(\%\text{Vβ8}^+/100)\times4\times105}$$

Statistical analysis

Levels of expression for cell surface markers by flow cytometry and chemokine levels are presented as mean±SEM. These data were analyzed by Student's t test and considered significant at $p<0.05$. For proliferation and ELISPOT analyses, a non-parametric ANOVA with Tukey's post-hoc test was performed to determine differences between groups. Groups were considered significantly different at $P<0.05$.

Results

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

We reported previously that the Vα2.3/Vβ8.2 and Vα4/Vβ8.2 Tg strains differed with respect to protection from EAE following oral administration of MBP [12, 35]. MBP administered orally 10 days before immunization, protected Vα2.3/Vβ8.2 Tg mice from EAE and this was the only time at which protection was observed [12]. In contrast, oral MBP protected the Vα4/Vβ8.2 Tg mice from EAE only when administered one day prior to immunization and not at other times (including 10 days) [35]. To understand the disparity with respect to oral tolerance induction, we compared the phenotypic and functional properties of the peripheral (spleen) and mucosal lymphoid tissue in the two MBP TCR Tg mouse strains.

MBP specific Tg cells (Vβ8 bearing cells) can potentially respond to orally administered MBP, but can be regulated by other cell types. We therefore enumerated T cell (CD3, CD4, TCRαβ, and TCRγδ), as well as B cell subsets in the peripheral lymphoid compartment and GALT from the two transgenic strains. Table 1 shows that there are equivalent numbers of TCRγδ⁺, and CD19⁺ cells, but the CD4⁺/CD8⁺ ratios differ between the strains. The CD4⁺/CD8⁺ ratio in the spleen and PP of Vα2.3/Vβ8.2 Tg mice is lower than that observed for the Vα4/Vβ8.2 Tg mice, since Vα4/Vβ8.2 Tg mice have more CD4⁺ and fewer CD8⁺ T cells in these compartments. On the other hand, the CD4⁺/CD8⁺ ratio is higher in the LPL of the Vα2.3/Vβ8.2 Tg mice. The total number of Vβ8.2⁺ cells in the IEL and LPL of Vα2.3/Vβ8.2 Tg mice is three–fourfold higher than in the Vα4/Vβ8.2 Tg mice. To determine the overall frequency of Tg T cells between the two strains, we compared the percentage of T cells staining for both chains of the TCR (Vα2⁺Vβ8.2⁺ for the Vα2 strain and G19⁺Vβ8.2⁺ for the Vα4 strain). Our results showed 11% double positive (DP) cells in the spleen of the Vα2 strain and 16–17.5% DP cells in the Vα4 strain. It should be noted that these values directly correspond with the values for Vβ8 positively alone (Table 1). We have therefore used Vβ8 subsequently to mark Tg T cells.

To determine the activation status 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 resting mature T cells. Figure 1 shows that both Tg strains express high levels of CD69 on Vβ8⁺ cells in the LPL and IEL compartments. When compared to cells from Vα4/Vβ8.2 Tg mice, Vβ8⁺ cells from Vα2.3/Vβ8.2 Tg mice have significantly higher levels of CD69 expression in the Peyer's patch. Interestingly, a much higher level of CD69 expression on Vβ8⁺ cells was observed in the GALT than in the periphery of both strains, likely due to antigen non-specific activation by intestinal flora and/or LPS. In addition to high levels of CD69 expression, we also observed a decrease in CD45RB expression in the GALT of Vα2.3/Vβ8.2 Tg mice (Figure 2), which is also indicative of prior
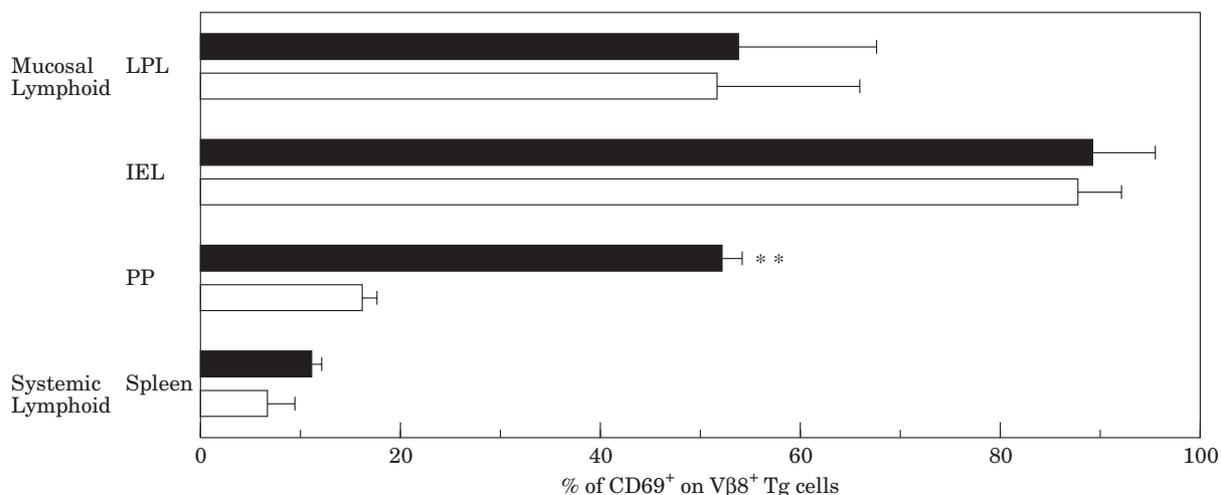
Figure 1. CD69 expression on Vβ8.2 Tg cells from systemic and mucosal lymphoid tissue in Vα2.3/Vβ8.2 Tg and Vα4/Vβ8.2 Tg mice. Cells from mucosal (LPL, IEL, and PP) and systemic (spleen) lymphoid tissue were collected from Vα2.3/Vβ8.2 and Vα4/Vβ8.2 Tg mice ($n=4$ mice/group). Cells were stained with FITC-conjugated anti-Vβ8.1/8.2 and PE-conjugated anti-CD69. Analysis gates were set on the Vβ8.1/8.2$^+$ population, and expression of CD69 on these cells is shown. **, Different between the two strains of Tg mice at $P<0.01$ by t test. Data is shown from one of two representative experiments. ■, Vα2.3/Vβ8.2 Tg; □, Vα4/Vβ8.2 Tg.
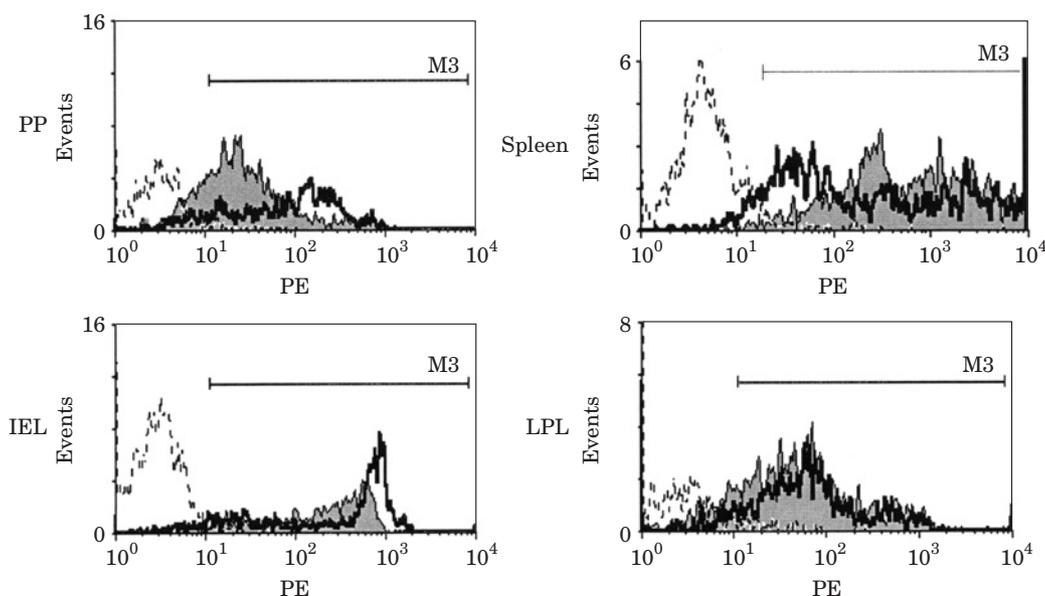

Figure 2. Decreased CD45RB expression on Vβ8.2 Tg cells from GALT in Vα2.3/Vβ8.2 Tg mice. Lymphocytes from the spleen and GALT were collected from Vα2.3/Vβ8.2 and Vα4/Vβ8.2 Tg mice ($n=4$ mice/group). The cells were stained with FITC-conjugated anti-Vβ8.1/8.2 and PE-conjugated anti-CD45RB. Analysis gates were set on the Vβ8.1/8.2$^+$ population, and expression of CD45RB on these cells is shown. Data is shown from one of two representative experiments. □, Vα2.3/Vβ8 Tg; ——, Vα4/Vβ8 Tg; – – – –, Isotype.

activation. Decreased CD45RB expression is most pronounced in the PP compartment (Figure 2).

The proliferative response of spleen and PP lymphocytes was determined as another indication of activation (Figure 3). Splenocytes were cultured in the presence of a wide range of concentrations of MBP peptide (NAc1-11) while there were sufficient PP lymphocytes for only a single concentration of MBP peptide. Proliferative responses were calculated as

cpm per Vβ8$^+$ T cells. The purpose of performing such a wide range of MBP peptide concentration for spleen cell responses was to determine whether there were avidity differences between the TCR of the two Tg mouse strains. Figure 3A shows that spleen cells from the Vα2.3/Vβ8.2 strain responded more robustly than spleen cells of the Vα4/Vβ8.2 strain (two fold greater response). This difference suggests that the Vα2.3/Vβ8.2 TCR binds MBP peptide with greater avidity
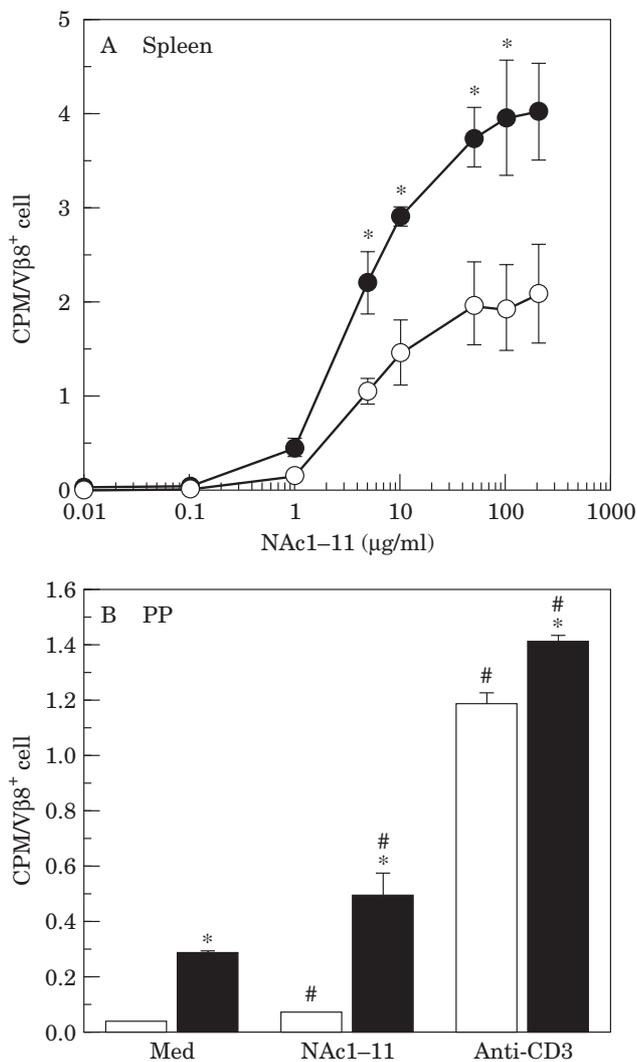
Figure 3. Increased proliferative response to MBP peptide (NAc1-11) on Vβ8^{+} cells in Vα2.3/Vβ8.2 mice. Lymphoid cells from individual Vα2.3/Vβ8.2 and Vα4/Vβ8.2 Tg mice (*n*=3 mice/group) were cultured with medium alone, NAc1-11 (0.01–200 µg/ml for the spleen, A; 10 µg/ml for the PP, B), and anti-CD3 mAb (2 µg/ml) for 72 h and ^{3}H-thymidine was added for the final 18 h of culture. Data is shown from one of two representative experiments. *, different between the two strains of Tg mice at *p*<0.05 by ANOVA; #, different from the medium control culture in the same Tg strain at *p*<0.05 by ANOVA. A, —●— Vα2.3/Vβ8 Tg; —○— Vα4/Vβ8 Tg. B. ■ Vα2.3/Vβ8 Tg; □ Vα4/Vβ8 Tg.

Figure 4. Increase in the frequency of Th1 and Th2 producing cells in the Vα2.3/Vβ8.2 Tg mice. Lymphoid cells from the spleen, PP, IEL and LPL were isolated from Vα2.3/Vβ8.2 (black bar) and Vα4/Vβ8.2 Tg (white bar) mice (*n*=4 mice/group) as described in *Materials and Methods*. Peritoneal exudate cells (PEC) from B10.PL mice were used as APC (5×10^{4} cells/well) for culture of IEL and LPL. Spleen cells (4×10^{5} cells/well), PP cells (1×10^{6} cells/well), IEL, and LPL (each at 5×10^{5} cells/well) were cultured with medium alone, and MBP (40 µg/ml) for 24 h (IL-2, IFN-γ) or 48 h (IL-4, IL-5 and TGF-β). Wells were then processed for the detection of IL-2, IFN-γ, IL-4, IL-5 and TGF-β by ELISPOT. Data is 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.

than the Vα4/Vβ8.2 TCR. PP lymphocytes of Vα2.3/Vβ8.2 mice proliferated significantly more in response to NAc1-11 and spontaneously (without any Ag stimulation) (Figure 3B). Although there was a similar trend in the IEL with greater proliferative responses exhibited by Vα2.3 cells, the difference between strains did not reach statistical significance (data not shown). These results, taken together, suggest that Vβ8^{+} cells from peripheral and gut lymphoid tissue in Vα2.3/Vβ8.2 Tg mice exhibit a higher level of T cell activation and greater avidity for MBP peptide than cells from Vα4/Vβ8.2 Tg mice.
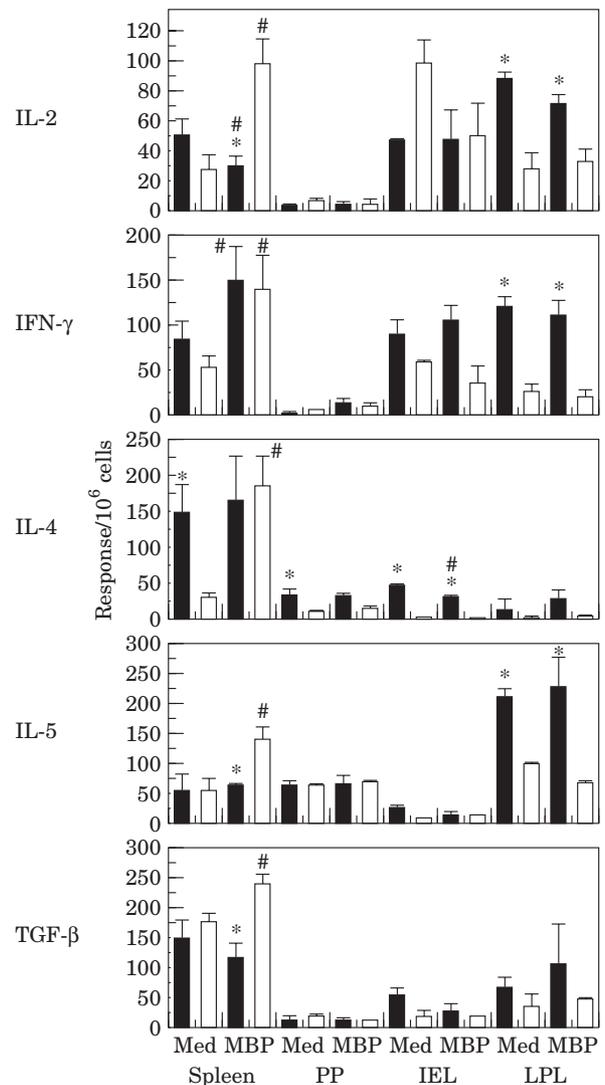
Cytokine production in TCR Tg mice

Previous studies have shown that cytokine production is critically linked with the induction of oral tolerance [12, 36]. We, therefore, compared the frequency of cytokine-producing cells in the spleen and the GALT of both Vα2.3/Vβ8.2 and Vα4/Vβ8.2 Tg mice. As shown in Figure 4, IL-4 production is significantly
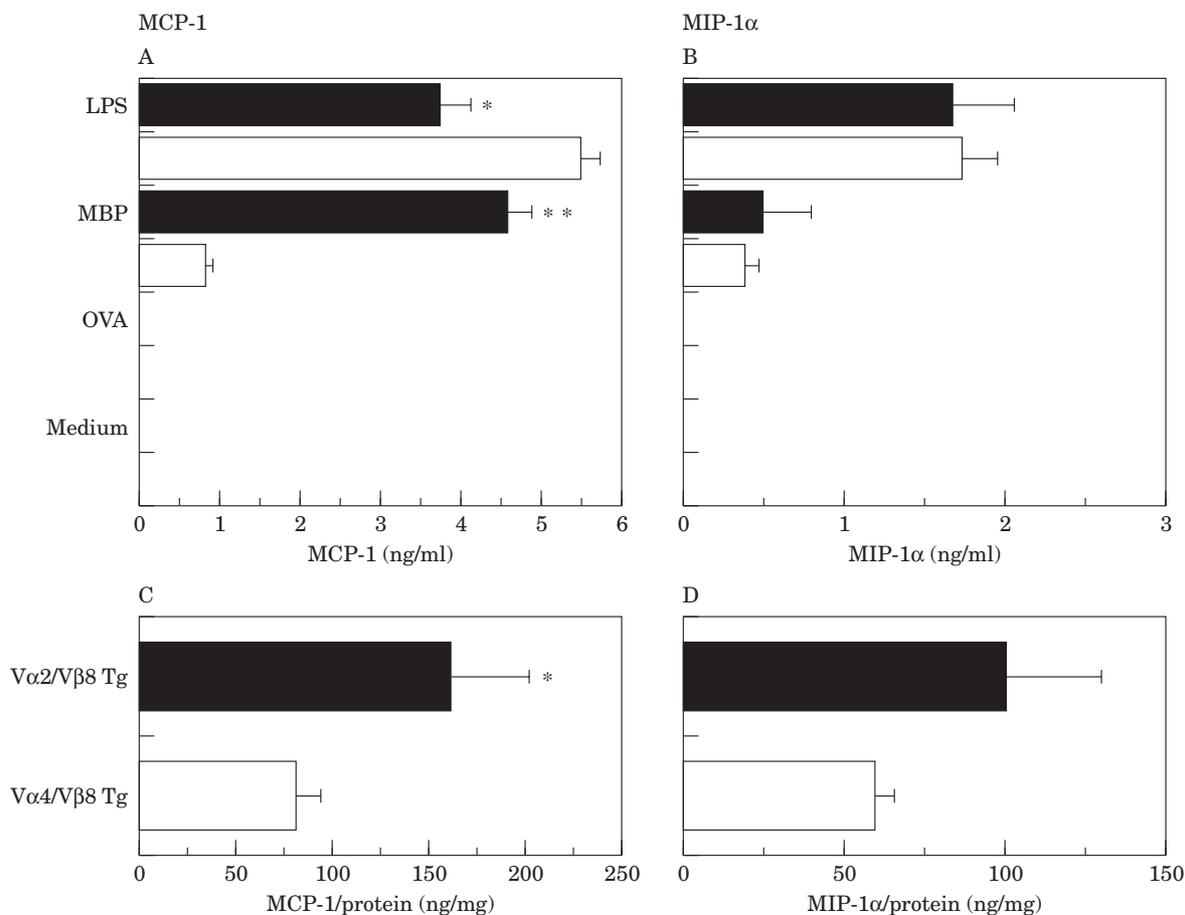
Figure 5. Increase in chemokine MCP-1 in Vα2.3/Vβ8.2 Tg mice. Supernatants from the culture of spleen cells (A and B) and small intestine tissues (C and D) from the two strains of Tg mice (*n*=3 mice/group) were collected, and assayed by ELISA as described in Materials and Methods. Values are expressed as ng chemokine/mg protein. *, different between the two strains of Tg mice at *p*<0.01 by *t* test. Data is shown from one of two representative experiments. ■, Vα2/Vβ8 Tg; □, Vα4/Vβ8 Tg.

increased in the spleen, PP, and IEL from Vα2.3/Vβ8.2Tg mice, compared to Vα4/Vβ8.2Tg mice. However, IL-4 production was not observed to be increased in response to MBP stimulation in most cases. IL-2, IFN-γ and IL-5 levels are significantly elevated in the LPL of Vα2.3/Vβ8.2 mice, observed with or without MBP stimulation. Interestingly, in the spleen compartment, cells from Vα4/Vβ8.2 mice show an increased frequency of all cytokine producing cells following MBP stimulation. However, a similar analysis of the Vα2.3/Vβ8.2 mice shows a decrease in IL-2 and an increase in IFN-γ-secreting cells in response to MBP. Thus, these results suggest that Th1 and Th2 cytokines are spontaneously produced by both peripheral and gut activated lymphocytes from Vα2.3/Vβ8.2 Tg mice, but only IFN-γ is elevated with MBP stimulation in the spleen.

Increase in MCP-1 in Vα2.3/Vβ8.2 Tg mice

CC chemokines have been shown to play a role in the induction of tolerance [21, 25, 26]. It has also been shown that the number of Tg cells increases in the lamina propria of Vα2.3/Vβ8.2 Tg mice after MBP

feeding [12]. As shown in Figure 5, MCP levels in spleen cell cultures stimulated with MBP (Figure 5A) and in gut tissue (Figure 5C) were significantly increased in Vα2.3/Vβ8.2 Tg compared to Vα4/Vβ8.2 Tg mice. Interestingly, MCP-1 production was decreased in the spleen with LPS stimulation (Figure 5A). MIP-1α production was observed to be somewhat increased in the gut although not significantly (Figure 5D), but not in the spleen (Figure 5B). These results suggest that Vα2.3/Vβ8.2 Tg mice produce higher levels of MCP-1 in both the periphery and gut compartment, which may affect the induction of oral tolerance.

More regulatory T cells in Vα4/Vβ8.2 Tg mice

Recently, it has been reported that CD4^{+} T cells are a critical cell population in controlling the self-reactive T cells in Vα4/Vβ8.2 Tg mice [37–39]. The putative regulatory cell was shown to be CD4^{+}Vβ8^{−}. As shown in Figure 6, approximately six times more CD4^{+}Vβ8^{−} cells are observed in the PP of Vα4/Vβ8.2 Tg mice. This higher proportion of CD4^{+}Vβ8^{−} cells is accompanied by a low rate of sEAE [40] and a shorter
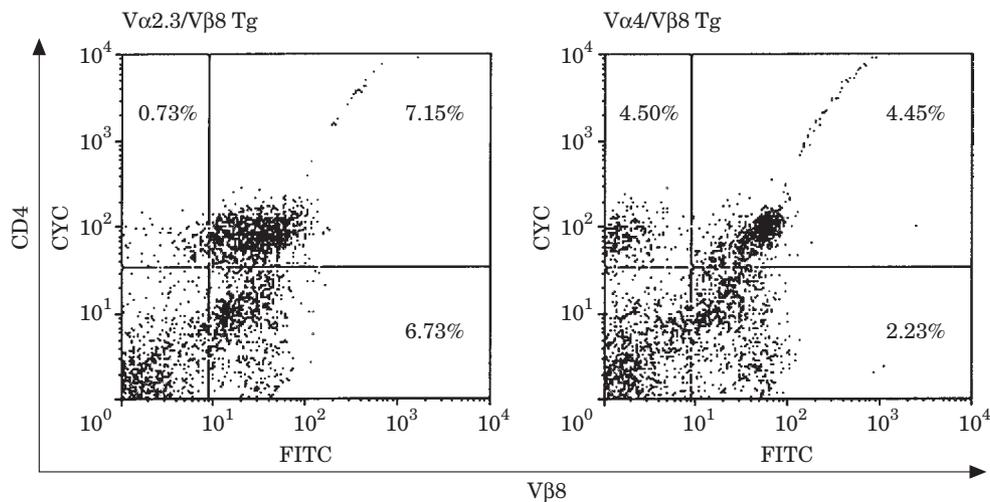
Figure 6. Increase in CD4$^+$Vβ8$^-$ regulatory T cells in the PP from Vα4/Vβ8.2 Tg mice. Lymphocytes from the PP were collected from Vα2.3/Vβ8.2 and Vα4/Vβ8.2 Tg mice (n=3 mice/group). The cells were stained with FITC-conjugated anti-Vβ8.1/8.2, PE-conjugated anti-CD3, and Cy-Chrome-conjugated anti-CD4. Analysis gates were set on the CD3$^+$ population, and expression of CD4 and Vβ8 on these cells is shown. Data is from one of two representative experiments.

time for induction of oral tolerance [35]. No significant differences in CD4$^+$Vβ8$^-$ cells were seen in the spleen (data not shown). The presence of this cell population in Vα4/Vβ8.2 mice may be one reason to explain why a larger number of Vβ8.2$^+$ cells (Table 1) does not correlate with the development of sEAE in these mice [40].

Discussion

Our studies have focussed most intensively on determining how oral tolerance is potentially affected by differences in the GALT of the Vα2.3/Vβ8.2 and Vα4/Vβ8.2 strains. Our results show that Tg$^+$ cells from the PP of Vα2.3/Vβ8.2 mice demonstrate higher levels of CD69 expression and lower CD45RB (Figure 1 and Figure 2). Furthermore, cells from the PP of the Vα2.3/Vβ8.2 mice show a higher proliferative response to NAc1-11 peptide than cells from the Vα4/Vβ8.2 mice (Figure 3). It is known that CD28 costimulation is necessary for MBP-specific T cell activation *in vivo* and the initiation of sEAE [41]. However, we found no significant differences in the expression of CD28 on Tg cells in the PP and LPL, and a decrease in expression in IEL of Vα2.3/Vβ8.2 mice (data not shown). We hypothesize, then, that the delay in oral tolerance induction in Vα2.3/Vβ8.2 mice may be explained by the presence of activated T lymphocytes in the GALT.

Delivery of antigen through the intestinal mucosa can either prime or suppress a peripheral immune response. The mechanisms responsible for non-responsiveness to orally introduced antigen are not completely understood at present. We have previously reported that feeding high doses of antigen results in clonal anergy and deletion of antigen-reactive T lymphocytes in the periphery [6]. In both

strains of MBP TCR Tg mice, we observe that within 24 h after feeding high doses of MBP, downregulation of the TCR β chain occurs [12, 35]. This is followed by a return of the TCR to pre-feed levels by 3 days after feeding. By 10 days after feeding, there is a further decrease in the total number of Tg T cells observed in the Vα2.3/Vβ8.2 strain but not in the Vα4/Vβ8.2 Tg mice [12]. EAE challenge of Vα2.3/Vβ8.2 Tg mice 10 days after high dose MBP feeding protects these mice from EAE. However, the Vα4/Vβ8.2 Tg mice are only protected when challenged one day after feeding. Therefore, a longer time interval after feeding is required to achieve protection in the Vα2.3/Vβ8.2 Tg mouse.

The environment of the intestinal mucosa at the time of antigen delivery dictates the nature of the immune response. The environment of the GALT favors the induction of both Th2 cells and T cells that secrete TGF-β. However, Marth *et al.* [42] have reported increased IFN-γ secretion in the PP when high dose antigen is fed to mice transgenic for an OVA-specific TCR. Our data contend that the two lines of MBP-specific TCR Tg animals have different conditions in the GALT. Th1 and Th2 type cytokines (IFN-γ, IL-2, IL-4, and IL-5) are secreted by cells from the PP, IEL and LPL of Vα2.3/Vβ8.2 Tg mice. In contrast, a more naïve T cell response is observed in the GALT and the spleen of Vα4/Vβ8.2 Tg mice (Figure 4). Taken together, the cytokine profiles and the large amount of spontaneous cytokine production in the Vα2.3/Vβ8.2 strain argue for a pre-existing activated immune environment in this mouse.

CC chemokine family members have been implicated as functional mediators of immunopathology in EAE [43]. For example, MIP-1α controls mononuclear cell accumulation during acute EAE, while MCP-1 controls mononuclear cell infiltration during relapsing

EAE in SJL mice [27]. We observed that MCP-1 is increased in the periphery and GALT of Vα2.3/Vβ8.2 Tg mice (Figure 5), which may ultimately affect the induction of EAE and influence Tg cell trafficking.

The possibility exists that the two Tg mouse strains differ in terms of positive selection in the thymus, which could play a definite role in tolerance induction. This is suggested by the data shown in Table 1 wherein the Vα4 strain exhibits a CD4/CD8 ratio of 11.4 whereas the Vα2.3 strain shows a ratio of less than 1. It could be that positive selection occurs to a greater extent in the Vα4 than in the Vα2.3 mouse. It has been reported that there is strong skewing of the thymocyte population towards mature CD4$^+$ T cells expressing the transgenic TCR in both strains indicating that positive selection occurs strongly in both [4, 5]. However, these studies were carried out in separate laboratories under different conditions. We examined the question of thymocyte selection comparatively in both strains side-by-side. We examined single positive (CD4$^+$ and CD8$^+$) and double positive (CD4$^+$8$^+$) cells in the thymus and spleen of both strains. Our results showed that there were approximately twice as many double positive cells in the thymus of Vα4/Vβ8.2 mice as in Vα2.3/Vβ8.2 mice. An assessment of single positive CD4$^+$ cells in the spleen indicated that after positive selection, there were still approximately twice as many CD4$^+$ cells in the Vα4 relative to the Vα2.3 strain. Levels of CD8$^+$ T cells were more variable between individual animals of both strains. Thus, there did not appear to be differences in positive selection between the two strains, but rather a difference in frequency of thymic double positive cells, which extended to single positive cells in the periphery (spleen). It is noteworthy that more transgenic cells are localized to the gut lymphoid tissue in the Vα2.3 strain (IEL and LPL) which may contribute to the longer time needed to induce oral tolerance in these mice.

The development of autoimmune disease depends not only on the generation of self-reactive T cells, but also on the presence or absence of the appropriate number of functional regulatory cells [37]. Lafaille [5] and Olivares-Villagomez [38] observed a low incidence of sEAE in Vα4/Vβ8.2 Tg mice and a high incidence of sEAE when these mice were bred onto the RAG$^{-/-}$ background. Transfer of monoclonal OVA-specific CD4$^+$ T cells did not confer protection from sEAE even when present in high numbers [39]. However, protection was conferred by T cells bearing limited TCR diversity [39]. Our studies reported here (Figure 6) show that CD4$^+$ T cells bearing endogenous α and β TCR chains are present in greater numbers in Vα4/Vβ8.2 Tg mice. These cells have been postulated to prevent sEAE [40] and may also play a role in the induction of oral tolerance in MBP TCR Tg mice [38]. Therefore, these putative regulatory cells in Vα4/Vβ8.2 Tg mice may affect the lower rate of sEAE [40] and the shorter time to induce oral tolerance [35]. It would be most interesting to compare the induction of oral tolerance to MBP in the two strains bred onto a RAG ($-/-$) background, which would lack the regulatory cell population.

In summary, there are definite differences in the GALT between Vα2.3/Vβ8.2 and Vα4/Vβ8.2 Tg mice. In comparing the two strains, Vα2.3/Vβ8.2 Tg mice require a longer time to exhibit peripheral non-responsiveness following oral antigen administration. Activated T cells in the GALT compartment from Vα2.3/Vβ8.2 Tg mice produce Th1 and Th2 cytokines. In addition, high levels of the CC chemokine, MCP-1, are produced in both the periphery and the GALT of Vα2.3/Vβ8.2 mice. In contrast, there are more non-Tg CD4$^+$ regulatory T cells in the PP of Vα4/Vβ8.2 Tg mice. We are currently exploring the function of the regulatory cells and the therapeutic potential of manipulating the chemokine environment in the intestinal mucosa of both mouse strains during oral tolerance induction.

Acknowledgements

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