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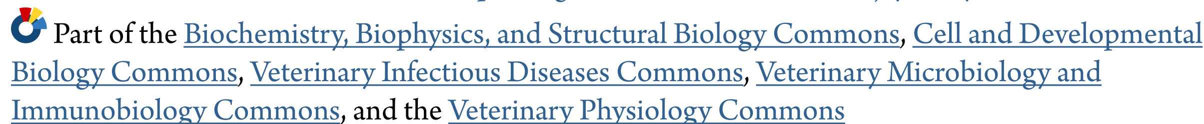
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Gender differences in CNS autoimmunity induced by mimicry epitope for PLP 139–151 in SJL mice

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Abstract

Development of multiple sclerosis (MS) is more prevalent in females than in males, but the underlying mechanisms are not clear. Microbial infections have been suspected as triggers of MS and it is not known whether gender differences in reactivity to environmental antigens contribute to the disease pathogenesis. We demonstrated that ACA 83–95, a mimicry epitope from *Acanthamoeba castellanii* for proteolipid protein (PLP) 139–151, induces clinical signs of encephalomyelitis in both male and female SJL mice. Conversely ACA 83–95-induced effector cells from males fail to induce disease in female mice. Although we found no gender differences in the frequencies of antigen-specific cells including cytokine production, PLP-specific cells induced with ACA 83–95 differed in T cell receptor vβ usage from those induced with PLP 139–151. The data suggest that cross-reactive T cell expansion occurs similarly in both males and females, but their disease-inducing ability is influenced by gender.

Keywords: Experimental autoimmune encephalomyelitis, multiple sclerosis, molecular mimicry, proteolipid protein, a*canthamoeba castellanii,* gender disparity

1. Introduction

Epidemiological data indicate that a number of autoimmune diseases are more prevalent in females than in males. These include systemic lupus erythematosus (9 to 13:1), Sjogren's syndrome (9:1), myasthenia gravis (2 to 14:1), scleroderma (3 to 4:1), Hashimoto's thyroiditis (3:1), multiple sclerosis (MS) (2 to 3:1), and rheumatoid arthritis (2:1) (Ahmed et al., 1999; Beeson, 1994; Lockshin, 2001; Whitacre, 2001). MS is a chronic demyelinating disease of the central nervous system (CNS). The disease severity in female patients is reduced during pregnancy, but the symptoms of MS return to their pre-pregnant state within six months post-delivery (Confavreux et al., 1998). Such an alteration in the disease course is attributed to elevated amounts of T helper (Th) 2 cytokines during pregnancy. In contrast, men are less likely to develop MS than women, but they tend to develop a more severe, chronic form of the disease (Compston et al., 1998) and the underlying mechanisms are unknown.

Three major myelin antigens (Ag), namely proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), have been identified as candidate autoantigens in MS pathogenesis. Healthy humans carry myelin-reactive cells in their naïve periphery, but CNS autoimmunity does not occur spontaneously. How this tolerance is maintained is a fundamental question. It has been suggested that the environmental Ag that bear structural similarities to self-Ag break self-tolerance by cross-reactivity. This is the basis for the molecular mimicry hypothesis, and several microbial mimics have been shown to induce CNS inflammation through the generation of cross-reactive T cells in an adjuvant protocol of experimental autoimmune encephalomyelitis (EAE) (Sospedra and Martin, 2005).

EAE can be induced in mice with whole myelin proteins or their peptides, such as MBP_{Ac} 1–11, MOG 35–55 and PLP 139–151, and the disease is primarily mediated by CD4 T cells. MBP $_{Ac}$ 1–11 induces the monophasic form of EAE in B10.PL or PL/J (IA^u) mice; MOG 35–55 induces the chronic progressive form in C57BL/6 (IA^b) mice; and PLP 139–151 induces relapsing–remitting paralysis in SJL (IA^s) mice (Miller and Karpus, 2007). These phenotypes resemble some of the clinical features of MS, making observations in mice relevant to humans. Gender bias in susceptibility to EAE exists for all of the above Ag, except for MOG 35–55-induced EAE in C57BL/6 mice (Okuda et al., 2002), suggesting that gender differences vary depending on the type of Ag or genetic backgrounds.

Our studies involve PLP 139–151-induced EAE in SJL mice. While both male and female mice develop PLP-induced EAE with equal severity, relapses occur only with females, and the disease is restricted to the monophasic form in males (Bebo et al., 1996). We recently identified a mimicry epitope from *Acanthamoeba castellanii* (*A. castellanii*), called ACA 83–95, that induces autoimmune encephalomyelitis by generating T cells that cross-react with PLP 139–151 (Massilamany et al., 2010). Using ACA 83–95 as a test antigen, we addressed the hypothesis that CNS autoimmunity induced by cross-reactive environmental Ag differs by gender. We provide evidence that in response to ACA 83–95 both male and female SJL mice generate similar frequen-

cies of PLP 139–151-reactive T cells, but encephalitogenicity differs by gender during the effector phase of disease induction.

2. Materials and methods

2.1. Mice

Five-to-six-week-old male and female SJL/J (H-2^s) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained in accordance with the animal protocol guidelines of the University of Nebraska-Lincoln, Lincoln, NE.

2.2. Peptide synthesis and immunization procedures

PLP 139–151 (HSLGKWLGHPDKF), ACA 83–95 (YFLLKWLGHPNVS) and neuraminidase (NASE) 101–120 (EALVRQGLAKVAYVYKPNNT) were synthesized on 9-fluorenylmethyloxycarbonyl chemistry (Neopeptide, Cambridge, MA). All peptides were HPLC-purified (> 90%), identity was confirmed by mass spectroscopy, and the peptides were dissolved in 1 × PBS. To measure recall responses, peptides were emulsified in complete Freund's adjuvant (CFA) and administered s.c. in multiple sites in the flank and sternum (100 μg per mouse). For disease induction, *Mycobacterium tuberculosis* H37RA extract (Difco Laboratories, Detroit, MI) was added as an additional component to a final concentration of 5 mg/ml. Pertussis toxin (PT) (List Biological Laboratories, Campbell, CA) was administered (100 ng per mouse) i.p. on day 0 and day 2 postimmunization.

2.3. Induction of adoptive transfer EAE (AT-EAE)

Groups of mice were immunized with 100 μg of either PLP 139–151 or ACA 83–95 per mouse. After ten days, mice were killed and the inguinal, axillary, cervical, maxillary, and mandibular draining lymph nodes (LN) were harvested, and single cell suspensions were prepared after lysing the erythrocytes with 1 × ammonium chloride potassium buffer (Lonza, Walkersville, MD). Lymph node cells (LNC) were stimulated with peptides (20 μg/ml) and rested for 14 days in medium containing interleukin (IL)-2. The cells were restimulated with PLP 139–151 or ACA 83–95 (20 μg/ml) in the presence of syngeneic antigen-presenting cells for two days, and the cultures were maintained in IL-2 medium. Viable lymphoblasts were harvested on day 4 postimulation by Ficoll-hypaque density gradient centrifugation, and groups of naive male and female SJL mice were injected i.p. with 10 × 10^6 cells per mouse (Whitham et al., 1991). PT (100 ng) was administered i.p. on days 0 and 2 posttransfer and the disease was scored.

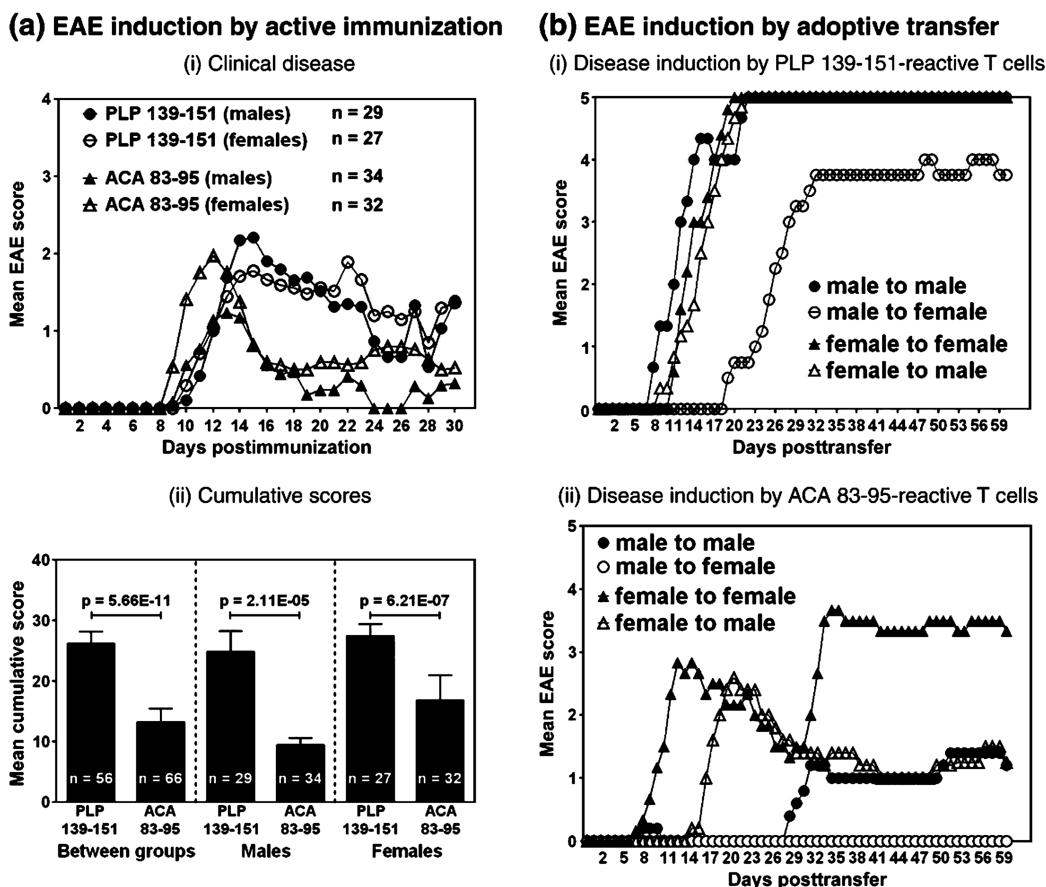


Figure 1. Gender differences in the susceptibility to autoimmune encephalomyelitis induced with ACA 83–95. (a) EAE induction by active immunization. Groups of male and female SJL mice were immunized with PLP 139–151 or ACA 83–95 in CFA s.c., and PT was administered i.p. on day 0 and day 2 postimmunization. The mice were monitored for signs of EAE and scored (i), and the cumulative scores obtained by adding the scores from each mouse in a group were compared between different groups (ii). Data represent mean ± SEM values of four individual experiments and in one experiment, animals were not scored from days 24 to 26. (b) EAE induction by adoptive transfer. Short-term T cell lines were derived from male and female SJL mice immunized with PLP 139–151 or ACA 83–95 as described in methods. Viable lymphoblasts were harvested on day 4 and administered into naïve recipients i.p., and PT was given i.p. on day 0 and day 2 posttransfer. The mice were monitored for clinical signs of EAE and scored (PLP groups, n = 3 to 6; ACA groups, n = 5–6).

Table 1. Clinical and histologic EAE in SJL male and female mice induced with PLP 139–151 and ACA 83–95. (Numbers are mean ± SEM.)

Treatment	Clinical disease			No. of inflammatory foci		
	Incidence (%)	Mean day of onset*	Mean maximum score*	Meninges	Parenchyma	Total
PLP 139–151						
Males	29/30 (96.6)	12.34 ± 0.29	2.86 ± 0.18	48.03 ± 9.54	23.50 ± 6.44	71.23 ± 15.56
Females	27/29 (93.1)	13.59 ± 0.61	3.19 ± 0.15	75.62 ± 9.88	34.83 ± 6.71	112.69 ± 16.07
p values				2.89E–10	2.59E–05	4.84E–11
ACA 83–95						
Males	34/40 (85.0)	11.56 ± 0.28	1.91 ± 0.13	8.63 ± 2.92	5.83 ± 2.32	14.38 ± 5.17
Female	32/38 (84.2)	11.56 ± 0.62	2.59 ± 0.18	19.71 ± 4.94	12.37 ± 4.34	32.37 ± 8.92
p values				2.06E–06	2.34E–04	4.54E–07

Scoring scale: 0, healthy; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial paralysis of hind limbs; 4, complete paralysis of hind limbs; 5, moribund or dead.
* Represents only mice that showed clinical disease.

2.4. Clinical scoring and histopathology

Following EAE induction, the mice were monitored for clinical signs of disease and scored as described previously (Tuohy et al., 1989): 0 – healthy; 1 – limp tail or hind limb weakness but not both; 2 – limp tail and hind limb weakness; 3 – partial paralysis of hind limbs; 4 – complete paralysis of hind limbs; 5 – moribund or dead. The neurological scores obtained from each mouse in a group were added to obtain cumulative scores and compared between groups. Animals were euthanized upon termination and brain and spinal cords were collected in 10% phosphate buffered formalin and analyzed for histological evidence of the disease (Massilamany et al., 2010; Sobel et al., 1990). After fixation, brain sections were made through the cerebrum, hippocampus, cerebellum, and brainstem. In the spinal cord, the cervical, thoracic, lumbar, and sacral regions were each made into three sections, and the tissues were stained by Hematoxylin and Eosin staining. Tissues were blinded to treatment and scored for lesion type and severity and counts were added together. Total scores were obtained by adding counts of inflammatory foci in both meninges and parenchyma. Inflammation was primarily classified as lymphocytic, suppurative, or mixed (Massilamany et al., 2010).

2.5. T cell proliferation assay

Ten days after immunization with the indicated peptides, mice were killed and the draining LN were harvested to obtain single cell suspensions. LNC were stimulated with PLP 139–151, ACA 83–95, and NASE 101–120 (control) at a cell density of 5×10^6 cells/ml for two days in RPMI medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 4 mM L-glutamine, 1 × each of non-essential amino acids and vitamin mixture, and 100 U/ml penicillin–streptomycin (Lonza). Cultures were then pulsed with 1 μCi of tritiated ^{3}[H] thymidine per well; 16 h later, proliferative responses were measured as counts per minute (cpm) using the Wallac liquid scintillation counter (Perkin Elmer, Waltham, MA).

2.6. IA^s tetramer staining

To determine the frequency of antigen-specific T cells, IA^s/PLP 139–151, ACA 83–95 and Theiler's murine encephalomyelitis virus (TMEV) 70–86 tetramers were created as described (Massilamany et al., 2010). LNC obtained from the mice immunized with PLP 139–151 or ACA 83–95 were stimulated with the corresponding peptides (20 μg/ml) for six days. Viable lymphoblasts were incubated with phycoerythrin (PE)-conjugated IA^s tetramers (30 μg/ml) at room temperature for 3 h (Massilamany et al., 2010; Reddy et al., 2003). After washing twice, cells were stained with anti-CD4 and 7-amino-actinomycin-D (7-AAD) (Invitrogen, Eugene, OR) and acquired by flow cytometer (FACS Scan, BD Pharmingen, San Diego, CA). The frequency of tetramer$^+$ (tet$^+$) cells was then enumerated using Flow Jo software (Tree Star, Ashland, OR) in a live (7-AAD$^-$) CD4$^+$ population. To determine the Ag specificity of cross-reactive T cell responses induced with PLP 139–151 and ACA 83–95, four-color flow cytometric analysis was performed to include allophycocyanin (APC)-conjugated PLP 139–151- and ACA 83–95-PE tetramers, anti-CD4, and 7-AAD. Cells were acquired by FACS Calibur (BD Pharmingen) and the frequency of tet$^+$ cells was analyzed using Flow Jo software in the live (7-AAD$^-$) CD4$^+$ subset.

2.7. Cytokine measurement

We measured the synthesis of IL-2, interferon (IFN)-γ, IL-4, IL-10, IL-17A, IL-17F, IL-22, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) by intracellular staining and/or cytokine ELISA. The frequency of cytokine-secreting cells was determined in LNC obtained from mice immunized with PLP 139–151 or ACA 83–95 by flow cytometry (Massilamany et al., 2010; Reddy et al., 2004). Briefly, LNC were stimulated with peptides (20 μg/ml) for two days and the cultures were maintained in IL-2 medium. Viable lymphoblasts were harvested on day 5 and stimulated

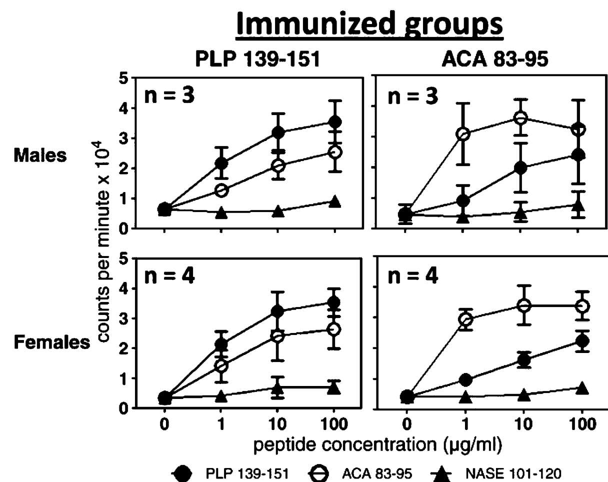


Figure 2. ACA 83–95 induces PLP 139–151-reactive T cell responses equally in both male and female SJL mice. Groups of male and female SJL mice were immunized with PLP 139–151 or ACA 83–95 in CFA. Ten days later, the mice were killed, LN were harvested, and single cell suspensions were prepared. LNC were stimulated with PLP 139–151, ACA 83–95, and NASE 101–120 (control) for two days. Cells were then pulsed with tritiated ^{3}[H] thymidine for 16 h, the incorporation of which was measured as cpm. Mean ± SEM values for a group of mice are shown.

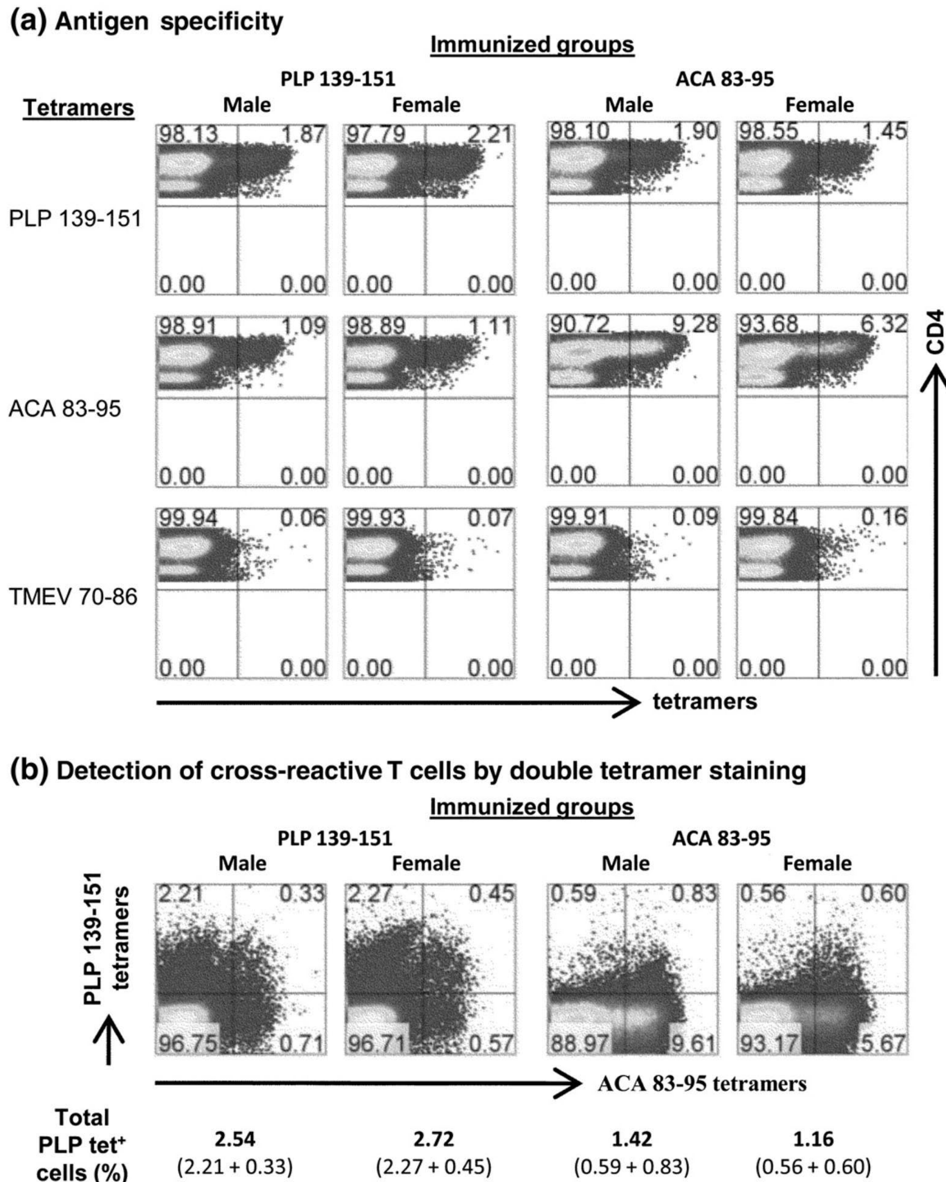


Figure 3. Antigen-specificity of cross-reactive T cells induced with PLP 139–151 or ACA 83–95. (a) Antigen specificity. Male and female SJL mice were immunized with PLP 139–151 or ACA 83–95 in CFA. After ten days, mice were killed and LNC were prepared from the draining LN and stimulated with the indicated peptides. Viable lymphoblasts were harvested on day 6 poststimulation and stained with IA^s/tetramers, namely PLP 139–151, ACA 83–95, and TMEV 70–86 (control), followed by staining with anti-CD4 and 7-AAD. Cells were acquired by flow cytometry and the percentages of tet^+ cells were analyzed in the live ($7\text{-}AAD^-$) CD4 subset. (b) Detection of cross-reactive T cells by double tetramer staining. Cells from the above cultures were stained with PLP 139–151-APC and ACA 83–95-PE tetramers, followed by staining with anti-CD4 and 7-AAD. After acquiring the cells by flow cytometry, double tet^+ cells were analyzed in the live ($7\text{-}AAD^-$) CD4 subset.

for approximately 5 h with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) and ionomycin (150 ng/ml) (Sigma-Aldrich, St. Louis, MO) in the presence of monensin 2 mM (GolgiStop, BD Pharmingen). After staining with anti-CD4 and 7-AAD, cells were fixed, permeabilized and stained with cytokine antibodies or isotype controls (eBioscience, San Diego, CA). The frequency of cytokine-secreting cells was then determined in the live ($7\text{-}AAD^-$) $CD4^+$ subset by flow cytometry (FACS Scan, BD Pharmingen). To estimate the frequency of cytokine-producing PLP 139–151-specific cells, lymphoblasts were first stained with PLP 139–151 tetramers, followed by staining with anti-CD4, 7-AAD and cytokine antibodies, and the cells were acquired by flow cytometry. Frequencies of cytokine-producing PLP 139–151 tet^+ cells were then determined in the live ($7\text{-}AAD^-$) CD4 population using Flow Jo software. Culture supernatants harvested on day 2 poststimulation with peptides were analyzed by cytokine ELISA. The clones of antibodies used for cytokine measurement were as previously published (Massilamany et al., 2010).

2.8. Analysis of T cell receptor (TCR) vβ usage

LNC obtained from the mice immunized with PLP 139–151 or ACA 83–95 were stimulated with peptides for two days (20 μg/ml) and maintained in medium containing IL-2 for five days. Cells were stained with a panel of anti-mouse TCR vβ antibodies: vβ 2, 3, 4, 5.1, 5.2, 6, 7, 8.1, 8.2, 8.3, 9, 10b, 11, 12, 13, 14 and 17a (BD Pharmingen), anti-CD4, and 7-AAD. After washing, cells were acquired by flow cytometer and the percentages of TCR $v\beta^+$ cells were enumerated in the live ($7\text{-}AAD^-$) CD4 population. To determine the frequency of TCR $v\beta^+$ PLP 139–151-specific cells,

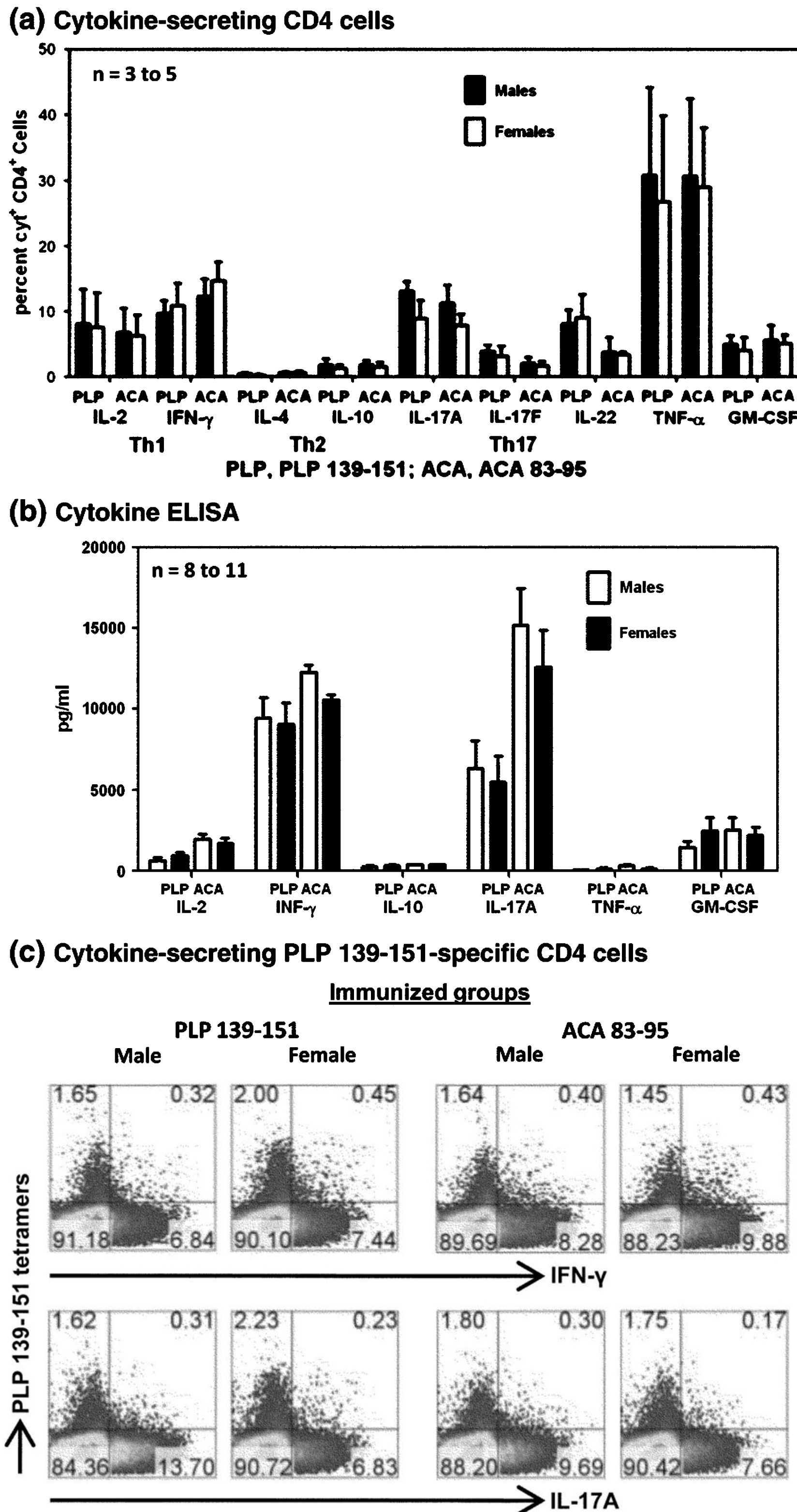
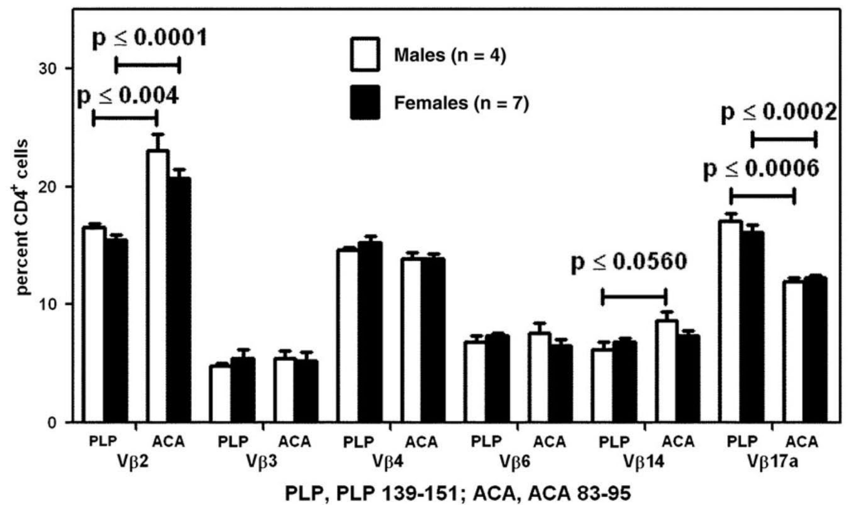


Figure 4. ACA 83–95-induced PLP 139–151-reactive T cells produce similar patterns of cytokines in both male and female SJL mice. (a) Detection of cytokine-secreting cells by intracellular staining. Groups of male and female SJL mice were immunized with PLP 139–151 or ACA 83–95; the mice were killed ten days later and LN were harvested to prepare single cell suspensions. LNC were stimulated with peptides for two days and the cultures were maintained in IL-2 medium. Viable lymphoblasts were harvested on day 5 poststimulation and stimulated with PMA and ionomycin for approximately 5 h, followed by staining with anti-CD4 and 7-AAD. After fixation and permeabilization, the cells were incubated with cytokine antibodies and the frequency of cytokine$^+$ cells was enumerated by flow cytometry in the live (7-AAD$^-$) CD4$^+$ T cells. Mean ± SEM values are shown (n = 4). (b) Cytokine ELISA. Cytokines in the supernatants harvested on day 2 from the above cultures were determined by ELISA. (c) Enumeration of the frequency of cytokine-secreting PLP 139–151 tet$^+$ cells. Viable lymphoblasts were harvested from peptide-stimulated cultures and the cells were first stained with PLP 139–151-APC tetramers followed by staining with anti-CD4, 7-AAD and cytokine antibodies. After acquiring the cells by flow cytometry, percentages of cytokine-secreting PLP 139–151 tet$^+$ cells were determined in the live CD4 subset. A representative from three individual experiments involving three mice in each group is shown.



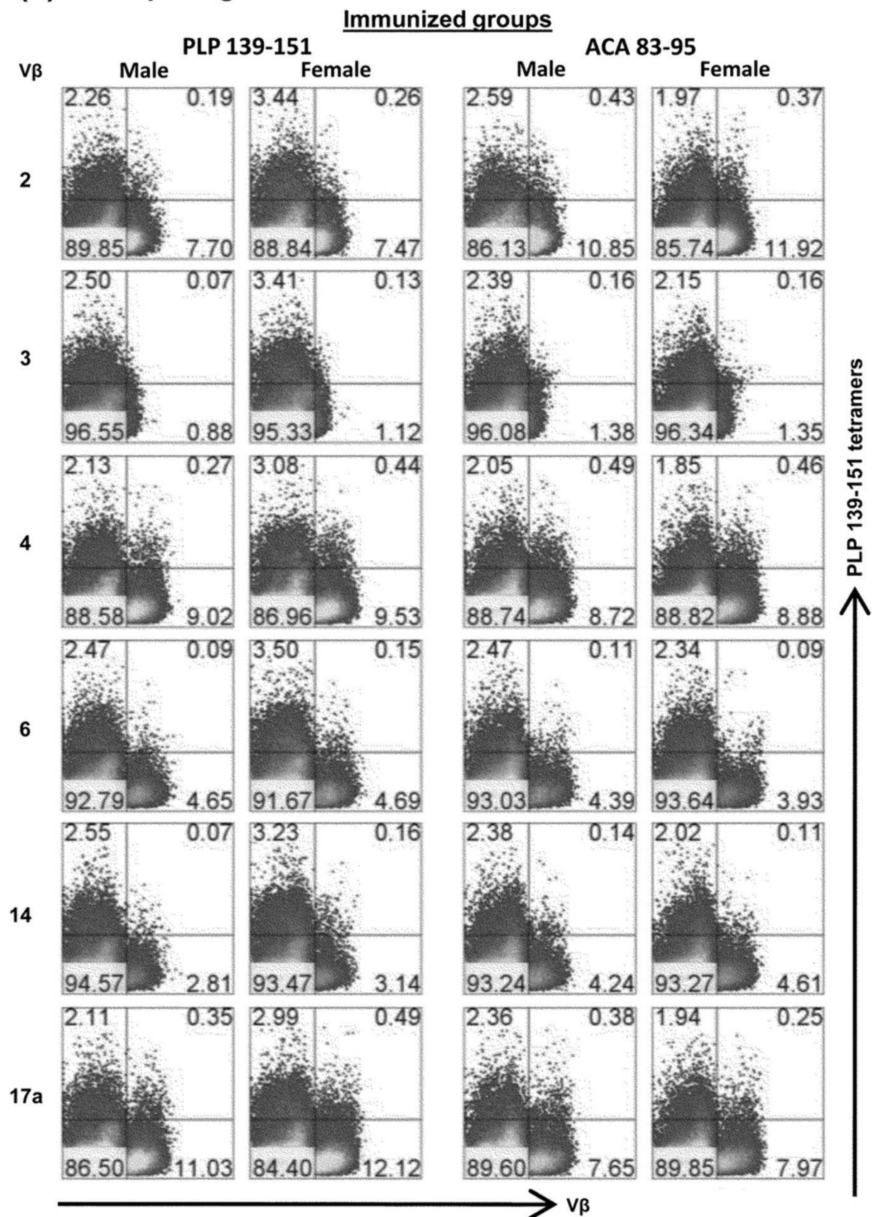


Figure 5. TCR vβ usage in PLP 139–151-reactive T cells generated in response to ACA 83–95 differs from PLP 139–151. (a) TCR vβ usage in CD4 T cells. Groups of male and female SJL mice were immunized with PLP 139–151 or ACA 83–95; ten days later, mice were killed and LN were harvested. LNC were stimulated with peptides for two days and maintained in IL-2 medium. Cells harvested on day 7 poststimulation were stained with a panel of anti-TCR vβ and anti-CD4 and 7-AAD. After acquiring the cells by flow cytometer, percentages of TCR $v\beta^{+}CD4^{+}$ cells were determined in the live ($7\text{-}AAD^{-}$) population. (b) TCR vβ usage in PLP 139–151 tet^{+} cells. LNC cultures were prepared from the mice immunized with PLP 139–151 or ACA 83–95 as above. Viable lymphoblasts were harvested on day 7 poststimulation and stained with PLP 139–151 tetramers, followed by anti-CD4, anti-TCR vβ and 7-AAD. Cells were acquired by flow cytometer and the percentages of TCR $v\beta^{+}$PLP 139–151 tet^{+} cells were determined in the live ($7\text{-}AAD^{-}$) CD4 subset.

lymphocytes were stained first with PLP 139–151 tetramers, followed by anti-CD4 and 7-AAD (Greve et al., 2004). After the cells were acquired by flow cytometry, the frequency of PLP 139–151 tet^+ cells was enumerated in relation to TCR vβ expression in the live (7-AAD^-) CD4 subset using Flow Jo software.

2.9. Statistics

The Wilcoxon rank sum test was used to compare differences in the severity of clinical and histological EAE induced with PLP 139–151 or ACA 83–95. Time-course analysis of clinical EAE induced by active immunization involved the use of median values of clinical scores taken within individual animals for two time periods, days 9–15 and days 1–30 postimmunization, and the data were analyzed using Matlab software. Cumulative scores for each animal within a group were obtained by adding the scores of all the days and comparisons were then made between groups. Gender differences in T cell proliferation, cytokine-secretion, and TCR vβ usage were analyzed by Student's *t*. $p \leq 0.05$ values were used to determine significance.

3. Results

3.1. ACA 83–95 induces autoimmune encephalomyelitis in both male and female SJL mice by active immunization, but the effector cells generated in males are less pathogenic than those generated in females

We used active immunization and AT-EAE protocols to determine gender differences in the susceptibility to autoimmune encephalomyelitis induced with ACA 83–95. These protocols involved PLP 139–151-induced EAE as a positive control system to which the disease phenotype of ACA 83–95 was compared. As shown in Figure 1a, both PLP 139–151 and ACA 83–95 induced clinical signs of EAE in male and female SJL mice. Although clinical signs induced by both peptides were identical, the disease onset occurred relatively earlier with ACA 83–95 (males and females: 11.56) than with PLP 139–151 (males, 12.34; females, 13.59) (Table 1). Time-course comparison of clinical EAE revealed that the EAE severity induced with ACA 83–95 was significantly lower than that induced with PLP 139–151 in both males (mean maximum scores: 1.91 vs. 2.86; $p = 2.94E{-}05$) and females (mean maximum scores: 2.59 vs. 3.19; $p = 3.49E{-}05$) (Table 1). These differences were also confirmed by comparing the cumulative scores between groups [Figure 1a (ii)]. EAE severity induced with ACA 83–95 was significantly greater in female mice during peaking of disease (9–15 days postimmunization) than in males ($p = 0.022$) (Figure 1a). Histologically, inflammatory infiltrates comprising predominantly mononuclear cells were also significantly higher in the brains and spinal cords of females than in males, regardless of immunogens used (Table 1).

We next studied gender-biased differences in the adoptive transfer system by administering ACA 83–95-stimulated cells into naïve recipients and comparing the disease severity with mice in which EAE was induced with PLP 139–151-reactive cells. This was to better discern gender-disparity in T cell functions at the level of the effector phase. Figure 1b (i) shows that PLP 139–151-reactive cells from males and females induced EAE with a comparable severity when cells were transferred into their corresponding genders and also into opposite sexes in criss-cross experiments, with one exception. That is, PLP-reactive T cells generated in males induced relatively mild disease in females (mean maximum scores: 4 vs. 5), and the mean day of disease-onset was delayed (male cells to males: day 9.33; male cells to females: day 30). One of the four female mice (25%) that received male cells showed a mild relapse (score 0 to 1) during days 55 to 58 posttransfer. The disease phenotype produced with ACA 83–95-reactive T cells, in contrast to PLP 139–151-reactive cells, differed in several aspects [Figure 1b (ii)]. (a) Cells from females induced EAE in both genders with a comparable severity (mean maximum scores: males, 3.5; females, 4.0), but the mean day of disease onset was delayed in male recipients (day 17.25) compared to female recipients (day 9.83). Relapses occurred in 83% (5/6) of females that received cells from females. Likewise 40% (2/5) of males that received female cells also showed relapses. Among these, one animal showed relapses on two occasions, days 29–33 and 57–59 and both from scores 0 to 1. Another animal showed a relapse with a score from 1 to 2 during days 35–38. (b) Cells from males could induce disease in only 40% (2/5) of male recipients, the onset of which occurred on day 18.5 posttransfer, and one of them showed relapses; however, these cells failed to induce EAE in female recipients. Together the data suggest both male and female SJL mice are equally susceptible to EAE induced with ACA 83–95 by active immunization, but the effector cells generated in males are less encephalitogenic than those generated in their female counterparts.

3.2. Male and female SJL mice respond equally to ACA 83–95

Groups of male and female SJL mice were immunized with ACA 83–95 or PLP 139–151, and ten days later, recall responses were measured based on tritiated ^{3}[H] thymidine incorporation using NASE 101–120 as a negative control (Figure 2). ACA 83–95 and PLP 139–151 induced T cell responses of a similar magnitude in both males and females in a dose-dependent manner, and a proportion of this response was also attributed to the generation of T cells that cross-react with unimmunizing peptides. That is, the mice immunized with PLP 139–151 showed proliferative response to ACA 83–95, and vice versa, and the T cell responses were specific, since there was no response to NASE 101–120 (Figure 2).

3.3. ACA 83–95 induces similar frequencies of cross-reactive T cells in both male and female SJL mice

We determined antigen specificity and the precursor frequency of cross-reactive T cells induced with PLP 139–151 and ACA 83–95 by flow cytometry using IA^s/tetramers for PLP 139–151 and ACA 83–95 (Figure 3). LNC from mice immunized with ACA 83–95 and PLP 139–151 were restimulated with corresponding peptides and the cultures were maintained in IL-2 medium. Lymphoblasts harvested on day 6 poststimulation were used for tetramer staining. In all of the staining reactions, TMEV 70–86 tetramers were used as negative controls, for which there was negligible binding in cultures stimulated with PLP 139–151 or ACA 83–95 (Figure 3a). Tetramer analysis indicated that in mice immunized with PLP 139–151, PLP-reactive T cells expanded with comparable proportions in both males (1.87%) and females (2.21%), and a subset of CD4 T cells also stained with ACA 83–95 tetramers expanded proportionally (males, 1.09%; females, 1.11%) (Figure 3a). In a similar analysis with ACA 83–95-immunized mice, it was found that males tended to respond relatively better to ACA 83–95 than females, and the percentages of ACA tet^+ cells in male and female mice were estimated to be 9.28% and 6.32%, respectively. Importantly, CD4 T cells from ACA 83–95-immunized mice also bind to PLP 139–151 tetramers, and the frequency of PLP tet^+ cells tended to be higher in males (1.90%) than females (1.45%) (Figure 3a). We further verified the precursor frequencies of cross-reactive T cells by double tetramer staining using PLP 139–151 and ACA 83–95 tetramers (Figure 3b). First, we compared the frequencies of total PLP 139–151-specific T cells generated in response to PLP 139–151 and ACA 83–95 by combining

the percentages of CD4 T cells that bind to PLP 139–151 tetramers alone (upper left quadrant, Figure 3b) and those that bind to both PLP 139–151 and ACA 83–95 tetramers (upper right quadrant, Figure 3b). The mice immunized with PLP 139–151 showed two-fold increase in the proportion of PLP-reactive T cells (males, 2.54%; females, 2.72%) over those immunized with ACA 83–95 (males, 1.42%; females, 1.16%). Second, we estimated the frequency of cross-reactive T cells that bind to both PLP 139–151 and ACA 83–95 tetramers; the frequency was higher in mice immunized with ACA 83–95 (males, 0.83%; females, 0.60%) than in PLP 139–151-immunized mice (males, 0.33%; females, 0.45%) (Figure 3b). The tetramer analysis thus enabled us to conclude that ACA 83–95 is relatively more immunogenic in males than in females, which might have allowed the males to generate a marginally greater number of cross-reactive T cells than females.

3.4. Cytokine production in ACA 83–95-induced PLP 139–151-reactive T cells does not differ by gender

We measured cytokines as readouts to determine the pathogenicity of effector cells induced with PLP 139–151 and ACA 83–95 (Figure 4). Cytokine secretion was measured in LNC cultures obtained from mice immunized with the corresponding peptides by intracellular staining and cytokine ELISA using a panel of Th1 (IL-2 and IFN-γ), Th2 (IL-4 and IL-10), Th17 (IL-17A, IL-17F, and IL-22) and other inflammatory cytokines, namely, TNF-α and GM-CSF. As shown in Figure 4a, CD4 T cells capable of secreting both Th1 and Th17 cytokines were dominant in cultures stimulated with ACA 83–95 or PLP 139–151, but there were no significant gender differences in the secretion of any of the cytokines tested. Similar patterns were also noted when cytokines were analyzed by ELISA (Figure 4b). Gender differences in the ability to secrete IFN-γ and IL-17 were further verified at a single cell level in PLP-specific T cell populations by using PLP 139–151 tetramers. We calculated the percentages of cytokine-producing PLP tet^+ cells using the formula: frequencies of $cytokine^+tet^+$ cells (upper right quadrant)/[frequencies of $cytokine^+tet^+$ cells (upper right quadrant) **+** frequencies of $cytokine^-tet^+$ cells (upper left quadrant)] × 100 (Figure 4c). The tetramer analysis indicated that the frequencies of PLP tet^+ cells capable of producing IFN-γ did not differ between genders, regardless of the immunogens used (PLP 139–151: males,13.02%; females, 12.31%; ACA 83–95: males, 13.38%; females, 14.05%) On the contrary, frequencies of IL-17-secreting PLP tet^+ cells tended to be higher in males than females (PLP 139–151: males, 13.85%; females, 8.55%; ACA 83–95: males, 10.61%; females, 7.26%). Collectively, the data indicate that both male and female mice can generate pathogenic cross-reactive T cells in response to ACA 83–95, suggesting that these cells have a potential to induce CNS inflammation regardless of their gender origin.

3.5. TCR vβ usage differs in PLP 139–151-reactive T cells induced with ACA 83–95 and those induced with PLP 139–151

To determine whether effector cells induced with PLP 139–151 and ACA 83–95 use similar or alternate TCR vβs, we analyzed their surface expression in LNC cultures derived from male and female mice immunized with PLP 139–151 or ACA 83–95. Lymphoblasts harvested on day 7 poststimulation were used for analysis by flow cytometry (Figure 5a). In cultures stimulated with PLP 139–151 or ACA 83–95, expression of TCR vβ2, vβ17a, vβ4, vβ14, vβ6 and vβ3 occurred predominantly but in a decreasing order and together represented 65% of the total CD4 cell population (Figure 5a). TCR vβ2 was expressed significantly more in ACA 83–95-stimulated cultures (males, p = 0.004; females, p = 0.0001) than in PLP 139–151-stimulated cultures, with a corresponding decrease in TCR vβ17a (males, p = 0.0006; females, p = 0.0002). Likewise, TCR vβ14 expression was also marginally more in ACA 83–95-stimulated cells than in cells stimulated with PLP 139–151 in males (p = 0.056). We verified these differences and confirmed antigen-specificity of cross-reactive cells by tetramer analysis. We calculated the percentages of TCR vβ-expressing PLP-specific cells using the following formula: frequencies of $v\beta^+tet^+$ cells (upper right quadrant)/[frequencies of $v\beta^+tet^+$ cells (upper right quadrant) + frequencies of $V\beta^-tet^+$ cells (upper left quadrant)] × 100 (Figure 5b). PLP 139–151 tet^+ cells generated with both PLP 139–151 and ACA 83–95 immunizations contained T cells expressing all of the above TCR vβs, but tetramer analysis revealed a few differences (Figure 5b and Table 2). (a) TCR vβ2 was expressed more in PLP tet^+ cells generated by ACA 83–95 in both males (p = 0.058) and females (p = 0.026), but frequencies of PLP tet^+ cells expressing TCR vβ17a did not differ. (b) While TCR vβ3 expression was insignificant in PLP tet^+ cells, TCR $v\beta14^+$ PLP tet^+ cells tended to be higher in cells stimulated with ACA 83–95 than PLP 139–151-stimulated cells within males (p = 0.067). **(c)** PLP tet^+ cells from female mice immunized with PLP 139–151 showed a marginal increase in the expression of TCR vβ6 as compared with males (p = 0.06). Overall, the expression pattern of TCR vβ usage revealed no gender differences, but PLP–specific cells induced with ACA 83–95 utilize different pattern of TCR vβs as opposed to those induced with PLP 139–151, raising the question whether such variations contribute to disease susceptibility.

4. Discussion

By using ACA 83–95, we investigated whether disparity in T cell reactivity to microbial mimics contributes to gender differences in CNS autoimmunity in SJL mice. Our data revealed that ACA 83–95-induced EAE was less severe in males than in females clinically (Figure 1a) and histologically (Table 1). In AT-EAE, we noted that PLP-reactive T cells obtained from female mice induced EAE with equal severity in both genders, whereas cells from males induced relatively mild EAE in females, the onset of which was significantly prolonged [Figure 1b (i)]. These data are consistent with the findings of others (Bebo et al., 1996, 1998; Voskuhl et al., 1996). In contrast, gender disparity was more pronounced with the disease produced by ACA 83–95-stimulated cells. First, cells from female mice induced EAE in both male and female recipients [Figure 1b (ii)]. Second, cells from males induced only mild EAE in male recipients, but they failed to induce disease in female recipients. These findings indicate that sex of both the donor and the recipient are critical for EAE induction with cross-reactive microbial Ag. Alternatively, the inherent ability of males to resist the development of EAE could explain the occurrence of a mild form of the disease in them. But it is hard to explain why females receiving male cells stimulated with ACA 83–95 did not develop EAE. To

Table 2. Analysis of TCR v -expressing PLP 139–151 $tetramer^+$ cells.

TCR v	Immunized groups[a]			
	PLP 139–151		ACA 83–95	
	Male	Female	Male	Female
v 2	11.78 ± 3.76[b]	10.61 ± 2.98[c]	17.33 ± 1.63[b]	18.06 ± 2.27[c]
v 3	2.38 ± 0.26	2.18 ± 0.74	10.02 ± 5.42	4.32 ± 1.35
v 4	13.25 ± 1.44	11.43 ± 0.86	10.76 ± 4.12	15.66 ± 2.28
v 6	3.90 ± 0.15[d]	4.93 ± 0.37[d]	6.06 ± 2.58	5.04 ± 0.61
v 14	3.42 ± 0.35[e]	4.22 ± 0.16	5.13 ± 0.40[e]	3.93 ± 0.78
v 17a	11.51 ± 1.24	11.14 ± 1.35	9.52 ± 3.00	8.00 ± 1.47

a. n = 3 each involving 2 to 3 mice per group.
b: p = 0.058 ; c: p = 0.026 ; d: p = 0.061 ; e: p = 0.067

explain these differences, we investigated whether variations in the generation of the frequencies of antigen-specific cells, cytokine secretion and TCR vβ usage in effector cells contribute to gender-disparity in EAE induced by ACA 83–95.

Induction of EAE by active immunization with myelin Ag first involves the generation of encephalitogenic T cells in the periphery followed by their migration into the CNS, which then mediates the inflammatory process. We verified that T cell responses of male and female SJL mice to both Ag were similar, including the magnitude, dose–response patterns, and cross-reactivity (Figure 2). We confirmed these findings by tetramer analysis, which indicated that the frequency of total number of PLP tet$^+$ cells generated in response to ACA 83–95-immunization was two-fold lower than the frequency obtained with PLP 139–151, regardless of gender (Figure 3b). This may be the reason ACA 83–95-induced EAE was less severe than EAE induced by PLP 139–151. However, if gender bias exists at all, male mice immunized with ACA 83–95 have a tendency to generate more PLP 139–151-reactive T cells, as assessed by using PLP 139–151 tetramers alone (Figure 3a) or a combination of both PLP 139–151 and ACA 83–95 tetramers to determine double tet$^+$ cells that represent cross-reactive T cell populations (Figure 3b). Therefore logically, ACA 83–95-induced PLP-reactive cells from males are expected to produce disease similar to that produced by female cells, but this was not the case. By analyzing cytokine production, we noted that Th1 and Th17 cytokine-producing cells were dominant in cultures stimulated with PLP 139–151 or ACA 83–95, with a tendency for males to show a higher frequency of IL-17-secreting cells than females (Figure 4). Therefore, we think that variation in cytokine synthesis in male mice was also not a contributing factor in their development of a mild EAE phenotype. Taken together, our data suggest that both male and female mice respond equally to ACA 83–95 and generate similar frequencies of pathogenic cells, while the effector cells from males but not females lose their encephalitogenicity.

We then asked whether inherent variations in the encephalitogenicity of PLP-reactive T cells induced with cognate (PLP 139–151) and mimicry (ACA 83–95) epitopes in males and females were due to differences in TCR vβ usage. It has been shown that the pathogenic T cell repertoires induced by PLP 139–151 are heterogenic in which TCR vβ2, 4, 6, 7, 14, and 17a are predominantly expressed (Kuchroo et al., 1992, 1994). Since the encephalitogenicity was weaker in effector cells induced by ACA 83–95 than in those induced by PLP 139–151, we asked whether ACA 83–95-induced effector cells use alternate TCRs such that the newly generated T cell clones are weakly encephalitogenic. We addressed this possibility by using PLP 139–151 tetramers. Although there were no gender differences, we noted that PLP 139–151 tet$^+$ cells expressing TCR vβ2 were skewed in LNC cultures from mice immunized with ACA 83–95 with no significant variations in the other vβs (Table 2). Previously it was shown that spinal cord infiltrates from PLP 139–151-induced EAE in SJL mice predominantly express TCR vβ2 and vβ 17a (Bebo et al., 1996). The fact that ACA 83–95-induced EAE was less severe than EAE induced by PLP 139–151 in both genders in spite of significant increase in vβ2-expressing cells suggests that they are not the major mediators of disease; rather, multiple T cell clones expressing heterogeneous TCR vβ chains are critical for disease induction by ACA 83–95.

Several factors contribute to gender bias in susceptibility to EAE. These include sex hormones, immune deviation, and functional disparity in antigen-presenting cells (Bebo et al., 1996, 2001; Offner, 2004; Voskuhl and Palaszynski, 2001). We previously demonstrated that regulatory T (Treg) cells mediate genetic resistance to the development of EAE in B10.S (H-2^s) mice (Reddy et al., 2005). We had proposed that males have a capacity to generate potent autoreactive T cells but their expansion is kept in check by Treg cells. Once this check point is released, autoreactive T cells freely expand and induce CNS inflammation. Whether a similar mechanism contributes to the reduced pathogenic potential of cross-reactive T cells induced by microbial products in males remains to be tested.

In summary, we show that both males and females generate similar frequencies of cross-reactive T cells for PLP, but their pathogenicity differs by gender during the effector phase of EAE induction. In contrast to PLP-reactive T cells obtained with PLP 139–151, encephalitogenicity of ACA 83–95-induced effector cells in males was weak, implying that environmental exposure to mimicry peptides can lead to the generation of more potent autoaggressive cells in females than in males. *A. castellanii* is a natural CNS pathogen of humans and the amoeba can induce encephalitis in infected mice; both mouse and human isolates of amoebae can induce histologically similar disease (Culbertson et al., 1959, Culbertson et al., 1958 and Marciano-Cabral and Cabral, 2003). This infection model can therefore be used to address gender-biased differences in the generation of autoreactive T cells and their ability to induce CNS autoimmunity.

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