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Antiviral CD8$^+$ T cells cause an experimental autoimmune encephalomyelitis-like disease in naive mice

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Abstract Major histocompatibility complex class I-restricted CD8$^+$ cytotoxic T lymphocytes are involved in the pathogenesis of multiple sclerosis (MS) and both autoimmune, experimental autoimmune encephalomyelitis, and viral, Theiler's murine encephalomyelitis virus (TMEV) infection, animal models of MS. Following TMEV infection, certain T cell hybridomas, generated from cloned TMEV-induced CD8$^+$ T cells, were able to produce clinical signs of disease (flaccid hind limb paralysis) upon adoptive transfer into naive mice. Dual T cell receptors (TCR) are present on the surface of these cells as both Vβ3 and Vβ6 were detected by polymerase chain reaction (PCR) screening and flow cytometry and multiple Vα mRNAs were detected by PCR screening. This is the first demonstration of antiviral CD8$^+$ T cells having more than one TCR initiating an autoimmune disease in the natural host of the virus. We hypothesize that this is a potential mechanism for virus-induced autoimmune disease initiated by CD8$^+$ T cells.

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Introduction

Multiple sclerosis (MS) is a common inflammatory, demyelinating disease of the central nervous system (CNS) of humans. Although the cause of MS is unknown, both organ-specific autoimmune responses and viral infections are thought to play important roles. As such, there are both autoimmune, experimental autoimmune encephalomyelitis (EAE), and viral, Theiler's murine encephalomyelitis virus (TMEV) infection, animal models of MS [reviewed in (Tsunoda and Fujinami 1996)]. Inflammatory demyelinating disease can be induced in EAE models through the administration of CNS proteins or peptides in adjuvant. A key feature demonstrating the autoimmune nature of EAE is through the adoptive transfer of myelin-specific T cells into naive animals resulting in CNS inflammation and demyelination with clinical disease. Intracerebral infection of susceptible strains of mice (SJL/J) with viruses from the less neurovirulent Theiler's Original subgroup, such as the Daniels (DA) strain, results in an inflammatory demyelinating disease, with chronic viral infection developing about 1 month postinfection.

In MS and two of the animal models (EAE and TMEV), autoreactive major histocompatibility complex (MHC) class II-restricted CD4$^+$ T cells are involved as effector cells mediating disease [reviewed in (Tsunoda and Fujinami 1996)]. However, in addition to the CD4$^+$ T cells, MHC class I-restricted CD8$^+$ T cells are also involved in pathogenesis. Both an association with MHC class I alleles and the expression of MHC class I molecules in the brain have been demonstrated in MS

[reviewed in (Friese and Fugger 2005)]. Also, $CD8^+$ T cells are found in significant numbers in MS lesions (Hayashi et al. 1988; McCallum et al. 1987; Sobel 1989) and actually outnumber the $CD4^+$ T cells in active lesions (Babbé et al. 2000). In EAE, $CD8^+$ suppressor T cells are thought to influence/regulate the pathogenicity of the disease (Ofosu-Appiah and Mokhtarian 1991; Sun et al. 1988; Weiner et al. 1994). More recently, an effector role for $CD8^+$ T cells in EAE has been suggested based on the ability of adoptively transferred myelin-specific $CD8^+$ cytotoxic T lymphocytes (CTL) to mediate CNS disease (Huseby et al. 2001; Sun et al. 2001). In TMEV, resistance to chronic infection maps genetically to the MHC class I H-2D locus (Clatch et al. 1985; Rodriguez et al. 1986) and MHC class I-restricted viral capsid-specific $CD8^+$ CTL have been found in the CNS of TMEV-infected mice resistant to the development of the TMEV-induced demyelinating disease (Lin et al. 1995, 1997), indicating the importance of $CD8^+$ CTL in viral clearance. In addition, the CD4/CD8 ratio in the CNS of TMEV-infected mice was lower than the ratio in the peripheral blood, indicating preferential infiltration of $CD8^+$ T cells into the CNS (Tsunoda et al. 1996). Evidence supporting an effector role of $CD8^+$ T cells in TMEV-induced demyelination includes parenchymal infiltration of these cells during chronic infection (Lindsley and Rodriguez 1989) and a decrease in demyelination upon infection of mice depleted of these cells (Rodriguez and Sriram 1988).

One possible scenario for the initiation and/or exacerbations of MS is that peripheral viral infections occurring outside of the CNS could start an immune response down the pathway which leads to CNS inflammation and demyelinating disease. To test this hypothesis, we previously found that peripheral infection of SJL/J mice with DA generated anti-DA-reactive $CD8^+$ T cells that could kill both infected and uninfected target cells (Tsunoda et al. 2002). Transfer of these antiviral and autoreactive $CD8^+$ T cells could induce CNS inflammatory changes in naive mice. In subsequent studies, we demonstrated that cloned antiviral and autoreactive $CD8^+$ T cells could kill both infected and uninfected target cells and also induce inflammatory changes such as perivascular infiltration and demyelination when transferred into naive mice (Tsunoda et al. 2005). T cell hybridomas were made from the cloned antiviral and autoreactive $CD8^+$ T cells that produced interferon-γ upon co-culture with DA-infected or uninfected syngeneic target cells (Tsunoda et al. 2008).

In the present study, we extended our initial characterization of the antiviral $CD8^+$ T cell clones/hybridomas. We discovered that certain, but not all, hybridomas induced flaccid hind limb paralysis (clinical signs of disease) in the recipient mice following adoptive transfer. Inflammatory changes, such as cell infiltration, were evident in the brains in these mice. Three hybridomas, 8a-1A1, 8a-1A3, and 8a-1Ae, were chosen for further examination, since the 8a-1Ae hybridoma caused paralysis while the 8a-1A1 and 8a-1A3 hybridomas did not. Recipient mice receiving the 8a-1A1, 8a-1A3, and 8a-1Ae cells were scored for disease and the mean EAE scores correlated with the observed paralysis.

To explore a potential mechanism to explain how $CD8^+$ T cells generated after viral infection could cause disease in naive mice, polymerase chain reaction (PCR) screening was used to determine which T cell receptor (TCR) Vα and Vβ mRNAs were expressed within the cells. In addition, flow cytometry was used to detect which TCR Vβ chains were present on the surface of the $CD8^+$ T cells. Both PCR screening and flow cytometry detected Vβ3 and Vβ6 in/on the 8a-1A parental clone and the 8a-1A1, 8a-1A3, and 8a-1Ae cells. Multiple Vα mRNAs were detected, by PCR screening, within the 8a-1A parental clone and the 8a-1A1, 8a-1A3, and 8a-1Ae cells, suggesting the presence of dual TCRs on the cell surface of these clones/hybridomas.

Therefore in this study, we have demonstrated that TMEV infection of the mouse, its natural host, induces $CD8^+$ T cells that have more than one TCR. Such virus-induced $CD8^+$ T cells having multiple TCRs may be one mechanism involved in the development of CNS autoimmune disease.

Materials and methods

Animals

Female SJL/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The care and use of the above-mentioned mice were in accordance with the guidelines prepared by the committee on Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council.

Cell growth

T cell clones were grown, for flow cytometry, in RPMI 1640 media (Mediatech, Herndon, VA) containing 10% Cosmic Calf serum (Hyclone, Logan, UT), 2 mM L-glutamine (Mediatech), 10 mM HEPES (Invitrogen, San Diego, CA), 1 mM sodium pyruvate (Mediatech), 50 μM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 1% Antibiotic–Antimycotic solution (Mediatech) and supplemented with 20 U/ml recombinant mouse interleukin (IL)-2 as needed (BD Bioscience, San Jose, CA).

BW-1100.129.237 ($BW\alpha^-\beta^-$) cells (fusion partner cell line kindly provided by Dr. Philippa Marrack, Howard Hughes Medical Institute) and hybridomas, generated previously (Tsunoda et al. 2008), were grown, for adoptive transfer and flow cytometry, in RPMI 1640 media supplemented with 10% Cosmic Calf serum, 2 mM L-glutamine, 1% Antibiotic–Antimycotic solution, and 50 μM β-mercaptoethanol.

Adoptive transfer of antiviral T cells

The protocol of Huseby et al. (2001) was followed with slight modification. The 8a-1A1, 8b-1C5, 8b-1Cs, 8b-1CZ, 8a-1Am, 8a-1A3, and 8a-1Ae hybridomas and the control BW$\alpha^-\beta^-$ fusion partner were grown in vitro for adoptive transfer. On day −1, 6- to 7-week old SJL/J mice were sublethally irradiated at 400 rad with an X-RAD 320 Irradiator (Precision X-ray, North Branford, CT). On day 0, 2×10^7 cells were adoptively transferred to the irradiated mice (three to ten mice per experimental group) via intravenous injection and 1×10^4 U of IL-2 was administered to the mice intraperitoneally. Mice were weighed daily and scored for clinical signs of EAE. Clinical signs of EAE were assessed as follows: 0=no clinical disease; 1=loss of tail tonicity; 2=mild hind leg paresis; 3=moderate hind leg paralysis; 4=complete paraplegia; and 5=quadriplegia, moribund state, or death (Tsunoda et al. 1998).

Histology

Mice were euthanized with isoflurane when clinical signs were evident or 3 weeks post-transfer. Animals were perfused with phosphate-buffered saline, followed by a buffered 4% paraformaldehyde solution. Brain, spinal cord, lymph nodes, spleen, liver, kidney, lung, heart, sciatic nerve, and skeletal muscle were harvested and fixed in 4% paraformaldehyde. Brains were divided into five coronal slabs per brain, spinal cords were divided into 10–12 transverse slabs per spinal cord, organs were bisected, and all organs were embedded in paraffin. Multiple 4-μm-thick tissue sections were cut and mounted on slides. Brains and spinal cords were stained with Luxol fast blue and the other organs were stained with hematoxylin and eosin. Using the stained tissue sections, cell infiltration was examined in the various organs of the sublethally irradiated recipient mice adoptively transferred with multiple hybridomas and the BW$\alpha^-\beta^-$ cells (three to ten mice per experimental group). Damaged axons were visualized on serial sections with SMI 311 (Sternberger Monoclonal, Inc., Baltimore, MD), a cocktail of antibodies (SMI 32, 33, 37, 38, and 39) to nonphosphorylated neurofilament protein, with autoclave pretreatment, as previously described (Tsunoda et al. 2003, 2007).

TCR Vα and Vβ repertoire clone screening

The TCRs of the hybridomas were examined through the use of the SuperTCR*Express*™ Mouse T Cell Receptor Vα and Vβ Repertoire Clone Screening Assay Kits (BioMed Immunotech, Tampa, FL), as per the manufacturer's instructions. Briefly, RNA was purified, cDNA was synthesized and primary and nested PCR amplifications produced various PCR products which when separated on an agarose gel allowed for the screening of 22 individual Vα gene families (Vα1 to Vα23 minus Vα21) and 22 individual Vβ gene families (Vβ1 to Vβ20 with subfamilies Vβ8.1, Vβ8.2, and Vβ8.3). However, as the Vβ subfamilies 5, 8, 9, 11, 12, and 13 are deleted in the SJL/J stain of mouse (Singer et al. 1988), we only included Vβ5 of these Vβ subfamilies in the assay as a negative control. For Vα, the expression of the PCR products was quantified, compared to an internal control PCR product, by scanning the photographs of the agarose gels with a HP Scanjet 8200 series scanner and analyzing the images with the Odyssey Imaging System version 3.0 (Li-Cor Biosciences, Lincoln, NE).

Flow cytometry

The BW$\alpha^-\beta^-$ cells, the 8a-1A parental T cell clone and the 8a-1A1, 8a-1A3, and 8a-1Ae cells were activated for 3 h with phytohaemagglutinin (0.5 μg/ml; Sigma) and ionomycin (1 μg/ml; Sigma). Cells were stained with various combinations of anti-mouse Vβ6 TCR phycoerythrin (PE)-conjugated monoclonal antibody, anti-mouse Vβ3 TCR fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody, anti-mouse TCRαβ FITC-conjugated monoclonal antibody, anti-mouse CD8 PE-conjugated monoclonal antibody, and the general lymphocyte marker anti-mouse CD45 V500-conjugated monoclonal antibody (all obtained from BD Biosciences). Nonviable cells were excluded through the use of 7-amino-actinomycin D (7-AAD) (BD Biosciences) for each experiment. Appropriate isotype control antibodies (BD Biosciences) were used for the detection of nonspecific staining. Cells were fixed after staining and stored at 4°C until analyzed. Cells were analyzed on a BD FACSCanto II (BD Biosciences). Flow cytometry data analysis was performed using FlowJo software (Tree Star, Inc., Ashland, OR).

Statistical analysis

The StatView program (SAS Institute Inc., Cary, NC) was used for all statistical analyses performed. The unpaired two-group Mann–Whitney *U* test was performed for all nonparametric analyses (mean EAE score).

Results

Adoptive transfer of antiviral T cells

Previously, an effector role for $CD8^+$ T cells in EAE was suggested based on the ability of adoptively transferred myelin-specific $CD8^+$ CTL to mediate CNS disease (Huseby et al. 2001; Sun et al. 2001). Here, we demonstrate an effector role for $CD8^+$ T cells in TMEV-induced disease based on the ability of transferred virus-specific $CD8^+$ CTL to mediate CNS disease. Multiple hybridomas (8a-1A1, 8b-1C5, 8b-1Cs, 8b-

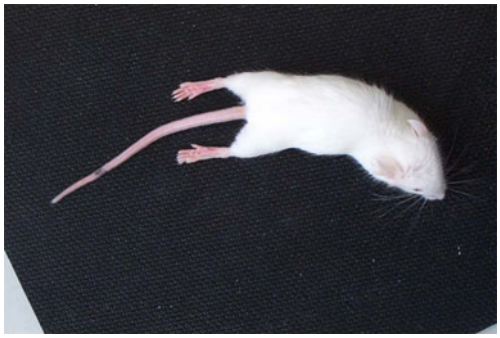

Fig. 1 Clinical signs of disease. Adoptive transfer of 8b-1C5 cells via the intravenous route into sublethally irradiated recipient mice resulted in flaccid hind limb paralysis

1CZ, 8a-1Am, 8a-1A3, and 8a-1Ae) were adoptively transferred into sublethally irradiated recipient mice. Clinical signs of disease, such as hind limb paralysis (Fig. 1), developed upon transfer of some (8b-1C5, 8b-1Cs, 8b-1CZ, 8a-1Ae), but not all (8a-1A1, 8a-1Am, 8a-1A3) of these hybridomas. Systematic evaluation of the clinical signs in recipient mice from day 8 through day 21 post-transfer for mice receiving the 8a-1A1, 8a-1A3 or 8a-1Ae cells or the control BWα$^-$β$^-$ cells showed that the mice receiving the 8a-1Ae cells scored in the range of 1.5 to 3 (days 10 through 21), while the mice receiving the other two hybridomas or the fusion partner scored very low or not at all (Fig. 2). The recipient mice receiving the 8a-1Ae cells had a significantly greater mean clinical score than the recipient mice receiving either the 8a-1A1 cells or the BWα$^-$β$^-$ cells at day 10 post-transfer and had a significantly greater mean clinical score than the recipient mice receiving the 8a-1A1 or 8a-1A3 cells or BWα$^-$β$^-$ cells at day 11 post-transfer ($p<$ 0.05, Mann–Whitney *U* test; Fig. 2). Of these three hybridomas, only the 8a-1Ae cells caused paralysis in the recipient mice, while the 8a-1A1 and 8a-1A3 cells and fusion partner did not cause paralysis.

Pathologic changes were present in the CNS (Fig. 3, Table 1) and other organs (Fig. 4) of mice receiving the various hybridomas. Changes observed in the CNS included cell infiltration of the meninges (all hybridomas) and perivascular regions (8b-1Cs) (Fig. 3a, Table 1), cell infiltration of the choroid plexus (8a-1Am) (Fig. 3b, Table 1) and hydrocephalus (8a-1Am) (Fig. 3c, Table 1) of the brain, as well as axonal degeneration in the spinal cord (8b-1C5) (Fig. 3d, Table 1). Changes observed in the other organs included cell infiltration

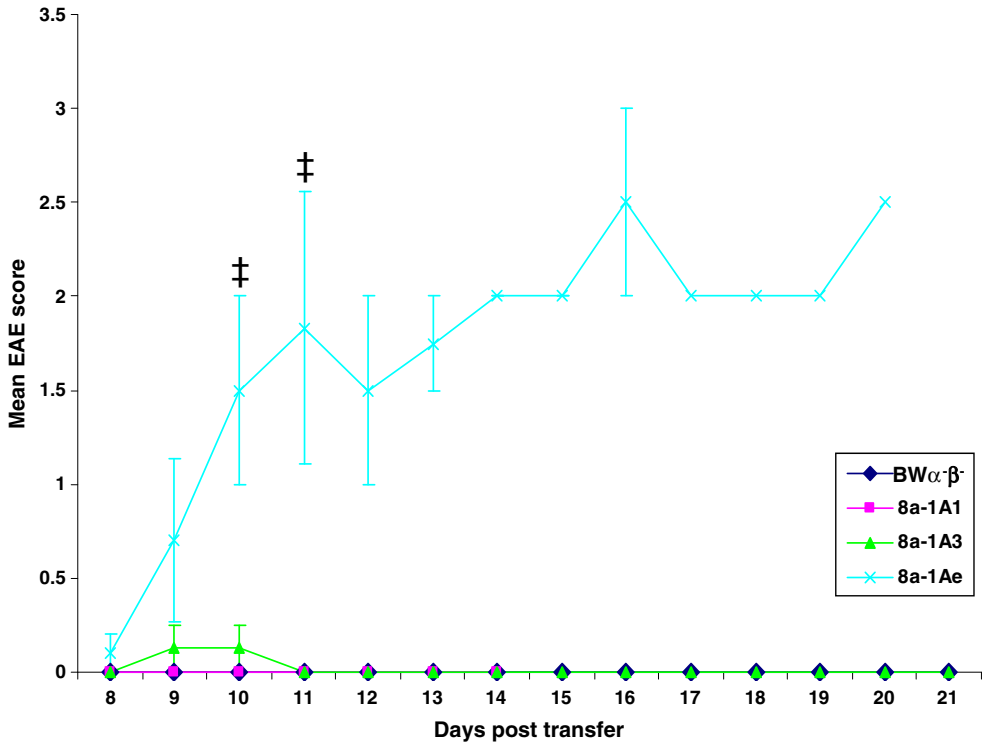

Fig. 2 Clinical score. EAE was scored, as described in the Methods, on days 8 through 21 post-transfer, in sublethally irradiated mice receiving the BWα$^-$β$^-$ cells or the 8a-1A1, 8a-1A3, or 8a-1Ae cells. Only the mice receiving the 8a-1Ae cells had an appreciable EAE score. At day 10 post-transfer, the mice receiving the 8a-1Ae cells (three mice) had a significantly greater mean EAE score than the mice receiving either the 8a-1A1 cells (four mice) or the BWα$^-$β$^-$ cells (five mice). At day 11 post-transfer, the mice receiving the 8a-1Ae cells (three mice) had a significantly greater mean EAE score than the mice receiving the 8a-1A1 or 8a-1A3 cells (four mice each) or the BWα$^-$β$^-$ cells (five mice). ‡, $p<0.05$ (Mann–Whitney *U* test). Results are mean± standard error of the mean of groups with one to five mice per group: three to five mice per group for days 8–11, two to five mice per group for days 12–16, and one to four mice per group for days 17–21

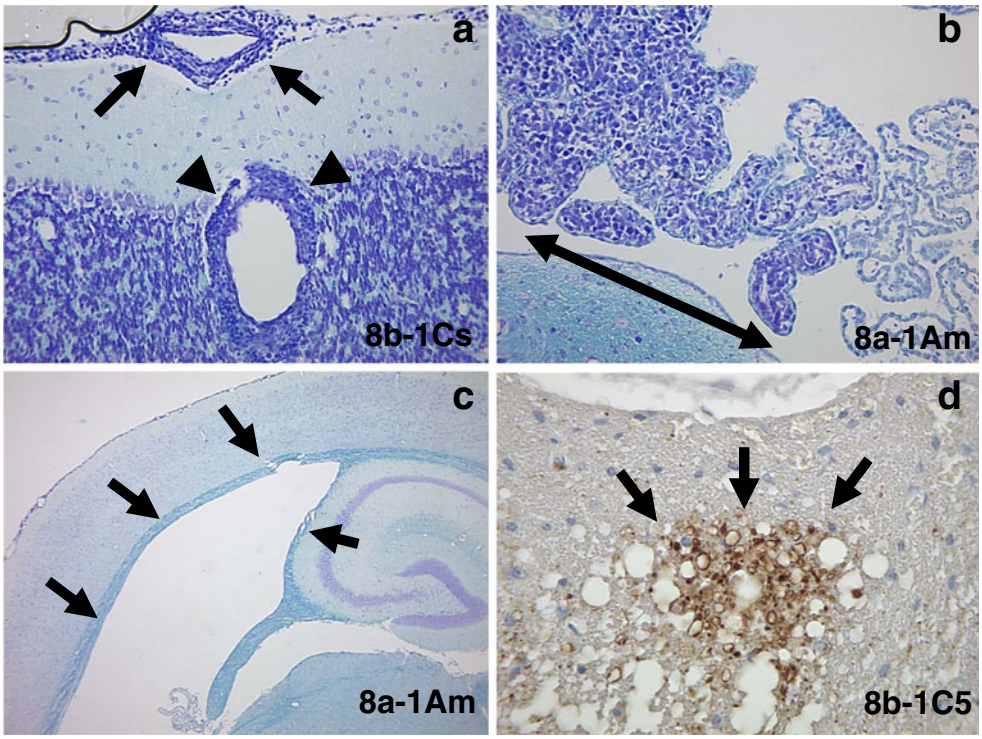

Fig. 3 Pathologic changes in the CNS. Adoptive transfer of 8b-1Cs cells via the intravenous route into sublethally irradiated recipient mice resulted in **a** meningeal (*arrows*) and perivascular (*arrowheads*) cell infiltration. Adoptive transfer of 8a-1Am cells via the intravenous route into sublethally irradiated recipient mice resulted in **b** choroid plexus cell infiltration (*double arrowhead*) and **c** hydrocephalus (*arrows*). Adoptive transfer of 8b-1C5 cells via the intravenous route into sublethally irradiated recipient mice resulted in **d** axonal degeneration (*arrows*) in the spinal cord. **a**–**c** Luxol fast blue staining, **d** Nonphosphorylated neurofilament protein immunohistochemistry

(some hybridomas) and necrosis of the liver (Fig. 4a), cell infiltration of the kidney (all hybridomas) (Fig. 4b) and cell infiltration and muscle fiber degeneration of the muscle (8a-1Ae and 8a-1A3 only) (Fig. 4c). Cell infiltration was also observed in the spleen, lymph node, and sciatic nerve (some hybridomas) but the lung and heart were not involved in most mice (histology not shown). The pattern and extent of cell infiltration in the CNS and other organs were examined in mice receiving the various hybridomas and control BW$\alpha^-\beta^-$ cells, and the results are presented in Table 2. No cell infiltration was found in the brains or other organs of mice receiving BW$\alpha^-\beta^-$ cells (Table 2, Fig. 4d). The pattern and extent of cell infiltration varied from hybridoma to hybridoma for any particular organ and from organ to organ for any particular hybridoma (Table 2). Overall, there was no correlation between the pattern or extent of the cell infiltration and the ability to induce paralysis.

TCR Vα and Vβ repertoire

A previous study reported single individual CD8$^+$ CTLs having multiple TCR Vα chains following influenza virus infection (Dash et al. 2011). The proportion of these cells increased over the course of the infection and these cells were found in the lungs, a nonlymphoid tissue site distal from lymphoid tissue, suggesting a role for the induction of autoimmunity (Dash et al. 2011). To determine whether the CD8$^+$ T cells induced following TMEV infection had mul-

Table 1 Pathological changes in the CNS

Cells	Meningeal infiltration	Perivascular infiltration	Choroid plexus infiltration	Hydrocephalus	Axonal degeneration[a]
BWα$^-\beta^-$	0/5 (0)[b]	0/5 (0)	0/5 (0)	0/5 (0)	0/1 (0)
8a-1Am	3/3 (100)	2/3 (66)	2/3 (66)	2/3 (66)	NA
8a-1Ae	5/5 (100)	0/5 (0)	0/5 (0)	1/5 (20)	1/1 (100)
8a-1A3	4/4 (100)	0/4 (0)	1/4 (25)	0/4 (0)	NA
8a-1A1	4/9 (44)	1/9 (11)	0/9 (0)	0/9 (0)	0/4 (0)
8b-1C5	5/10 (50)	0/10 (0)	0/10 (0)	0/10 (0)	2/3 (66)
8b-1Cs	4/5 (80)	3/5 (60)	0/5 (0)	1/5 (20)	1/1 (100)
8b-1CZ	4/4 (100)	2/4 (50)	1/4 (25)	1/4 (25)	0/1 (0)

NA not assayed

[a]Assayed via SMI 311 immunostain

[b]Number of mice affected/total number of mice examined (percent affected)

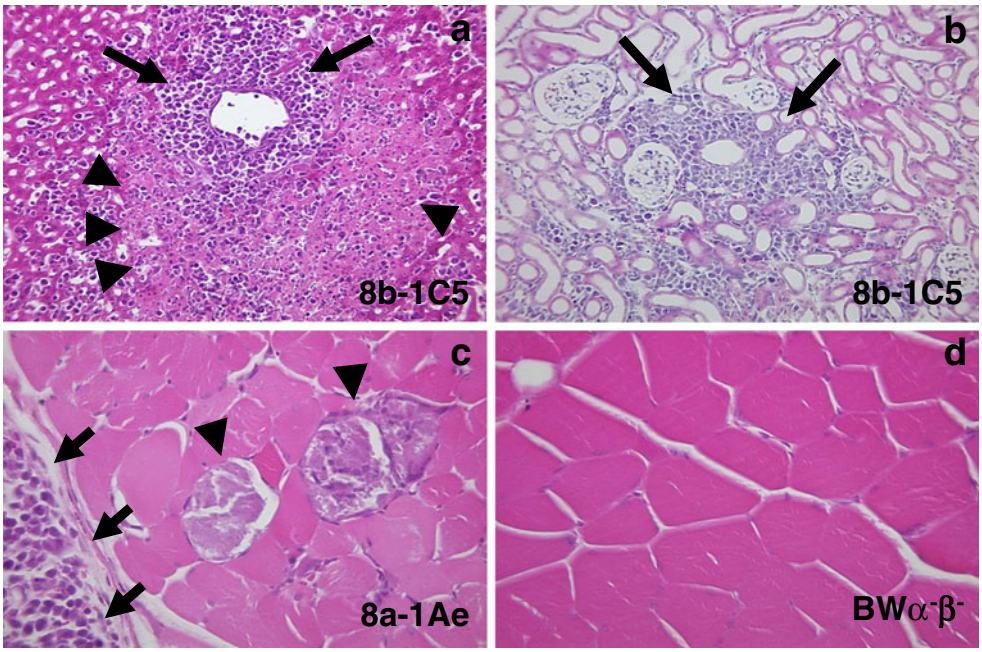

Fig. 4 Pathologic changes in other organs. Adoptive transfer of 8b-1C5 cells via the intravenous route into sublethally irradiated recipient mice resulted in **a** cell infiltration (*arrows*) and necrosis (*arrowheads*) of the liver and **b** cell infiltration (*arrows*) of the kidney. Adoptive transfer of 8a-1Ae cells via the intravenous route into sublethally irradiated recipient mice resulted in **c** degeneration (*arrowheads*) and cell infiltration (*arrows*) of the skeletal muscle. No muscle pathology was seen upon adoptive transfer of the BWα$^-$β$^-$ cells **d** via the intravenous route into sublethally irradiated recipient mice. **a**–**d** Hematoxylin and eosin staining

tiple TCRs which could confer reactivity to virus and self, TCR Vα and Vβ usage was determined.

No Vβ transcripts were detected in BWα$^-$β$^-$ cells for any of the Vβ gene families analyzed (data not shown). The 8a-1A parental CD8$^+$ T cell clone and the 8a-1A1, 8a-1A3, and 8a-1Ae CD8$^+$ T cells derived from 8a-1A all expressed both Vβ3 and Vβ6 (data not shown).

The Vα results for the BWα$^-$β$^-$ cells, 8a-1A parental CD8$^+$ T cell clone and 8a-1A1, 8a-1A3, and 8a-1Ae CD8$^+$ T cells are presented as histograms in Fig. 5. Our analysis of BWα$^-$β$^-$ cells found Vα3, Vα4, Vα13, Vα16, Vα17, and Vα22 mRNA (Fig. 5). Examination of the 8a-1A parental CD8$^+$ T cell clone demonstrated the presence of Vα's 1-8, 10, 11, 13, 15, 16, 18, and 19 mRNAs (Fig. 5). Our analysis of the 8a-1A1, 8a-1A3, and 8a-1Ae CD8$^+$ T cells detected Vα's 19 and 22 (8a-1A1), 1, 19, and 22 (8a-1A3) or 1, 4, and 22 (8a-1Ae) (Fig. 5). The lineages of the individual Vαs present in the 8a-1A1, 8a-1A3, and 8a-1Ae CD8$^+$ T cells can be traced back to either the BWα$^-$β$^-$ cells or the 8a-1A parental CD8$^+$ T cell clone or to both.

Since Vβ3 and Vβ6 mRNAs are present in all three CD8$^+$ T cells, it may be that a specific combination of a particular Vα chain with one or the other of the Vβ chains is the determining factor as to whether or not the CD8$^+$ T cell is able to induce disease. The Vα22 mRNA is present in all three CD8$^+$ T cells and, therefore, probably does not play a role in the induction of disease. The Vα19 mRNA is present in the 8a-1A1 and 8a-1A3 cells, which do not induce disease, and, therefore, also probably does not play a role in the induction of disease. The Vα1 mRNA is present in the 8a-1A3 cells which

Table 2 Homing patterns of antiviral CD8$^+$ T cells

Cells	Paralysis	Brain	Spinal cord	Spleen lymph	Liver	Kidney	Lung	Muscle	Heart	Sciatic nerve
BWα$^-$β$^-$	−	0/5 (0)[a]	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)	0/2 (0)
8a-1Am	−	3/3 (100)	3/3 (100)	0/3 (0)	1/3 (33)	3/3 (100)	0/3 (0)	0/3 (0)	0/3 (0)	2/2 (100)
8a-1Ae	+	5/5 (100)	5/5 (100)	0/5 (0)	0/5 (0)	3/5 (60)	1/5 (20)	3/5 (60)	1/5 (20)	2/2 (100)
8a-1A3	−	4/4 (100)	4/4 (100)	3/4 (75)	2/4 (50)	4/4 (100)	2/4 (50)	4/4 (100)	1/4 (25)	2/2 (100)
8a-1A1	−	4/9 (44)	1/9 (11)	4/9 (44)	5/9 (55)	6/9 (66)	0/9 (0)	1/9 (11)	0/9 (0)	NA
8b-1C5	+	5/10 (50)	4/10 (40)	0/10 (0)	4/10 (40)	3/9 (33)	0/10 (0)	0/10 (0)	0/10 (0)	NA
8b-1Cs	+	4/5 (80)	2/5 (40)	0/5 (0)	1/4 (25)	2/4 (50)	0/5 (0)	0/5 (0)	0/5 (0)	0/1 (0)
8b-1CZ	+	4/4 (100)	3/3 (100)	4/4 (100)	3/4 (75)	3/4 (75)	0/4 (0)	1/4 (25)	1/4 (25)	1/1 (100)

NA not assayed

[a] Number of mice affected/total number of mice examined (percent affected)

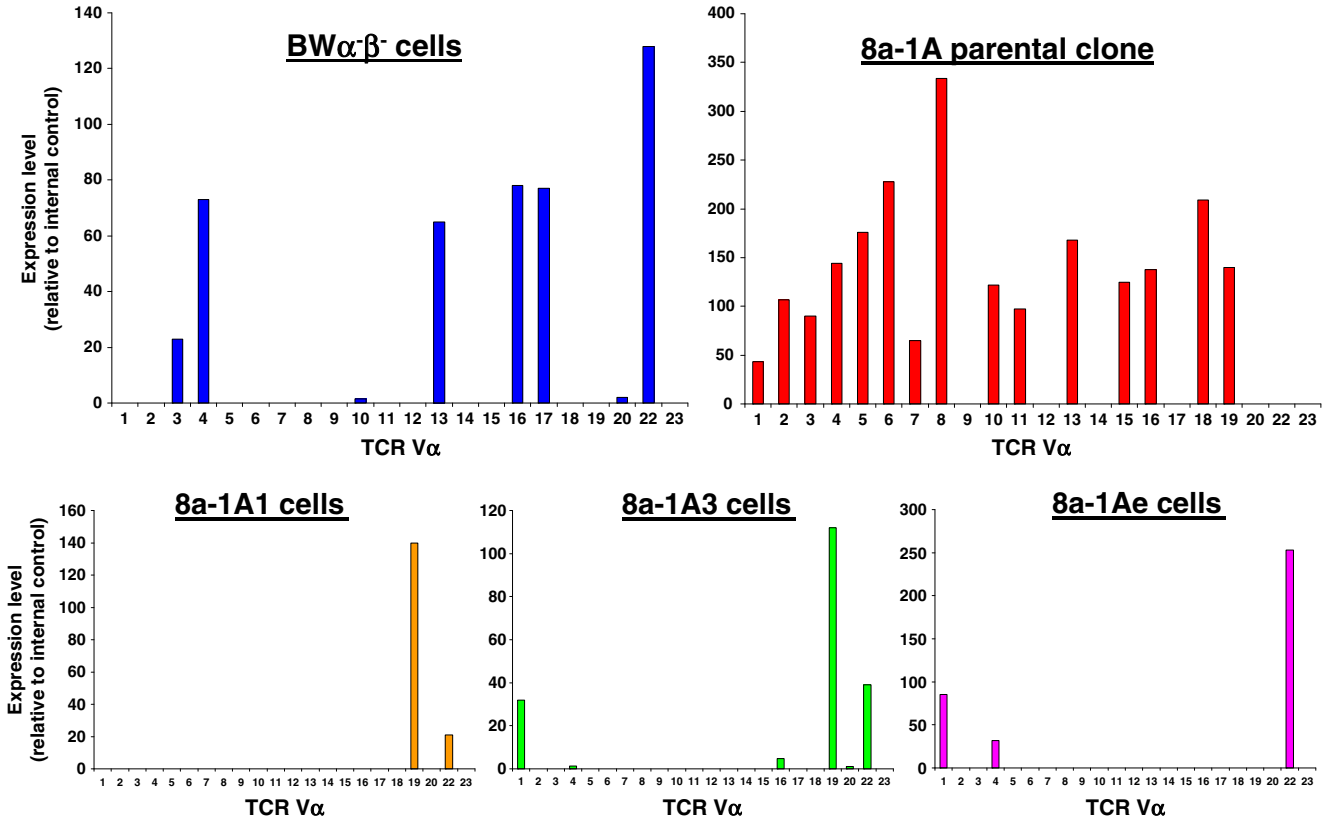

Fig. 5 Vα mRNA expression. The expression (relative to an internal control) of Vα mRNAs was analyzed and quantified, as described in the methods, and presented in histogram form for the BWα$^-$β$^-$ cells (*upper left histogram*), the 8a-1A parental clone (*upper right histogram*) and the 8a-1A1, 8a-1A3, and 8a-1Ae cells (*lower histograms from left to right,* respectively). Multiple Vα mRNAs were found to be present in all of these cell types

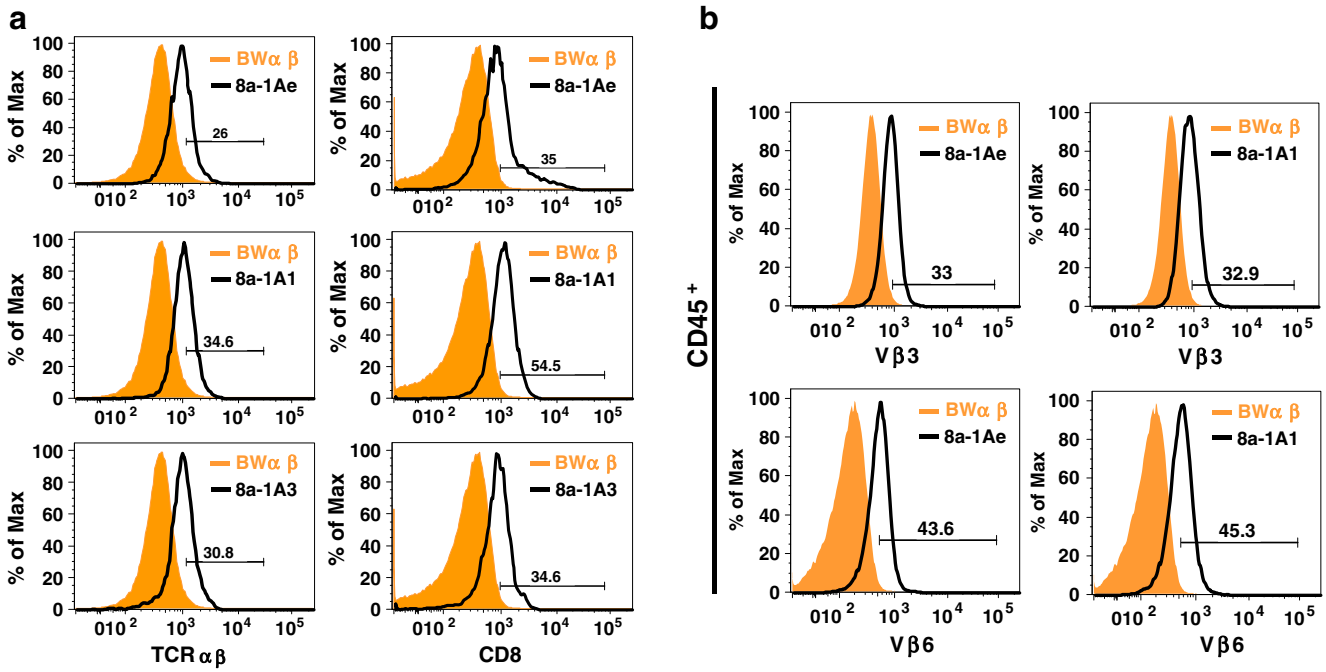

Fig. 6 Surface expression of T cell-specific markers. The BWα$^-$β$^-$ cells and the 8a-1A1, 8a-1A3, and 8a-1Ae cells were analyzed by flow cytometry for surface expression of **a** TCRαβ and CD8, and **b** Vβ3 and Vβ6. **a** All three hybridomas expressed both TCRαβ and CD8 in comparison to BWα$^-$β$^-$ cells. **b** The 8a-1A1, 8a-1A3 (not shown), and 8a-1Ae cells expressed both Vβ3 and Vβ6 compared to BWα$^-$β$^-$ cells. Cells were gated for CD45$^+$ cells

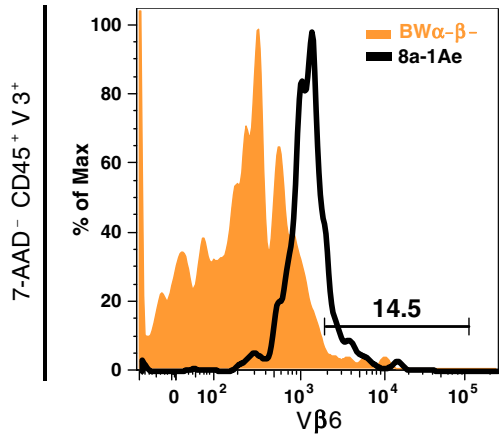

Fig. 7 Dual expression of Vβ3 and Vβ6. The BWα⁻β⁻ cells and the 8a-1Ae cells were analyzed by flow cytometry for dual surface expression of Vβ3 and Vβ6. Viable 8a-1Ae cells (7-AAD⁻), gated for CD45⁺ cells followed by Vβ3⁺ cells, expressed Vβ6 in comparison to BWα⁻β⁻ cells

do not induce disease and the 8a-1Ae cells which do induce disease, so, again, this Vα probably does not play a role in the induction of disease. The Vα4 mRNA is present only in the 8a-1Ae cells which do induce disease, and, therefore, this Vα probably does play a role in the induction of disease.

Cell surface phenotype

Flow cytometry was used to characterize the cell surface expression of T cell-specific markers (Fig. 6a) and to confirm the surface expression of Vβ3 and Vβ6 detected in the 8a-1A1, 8a-1A3, and 8a-1Ae cells by PCR (Figs. 6b and 7). All three hybridomas expressed both TCRαβ and CD8 in comparison to BWα⁻β⁻ cells (Fig. 6a). All three hybridomas (8a-1A3 not shown) expressed both Vβ3 and Vβ6 compared to BWα⁻β⁻ cells (Fig. 6b). Dual staining of CD45⁺ viable cells (7-AAD⁻) for both Vβ3 and Vβ6 demonstrated the presence of both Vβ3 and Vβ6 on the surface

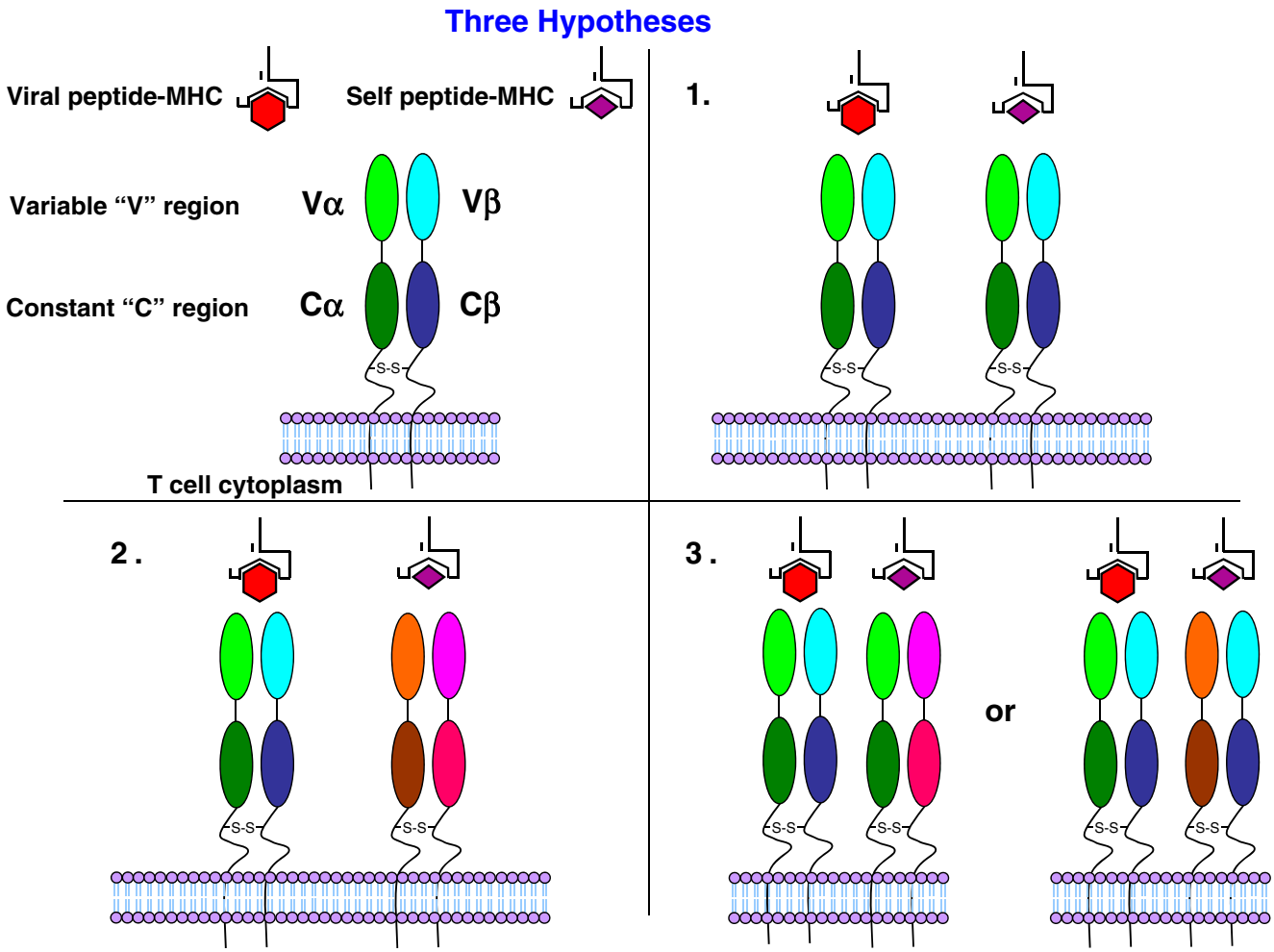

Fig. 8 Model for dual TCR reactivity. Three possible mechanisms exist for dual reactivity of the TCR in autoimmune disease: (*1*) Molecular mimicry, where one TCR recognizes virus and self; (*2*) dual TCRs, where one TCR is specific for virus and the other TCR is specific for self; and (*3*) a chimeric TCR, where different Vα and Vβ combinations on a single T cell are responsible for recognizing both virus and self

of the same 8a-1Ae cells (Fig. 7). Appropriate isotype control antibodies did not detect any nonspecific staining (data not shown).

Discussion

Epidemiological and molecular evidence implicates infectious agents (viral and bacterial) as the principal environmental insults responsible for the induction of autoimmune diseases [reviewed in (Ascherio and Munger 2007; Fujinami 2001; Libbey and Fujinami 2010)]. A variety of mechanisms have been suggested as the means by which infections can initiate and/or exacerbate autoimmune diseases [reviewed in (Cusick et al. 2011)]. One mechanism is molecular mimicry, where a foreign antigen shares sequence or structural similarities with self-antigens. Molecular mimicry has typically been characterized on an antibody or T cell level. However, structural relatedness between pathogen and self does not account for T cell activation in a number of autoimmune diseases. A proposed mechanism that could have been misinterpreted for molecular mimicry is the expression of dual TCRs on a single T cell. These T cells have dual reactivity to both foreign and self-antigens leaving the host vulnerable to foreign insults capable of triggering an autoimmune response.

Previously, we demonstrated that dual-reactive (viral and self) $CD8^+$ T cells were produced following a natural viral infection (Tsunoda et al. 2002, 2005, 2006, 2008). Here, we demonstrate that some (8a-1Ae) but not others (8a-1A1 and 8a-1A3) of these dual-reactive (viral and self) $CD8^+$ T cells could induce disease in naive mice. The TCR Vβ and Vα repertoires were than examined as a means of exploring a potential mechanism for how some of these dual-reactive (viral and self) $CD8^+$ T cells could induce disease. We found that these cells had more than one TCR (Vβ3Vα? and Vβ6Vα?) on their surface. The presence of dual TCRs made up of different combinations of Vβ3, Vβ6, and Vαs could explain how some dual-reactive (viral and self) $CD8^+$ T cells can induce disease while others cannot.

The concept that $CD8^+$ CTLs can express dual TCRs which play a role in the development of CNS autoimmune disease is relatively new (Ji et al. 2010). In this recent study, $CD8^+$ CTLs isolated from TCR-transgenic (Vβ8 Vα8) mice were shown to express, by flow cytometry, both Vβ8 proteins, specific for self, and Vβ6 proteins, specific for virus, and to express Vα8, Vα11, Vα13, and Vα14 mRNAs as determined by real-time PCR. The functionality of these Vα mRNAs was not determined and the specific pairing of Vα and Vβ chains on the T cell surface was not determined (Ji et al. 2010). In comparison, the $CD8^+$ CTLs harboring the dual TCRs in our studies occurred following viral infection of wild-type mice (Tsunoda et al. 2002, 2005, 2006, 2008) and were not generated artificially using transgenes. The mouse is the natural host for TMEV, which is the virus used in our studies. In contrast to the previous study in which a self-specific $CD8^+$ CTL was found to also express a viral-specific TCR (Ji et al. 2010), in our study, a viral-specific $CD8^+$ CTL was found to also react with self (Tsunoda et al. 2002, 2005, 2006, 2008). The identification of the viral and self epitopes recognized by the dual-reactive $CD8^+$ CTLs would increase our knowledge concerning the development of autoimmune disease. These studies are ongoing.

The role of the dual TCR in autoimmune disease could be through the activation of the T cell by a foreign antigen recognized by the first TCR which makes the cell competent to use the second TCR to attack self (Padovan et al. 1993). However, three possible mechanisms, including the above, exist for dual reactivity of the TCR in autoimmune disease: (1) molecular mimicry, where one TCR recognizes virus and self; (2) dual TCRs, where one TCR is specific for virus and the other TCR is specific for self; or (3) a chimeric TCR, where different Vα and Vβ combinations on a single T cell are responsible for recognizing virus and self (Fig. 8). The detection, via flow cytometry, of both Vβ6 and Vβ8 on the surface of a single T cell in the TCR-transgenic system (Ji et al. 2010) suggests that the dual reactivity occurs by either mechanism 2 dual TCRs or mechanism 3 chimeric TCR and not mechanism 1 molecular mimicry. Likewise, our ability to detect, via flow cytometry, both Vβ3 and Vβ6 proteins on the surface of a single T cell (Fig. 7) suggests that the dual reactivity in our system occurs by either mechanism 2 dual TCRs or mechanism 3 chimeric TCR and not likely mechanism 1 molecular mimicry. Studies are currently ongoing to determine the pairings of Vαs with Vβs on the surface of our hybridomas as a means of differentiating between mechanism 2 and mechanism 3.

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