

Short communication

Contrasting roles for V α 14⁺ natural killer T cells in a viral model for multiple sclerosis

Ikuo Tsunoda,^{1*} Tomoko Tanaka,¹ Masaru Taniguchi,² and Robert S. Fujinami¹

¹Department of Pathology, Division of Cell Biology and Immunology, University of Utah School of Medicine, 30 North 1900 East, Salt Lake City, Utah, USA; and ²Laboratory for Immune Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa, Japan

Most natural killer (NK) T cells express an invariant V α 14 T-cell receptor. To explore the contribution of NKT cells in an animal model for multiple sclerosis, Theiler's murine encephalomyelitis virus (TMEV) infection, TMEV-infected mice were treated with V α 14 antibody. Treatment during the early stage of infection delayed the onset of demyelinating disease with higher interleukin-4 production, whereas administration during the late stage or weekly resulted in more severe demyelination with enhanced virus persistence. The effect of *in vivo* depletion of NKT cells differed depending on the stage of infection, suggesting contrasting roles for NKT cells over the disease course. *Journal of NeuroVirology* (2009) 15, 90–98.

Keywords: CD1d; CNS autoimmune demyelinating diseases; cytokines; iNKT lymphocytes; *Picornaviridae* infections; Theiler's virus

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). Although the etiology of MS remains unclear, MS is considered to be an immune-mediated disease; CD4⁺ and CD8⁺ T cells and B cells have been shown to play potentially pathogenic as well as protective roles in MS (reviewed in Rose *et al*, 2004). MS has been suggested to develop in genetically susceptible individuals and requires environmental triggers, such as virus infection. Epidemiological studies and experimental models have suggested a role for viruses in MS (reviewed in Greenlee and Rose, 2000).

Natural killer (NK) T cells have both NK cell markers, such as CD161 (CD161c is known as NK1.1

in mice), and T-cell receptors (TCR) (Taniguchi *et al*, 1996). Most NKT cells express an invariant TCR encoded by V α 14 in combination with V β 2, V β 7, or V β 8 in mice and V α 24 in combination with V β 11 in humans (Makino *et al*, 1995). V α 14 TCR is expressed on murine NKT cells, but not on NK cells or conventional T cells (Apostolou *et al*, 1999; Dang *et al*, 2000; Schofield *et al*, 1999; Sharif *et al*, 2002; Makino *et al*, 1995). NKT cells recognize glycolipid antigens presented with CD1d (Kawano *et al*, 1997). NKT cells can produce large amounts of cytokines, particularly interleukin (IL)-4 and/or interferon (IFN)- γ , leading to modulation of a variety of immune cells; NKT cells can play an important role in bridging innate and acquired immunity (Yoshimoto *et al*, 1995; Taniguchi *et al*, 2003; Yamamura *et al*, 2007). NKT cells have been implicated in a variety of immune responses such as tumor immunity (Cui *et al*, 1997; Kawano *et al*, 1998) and immune responses against a variety of microbial pathogens, including viruses, in both humans and mice (Grubor-Bauk *et al*, 2003; Levy *et al*, 2003; Rigaud *et al*, 2006; Van Kaer, 2007).

It has been proposed that NKT cells play a protective role in the development of several immune-mediated diseases, such as lupus and diabetes, in humans and other animals (Miyake and Yamamura, 2005; Van Kaer, 2007; van der Vliet *et al*, 2001). In MS, several studies have suggested a

Address correspondence to Ikuo Tsunoda, MD, PhD, Department of Pathology, Division of Cell Biology and Immunology, University of Utah School of Medicine, 30 North 1900 East, MREB, Room 218, Salt Lake City, UT 84132, USA. E-mail: ikuo.tsunoda@hsc.utah.edu

The authors thank Jane E. Libbey, MS, and Takahisa Masaki, PhD, for helpful discussions, along with Nikki J. Kirkman, BS, Andy K. Luu, and Faris Hasanovic for excellent technical assistance. The authors are grateful to Ms. Kathleen Borick for preparation of the manuscript. This work was supported by the National Institutes of Health (R21NS059724, NS034497 and AI581501).

Received 22 April 2008; revised 25 July 2008; accepted 6 August 2008.

beneficial role for NKT cells in pathogenesis, although it may be difficult to compare the studies due to the differences in the proportion of NKT cells in normal individuals among the studies. Some investigators have reported that V α 24⁺ NKT cells were rare in plaque lesions and reduced in the peripheral blood (Illés *et al*, 2000; van der Vliet *et al*, 2001). However, others found no difference in the frequency of CD4⁻CD8⁻V α 24⁺ NKT cells between MS and control subjects, whereas frequencies of IL-4 secreting NKT cell clones were lower in relapsing-remitting MS than in progressive MS and controls (Gausling *et al*, 2001). The CD1d-restricted NKT cells expanded from MS patients in remission produced a larger amount of IL-4 than those from patients with active MS and control individuals (Araki *et al*, 2003). Similarly, in experimental allergic (autoimmune) encephalomyelitis (EAE), an autoimmune model for MS, NKT cells have been shown to play a protective role against demyelination, whereas others reported no role or a pathogenic role for NKT cells (Furlan *et al*, 2003; Jahng *et al*, 2001; Mars *et al*, 2002; Miyamoto *et al*, 2001; Singh *et al*, 2001; Teige *et al*, 2004). The discrepancies could be due to differences in mouse strains, encephalitogen, and methods of depletion/stimulation of NKT cells used in the studies.

Theiler's murine encephalomyelitis virus (TMEV) belongs to the family *Picornaviridae*, and the Theiler's original (TO) subgroup viruses include the Daniels (DA) and BeAn strains. Intracerebral injection of susceptible mice with DA virus leads to a persistent infection of the white matter in the spinal cord of susceptible mice. This leads to the development of inflammatory demyelinating lesions (Tsunoda and Fujinami, 1999), reminiscent of those observed in MS (Pirko *et al*, 2007). Although precise mechanisms of demyelination are unclear, direct virus infection of myelin-forming cells, oligodendrocytes, as well as immune-mediated pathomechanisms, including CD4⁺ and CD8⁺ T cells and demyelinating antibody, have been demonstrated to play important roles.

NKT cells have been shown to play a protective role in virus infection, including picornaviruses (Exley *et al*, 2001; Potvin *et al*, 2003). However, NKT cells could play a regulatory or pathogenic role in autoimmunity through the production of immunoregulatory cytokines (Yamamura *et al*, 2007). Thus, in TMEV infection, NKT cells could not only contribute to viral clearance but also modulate the immune-mediated demyelinating disease. It has been reported that administration of monoclonal antibody against V α 14 TCR, CMS-5 (Ito *et al*, 1991), to MRL *lpr/lpr* mice leads to a decrease in the number of V α 14⁺ NKT cells and acceleration of autoimmune disease (Mieza *et al*, 1996). Here, we examined clinically, histologically and immunologically whether NKT cells could modulate TMEV-

induced demyelinating disease, using the same depletion protocol and V α 14 antibody CMS-5.

Early V α 14 antibody treatment delays the onset of chronic disease

Infection of SJL/J mice with the DA strain of TMEV causes a biphasic disease. During the acute stage, 1 week post infection (p.i.), TMEV predominantly infects neurons in the gray matter of the brain. Infected mice generally do not develop paralysis of the limbs and had only mild weight loss and alterations in the righting reflex. Infected mice survive and develop an inflammatory demyelinating disease in the white matter of the spinal cord (chronic stage). During the chronic stage, TMEV persists in macrophages and glial cells but not in neurons. The onset of the chronic stage is around 3 to 4 weeks p.i., and after this time point, infected mice develop a spastic paralysis of the hind limbs due to inflammatory demyelinating changes in the spinal cord. Effects of immunomodulation therapies, such as T-cell depletion or cytokine antibody administration, have been shown to differ depending on the timing of treatment in TMEV infection (Tsunoda and Fujinami, 1999). Thus, we decided to administer V α 14 antibody: (1) before and at the time of infection (the early treatment group); (2) at 3 and 4 weeks p.i. (the late treatment group); or (3) weekly (the weekly treatment group) in TMEV-infected mice.

Purified V α 14 antibody (1 mg/ml) from the supernatant fluid of CMS-5 cell culture (previously described as CMS-1) (Ito *et al*, 1991) was used for the injection of female SJL/J mice (Jackson Laboratory, Bar Harbor, ME), as described previously (Mieza *et al*, 1996). NK and NKT cells in SJL/J mice have been shown to be undetectable or very few (Kaminsky *et al*, 1983, 1985; Kaminsky *et al*.); we have confirmed that NK1.1⁺CD3⁺ NKT cells were undetectable by flow cytometry in peripheral mononuclear cells (MNCs) in SJL/J mice (<0.5%). This made it difficult to assess the efficacy of NKT cell depletion by V α 14 antibody treatment in SJL/J mice. Thus, we took advantage of using A.SW mice whose NKT cells are inducible and their *H-2* haplotype (*H-2^s*) is the same as SJL/J mice (Kaminsky *et al*, 1983, 1985; Kaminsky *et al*.; Tsunoda *et al*, 2000). Weekly V α 14 antibody treatment of A.SW mice resulted in depletion of 96.3% of NK1.1⁺CD3⁺ NKT cells, whereas the treatment did not deplete NK1.1⁺CD3⁻ NK cells. Groups of mice were injected intraperitoneally with 100 μ g of V α 14 antibody: (1) on weeks -2, -1, 0, and 1 p.i. (four injections before and during the early stage of infection); (2) on 3 and 4 weeks p.i. (two injections during the late stage of infection); or (3) weekly (seven injections). Control mice received purified

mouse immunoglobulin (Ig) weekly, or no injections. All mice were infected intracerebrally with 2×10^5 plaque-forming units (PFU) of the DA strain of TMEV at 7 weeks of age. Clinical signs of disease were evaluated by means of an impaired righting reflex (Tsunoda *et al*, 2001).

During the first 3 weeks p.i., all groups of mice had mild impairment of the righting reflex, and there were no significant differences in clinical signs among the groups (Figure 1). Beginning around 3 and 4 weeks p.i., impairment of the righting reflex, i.e., the onset of demyelinating disease, was seen in control infected mice and groups of mice receiving V α 14 antibody weekly or during the late stage of infection. Interestingly, the onset of the clinical signs was delayed about 1 week in the group receiving V α 14 antibody during the early stage. Mice treated during the early stage had significantly lower righting reflex scores than mice treated during the late stage or weekly or control mice ($P < .05$: day 24, early versus weekly groups; day 27, early versus late or weekly groups; day 28, early versus weekly or control groups). However, at 5 weeks p.i., all groups of mice, including the ones treated during the early stage, had similar levels of impairment of the righting reflex.

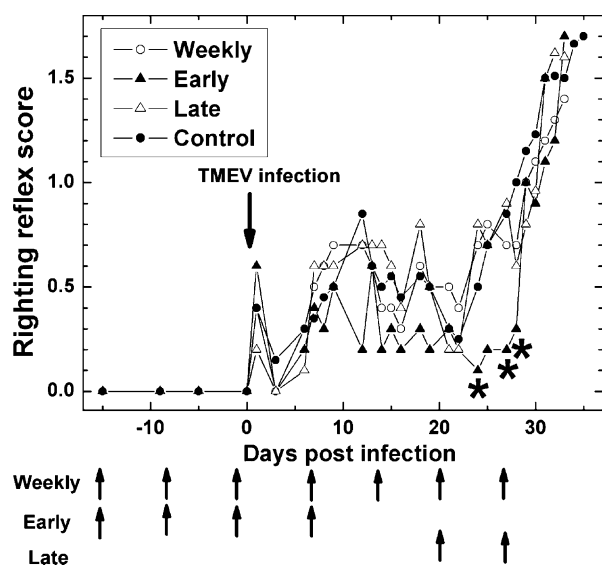


Figure 1 Modulation of clinical disease in TMEV-infected mice by administration of V α 14 antibody. TMEV was injected on day 0. TMEV-infected mice received V α 14 antibody: weekly (○); on weeks, -2, -1, 0, and 1 (early; π); or on weeks 3 and 4 post infection (late; \blacktriangle). Control mice received mouse immunoglobulin (Ig) or no injection (\bullet). Impaired righting reflex scores were compared between the groups. When the proximal end of the mouse's tail is grasped and twisted to the right and then to the left, a healthy mouse resists being turned over (score of 0). If the mouse is flipped onto its back but immediately rights itself on one side or both sides, it is given a score of 1 or 1.5, respectively. If it rights itself in 1 to 5 seconds, the score is 2. If righting takes more than 5 s, the score is 3. Three to four weeks after infection, mice treated during the early stage had significantly lower righting reflex scores, comparing with the other groups of mice ($*P < .05$). Shown are mean righting reflex scores of a group of 5 to 10 mice.

Late or weekly V α 14 antibody administration alters neuropathology and virus persistence in TMEV infection

We compared the neuropathology 5 weeks after TMEV infection, in mice that received control antibody or V α 14 antibody. Mice were perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). Brains were coronally divided into five slabs and spinal cords were transversely divided into 12 slabs, which were embedded in paraffin. Four micrometer thick sections were stained with Luxol fast blue for myelin visualization. Histological scoring was performed as previously described (Tsunoda *et al*, 2001). Viral antigen-positive cells were visualized by immunohistochemistry with TMEV antiserum using the avidin-biotin-peroxidase complex (ABC) technique (Vector, Burlingame, CA) with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) as chromogen (Tsunoda *et al*, 2007a). Enumeration of viral antigen-positive cells per quadrant in the spinal cord was carried out as described previously (Tsunoda *et al*, 2007b). The number of TMEV antigen-positive cells in the CNS has been shown to correlate with CNS virus titers measured by a plaque assay (McCright *et al*, 1999).

In the spinal cord, control infected mice receiving no antibody had inflammatory demyelinating lesions in the white matter, particularly in the ventral funiculus and the ventral root exit zone (Figure 2c). Using consecutive sections immunostained against viral antigen, we found small numbers of viral antigen-positive cells in the demyelinating lesions (Figure 2d). The mice receiving V α 14 antibody weekly or during the late stage had more severe inflammatory demyelination (Figure 2a) with more viral antigen-positive cells in the spinal cord (Figure 2b), compared with control mice. Spinal cord pathology scores and numbers of viral antigen-positive cells in mice treated weekly or during the late stage were significantly higher than those of control mice (Figure 2e, f). No significant difference was seen in neuropathology between control mice versus mice treated during the early stage. In contrast to the spinal cord, in the brain all groups of mice only had a mild degree of inflammation without accompanying demyelination. There was no difference in pathology scores or numbers of viral antigen-positive cells in the brain among groups ($P > .05$, analysis of variance [ANOVA], data not shown).

Effects of V α 14 antibody treatment on lymphoproliferative, antibody, and cytokine responses

We also compared cellular and humoral immune responses to TMEV, and monitored cytokine

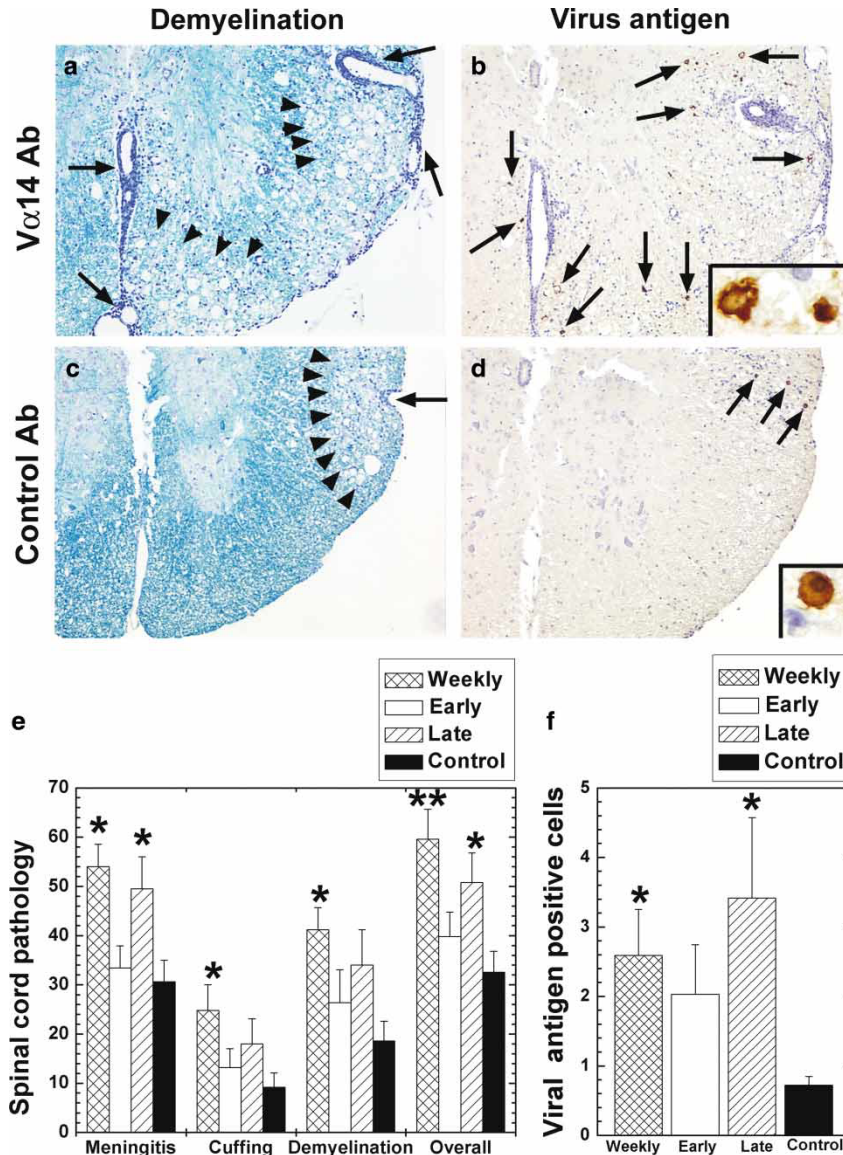


Figure 2 Spinal cord pathology 5 weeks after TMEV infection, in mice that received either control antibody or Vα14 antibody. (a–d) TMEV-infected mice were treated with Vα14 antibody (a, b) or control antibody (c, d) weekly. Mice receiving Vα14 antibody had more severe meningitis (arrow), perivascular cuffing and demyelination (arrow head) (a) with an increase in the number of virus antigen-positive cells (arrow) (b) compared with control mice (c, d). (a, c) Luxol fast blue stain. (b, d) Immunohistochemistry for TMEV antigen. Magnifications: a–d, 48, inset, ×410. (e) Spinal cord pathology scores. Mice receiving Vα14 antibody weekly had significantly higher pathology scores in all categories: meningitis, perivascular cuffing, demyelination, and overall pathology than control mice receiving mouse Ig (** $P < .01$; * $P < .05$), whereas mice treated during the late stage had higher meningitis and overall pathology scores than control mice. (f) Numbers of viral antigen-positive cells per quadrant of the spinal cord white matter. Higher numbers of viral antigen-positive cells were detected in mice receiving Vα14 antibody weekly or during the late stage of TMEV infection, compared with control mice receiving mouse Ig (* $P < .05$). (e, f) Values are mean + standard error of the mean (SEM) for five mice. For scoring of spinal cord sections, each spinal cord segment was divided into four quadrants: the ventral funiculus, the dorsal funiculus, and each lateral funiculus. Any quadrant containing meningitis, perivascular cuffing, or demyelination was given a score of 1 in that pathologic class. The total number of positive quadrants for each pathologic class was determined, divided by the total number of quadrants present on the slide and multiplied by 100 to give the percent involvement for each pathologic class. An overall pathologic score was also determined by giving a positive score if any pathology was present in the quadrant. This was also presented as a percent involvement.

production, at 5 weeks p.i., among infected mice treated with Vα14 antibody or with control antibody. Spleen MNCs were isolated with Histopaque-1083 (Sigma-Aldrich). A volume of 100 μl of 2×10^5 MNCs was incubated with a 100-μl solution containing either live TMEV at a multiplicity of

infection (MOI) of 5, 5 μg of purified ultraviolet-irradiated TMEV or 2×10^5 TMEV antigen-presenting cells (TMEV APCs). TMEV APCs were made from whole spleen cells infected *in vitro* with TMEV at an MOI of 1 and irradiated with 2000 rads (Tsunoda *et al*, 2007b). The MNCs were then

cultured for 5 days and TMEV-specific lymphoproliferative responses were measured using [^3H]thymidine. Regardless of the timing of V α 14 antibody administration, we found no significant differences in TMEV-specific lymphoproliferative responses. All groups showed substantial lymphoproliferation to TMEV, which was comparable to the control group (Figure 3a).

Using an enzyme-linked immunosorbent assay (ELISA), we titrated serum TMEV-specific antibodies among TMEV-infected mice treated with V α 14 antibody or control antibody, as described previously (Tsunoda *et al*, 2001). Ninety-six-well plates were coated overnight with TMEV antigen at 4°C. After blocking, two-fold dilutions of mouse sera beginning at 1:2⁷ were added to the plates and

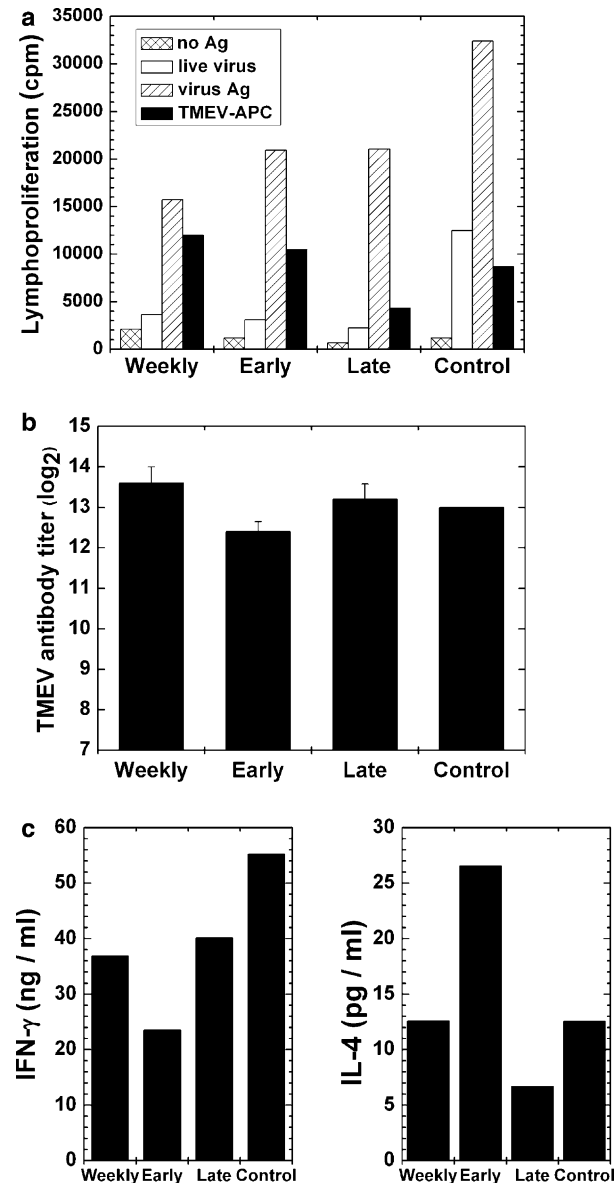


Figure 3

incubated for 90 min. After washing, the plates were incubated with a peroxidase-labeled goat anti-mouse antibody, were colorized with *o*-phenylenediamine dihydrochloride (Sigma-Aldrich), and were read at 492 nm. We found that all groups of mice had high titers of TMEV-specific antibody (Figure 3b). There were no significant differences in TMEV antibody titers among the groups ($P > .05$, ANOVA).

We measured the mitogen-induced production of IFN- γ versus IL-4 by spleen MNCs from TMEV-infected mice treated with V α 14 antibody, using the ELISA system, OptEIA Set (BD PharMingen, San Jose, CA), according to the manufacture's instruction (Tsunoda *et al*, 2005). MNCs were cultured at 2×10^6 cells/ml in six-well plates (Corning, Corning, NY) in the presence or absence of concanavalin A (5 $\mu\text{g}/\text{ml}$) or TMEV. Culture supernatant fluids were harvested 48 h after stimulation. In cultures stimulated with concanavalin A, we detected a large amount of IFN- γ in all groups, while IL-4 was low (Figure 3c). The group receiving V α 14 antibody during the early stage produced lower levels of IFN- γ but higher levels of IL-4, compared with the other groups. Cytokine production was not seen in cultures stimulated with TMEV or no stimulation (data not shown). Because V α 14⁺ NKT cells have been predicted to play a role during the early phase of several infectious diseases, the modulation of cytokine production in this group is consistent with this hypothesis.

Contrasting roles for NKT cells in SJL/J mice infected with TMEV

In the present study, we found that V α 14 antibody treatment during the late stage or weekly resulted in severe neuropathology with higher numbers of virus antigen-positive cells in the CNS versus other

Figure 3 Cellular and humoral immune responses at 5 weeks after TMEV infection, in mice that received V α 14 antibody weekly, during the early or late stage or control antibody. (a) Mononuclear cells (MNCs) were isolated from two to three spleens in each group and stimulated with no antigen (no Ag), live virus, UV-inactivated purified virus (virus Ag), or TMEV antigen-presenting cells (TMEV-APC) for 5 days. All groups of mice showed substantial lymphoproliferative responses to all stimuli. All cultures were performed in triplicate. Results are means of two independent experiments. (b) Serum anti-TMEV antibody titers were titrated from mice infected with TMEV using enzyme-linked immunosorbent assays (ELISAs). High antiviral antibody responses were detected in all groups of mice. There were no significant differences in antibody titers among groups. The end point of the assay was determined as the reciprocal of the highest dilution that gave an optical density reading that was two standard deviations above the control baseline from preimmune sera. Values are mean + SEM for five mice. (c) Interferon (IFN)- γ (left) and interleukin (IL)-4 (right) levels in spleen MNCs from TMEV-infected mice treated with V α 14 antibody. Cytokine concentrations of the supernatants from concanavalin A-stimulated MNCs were measured by ELISA. We detected lower IFN- γ and higher IL-4 production in the group receiving V α 14 antibody during the early time point, compared with the other groups. Each group was composed of five mice. ELISA was performed in duplicate, and shown are means of two independent experiments.

groups. This suggests a protective role for NKT cells during TMEV infection. In contrast, treatment of mice in the early stage of TMEV infection delayed the onset of clinical disease with an alteration of the Th1/Th2 profile. At 1 month p.i., although we did not observe differences in TMEV-specific lymphoproliferative responses or serum antibody titers among any groups treated with V α 14 antibody, V α 14 antibody treatment did modulate the clinical disease, the extent of virus persistence and pathology. Further studies, including time course analyses of virus replication and anti-viral immune responses, are required to understand why V α 14 antibody treatment differs depending on the timing of antibody administration. Currently, we hypothesize that disease modulation by V α 14 antibody could be due to alterations in the cytokine profile. The cytokine profile of NKT cells is likely to change during the course of TMEV infection leading to the differential effects of V α 14 antibody treatment. Indeed in MS, CD4⁺ NKT cell lines from MS patients in remission have been shown to produce a larger amount of IL-4 than those from MS patients in relapse (Araki *et al*, 2003).

Previously, it was reported that Th1/Th2 and proinflammatory/anti-inflammatory cytokines are important in virus clearance and demyelination in TMEV infection. The modulation of cytokines at different stages in TMEV infection leads to different outcomes (Kim *et al*, 2005). Here, cytokines have dual functions: a protective role in the early stage of infection and a pathogenic/regulatory role in inflammatory demyelinating disease in the late stage. Thus, the different outcomes of V α 14 antibody treatment could be due to (1) functional changes in the NKT cells during the course of TMEV infection, including cytokine production and cytotoxicity; and (2) different roles for the cytokines produced by NKT cells during the early versus the late stage of TMEV infection.

One concern in our current experiment could be that certain percentages of NKT cells have been shown to be V α 14 mRNA positive, but the cell surface expression of V α 14 TCR is negative in lymphoid organs, particularly in the thymus (Makino *et al*, 1995). This can be due to down-regulation of V α 14 TCR expression at a certain stage of differentiation in NKT cells. If this is the case, certain immature NKT cells would not be depleted by V α 14 antibody treatment; however, we do not know whether such a cell type is functional, or whether the cells become V α 14 TCR positive after maturation.

Another concern might be the fact that SJL/J mice have a low number of NKT cells and lack V β 8⁺NKT cells (Kaminsky *et al*, 1983; Kaminsky *et al*, 1985; Habu *et al*, 2007). The low NKT cell number in SJL/J mice was associated with defective early IL-4

responses to CD3 antibody challenge (Yoshimoto *et al*, 1995). However, despite the low number of NKT cells, *in vivo* and *in vitro* stimulation of NKT cells with a synthetic ligand of NKT cells, α -galactosylceramide (GalCer) (Kawano *et al*, 1997; Burdin *et al*, 1998), in SJL/J mice resulted in an increase of IL-4 and a decrease in IFN- γ (Serizawa *et al*, 2000; Singh *et al*, 2001). Stimulation of NKT cells in SJL mice has also been shown to modulate immune-mediated diseases including collagen-induced arthritis (Chiba *et al*, 2004) and pristine-induced lupus (Singh *et al*, 2005). When EAE was induced in SJL/J mice with myelin basic protein (Singh *et al*, 2001) or with myelin proteolipid protein peptide (PLP₁₃₉₋₁₅₁) (Miyake and Yamamura, 2005), α -GalCer treatment exacerbated the morbidity and mortality, while the onset of disease was delayed. Our current depletion studies of NKT cells by V α 14 antibody administration also support the presence of functional NKT cells *in vivo* in SJL/J mice. We are investigating whether the relative lack of NKT cells in SJL/J mice, compared with other strains of mice, can contribute to susceptibility to demyelinating disease in TMEV infection. This can be tested by adoptive transfer of NKT cells or by α -GalCer treatment (Berzofsky and Terabe, 2008) during the course of TMEV infection.

NKT cells can play different roles depending on the mouse strain. Singh *et al* (Singh *et al*, 2005) tested whether α -GalCer could modulate pristine-induced lupus in BALB/c and SJL/J mice. They found that NKT cells in SJL/J mice exhibited a Th1-biased cytokine profile while BALB/c mice had a mixed Th1/Th2 cytokine profile. They also found that repeated injections of α -GalCer were able to prevent pristine-induced lupus in BALB/c mice that was dependent on CD1d and IL-4 expression. In contrast, such a regimen augmented disease induced in SJL mice; suggesting that NKT cells can play both suppressive and pathogenic roles in the regulation of lupus in a strain-dependent manner.

In separate experiments, we have investigated the role of CD1d-restricted NKT cells in TMEV infection using CD1d-deficient mice (CD1^{-/-} mice) on a background of BALB/c mice, which are resistant to TMEV-induced demyelinating disease (Tsunoda *et al*, 2008). We found that only TMEV-infected CD1^{-/-} mice developed demyelination with neurologic deficits 3 to 5 weeks p.i., suggesting that CD1d-restricted NKT cells may play a protective role against demyelination around 3 to 5 weeks p.i. This is consistent with our current results in SJL/J mice, where mice depleted of NKT cells at 3 and 4 weeks p.i. had an enhancement of the demyelinating disease versus control mice.

Although CD1d-restricted cells may include not only V α 14⁺ NKT cells but also certain T cells and V α 14⁻ NKT cells (Amano *et al*, 1998; Baron *et al*,

2002; Huber *et al*, 2003), CD1d antibody treatment during the course of TMEV infection in SJL/J mice would give further insight into the role of NKT cells in TMEV-induced demyelination (Chiba *et al*, 2005). In addition, TMEV infection in CD1d- or J α 18-deficient mice with SJL/J background (currently not available) is an alternative method to see a role for NKT cells (Berzofsky and Terabe, 2008; Chiba *et al*, 2005; Cui *et al*, 1997), whereas it might not be the best way to see potentially contrasting roles for NKT cells, depending on the stage of TMEV infection.

Although the role of NKT cells has not been previously investigated in TMEV infection, the role of NK cells in TMEV infection was reported by Paya *et al* (Paya *et al.*, 1989). In their study, DA virus-infected resistant C57BL/10 mice were treated with NK1.1 antibody or asialo GM1 antibody throughout the course of infection. Both antibody modalities resulted in exacerbation of the acute gray matter disease without an increase in viral titers in the CNS, although only asialo GM1 treated mice developed demyelination. In addition, NK-defective beige/beige mice also showed exacerbation of gray matter disease without demyelination. The authors suggested that NK cells were important for defense against early neuronal damage in the gray matter and that depletion of NK1.1⁺ cells was not sufficient to induce demyelination in the white matter in a demyelination resistant mouse strain. However, these results may not be solely attributed to depletion of NK cells alone because (1) both NK1.1 and asialo GM1 are expressed on NK cells, NKT cells and some other cell types, including T cells (Byrne *et al*,

2004; Slifka *et al*, 2000; Trambley *et al*, 1999); and (2) although beige mice seem to have functional NKT cells, beige mice have other abnormalities in immune responses in addition to deficient NK cell function (Bannai *et al*, 2000; Cavarra *et al*, 1999; Nakagawa *et al*, 2001). In this context, changes in pathology by asialo GM1 and NK1.1 antibody treatments in Paya's study (Paya *et al*, 1989) could be attributed to depletion of not only NK cells but also NKT cells. If this is the case, NKT cells may play a protective role against demyelination during the late stage of TMEV infection in both resistant (Paya's study) and susceptible (current study) mouse strains, because we found exacerbation of demyelination in TMEV-infected susceptible SJL/J mice treated with V α 14 antibody weekly or during the late stage.

In summary, we found that the effect of V α 14 antibody injections in TMEV infection differed depending on the timing and numbers of the treatments. Treatment of mice during the early stage delayed the onset of TMEV-induced clinical signs that were associated with an alteration of the Th1/Th2 profile. In contrast, administration during the late stage or weekly resulted in enhanced neuropathology with higher levels of virus antigen-positive cells in the CNS. The timing of NKT cell modulation as well as cytokine profiles and numbers of NKT cells in hosts should be taken into account when planning therapeutic strategies for MS.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

- Amano M, Baumgarth N, Dick MD, Brossay L, Kronenberg M, Herzenberg LA, Strober S (1998). CD1 expression defines subsets of follicular and marginal zone B cells in the spleen: β_2 -microglobulin-dependent and independent forms. *J Immunol* **161**: 1710–1717.
- Apostolou I, Takahama Y, Belmont C, Kawano T, Huerre M, Marchal G, Cui J, Taniguchi M, Nakauchi H, Fournié J-J, Kourilsky P, Gachelin G (1999). Murine natural killer T(NKT) cells [correction of natural killer cells] contribute to the granulomatous reaction caused by mycobacterial cell walls. *Proc Natl Acad Sci U S A* **96**: 5141–5146.
- Araki M, Kondo T, Gumperz JE, Brenner MB, Miyake S, Yamamura T (2003). Th2 bias of CD4⁺ NKT cells derived from multiple sclerosis in remission. *Int Immunol* **15**: 279–288.
- Bannai M, Oya H, Kawamura T, Naito T, Shimizu T, Kawamura H, Miyaji C, Watanabe H, Hatakeyama K, Abo T (2000). Disparate effect of *beige* mutation on cytotoxic function between natural killer and natural killer T cells. *Immunology* **100**: 165–169.
- Baron JL, Gardiner L, Nishimura S, Shinkai K, Locksley R, Ganem D (2002). Activation of a nonclassical NKT cell subset in a transgenic mouse model of hepatitis B virus infection. *Immunity* **16**: 583–594.
- Berzofsky JA, Terabe M (2008). NKT cells in tumor immunity: Opposing subsets define a new immunoregulatory axis. *J Immunol* **180**: 3627–3635.
- Burdin N, Brossay L, Koezuka Y, Smiley ST, Grusby MJ, Gui M, Taniguchi M, Hayakawa K, Kronenberg M (1998). Selective ability of mouse CD1 to present glycolipids: α -galactosylceramide specifically stimulates V α 14⁺ NK T lymphocytes. *J Immunol* **161**: 3271–3281.
- Byrne P, McGuirk P, Todryk S, Mills KHG (2004). Depletion of NK cells results in disseminating lethal infection with *Bordetella pertussis* associated with a reduction of antigen-specific Th1 and enhancement of Th2, but not Tr1 cells. *Eur J Immunol* **34**: 2579–2588.
- Cavarra E, Martorana PA, de Santi M, Bartalesi B, Cortese S, Gambelli F, Lungarella G (1999). Neutrophil influx into the lungs of beige mice is followed by elastolytic damage and emphysema. *Am J Respir Cell Mol Biol* **20**: 264–269.
- Chiba A, Kaieda S, Oki S, Yamamura T, Miyake S (2005). The involvement of V α 14 natural killer T cells in the pathogenesis of arthritis in murine models. *Arthritis Rheum* **52**: 1941–1948.

- Chiba A, Oki S, Miyamoto K, Hashimoto H, Yamamura T, Miyake S (2004). Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of α -galactosylceramide. *Arthritis Rheum* **50**: 305–313.
- Cui J, Shin T, Kawano T, Sato H, Kondo E, Toura I, Kaneko Y, Koseki H, Kanno M, Taniguchi M (1997). Requirement for $V_{\alpha}14$ NKT cells in IL-12-mediated rejection of tumors. *Science* **278**: 1623–1626.
- Dang Y, Beckers J, Wang C-R, Heyborne KD (2000). Natural killer $1.1^{+} \alpha\beta$ T cells in the periimplantation uterus. *Immunology* **101**: 484–491.
- Exley MA, Bigley NJ, Cheng O, Tahir SMA, Smiley ST, Carter QL, Stills HF, Grusby MJ, Koezuka Y, Taniguchi M, Balk SP (2001). CD1d-reactive T-cell activation leads to amelioration of disease caused by diabetogenic encephalomyocarditis virus. (Erratum in *J Leukoc Biol* 2001, 70; 340). *J Leukoc Biol* **69**: 713–718.
- Furlan R, Bergami A, Cantarella D, Brambilla E, Taniguchi M, Dellabona P, Casorati G, Martino G (2003). Activation of invariant NKT cells by α GalCer administration protects mice from MOG35-55-induced EAE: critical roles for administration route and IFN- γ . *Eur J Immunol* **33**: 1830–1838.
- Gausling R, Trollmo C, Hafler DA (2001). Decreases in interleukin-4 secretion by invariant $CD4^{-}CD8^{-}V_{\alpha}24J_{\alpha}Q$ T cells in peripheral blood of patients with relapsing-remitting multiple sclerosis. *Clin Immunol* **98**: 11–17.
- Greenlee JE, Rose JW (2000). Controversies in neurological infectious diseases. *Semin Neurol* **20**: 375–386.
- Grubor-Bauk B, Simmons A, Mayrhofer G, Speck PG (2003). Impaired clearance of herpes simplex virus type 1 from mice lacking CD1d or NKT cells expressing the semivariant $V_{\alpha}14$ - $J_{\alpha}281$ TCR. *J Immunol* **170**: 1430–1434.
- Habu Y, Shinomiya N, Kinoshita M, Matsumoto A, Kawabata T, Seki S (2007). Mice deficient in $V\beta 8^{+}$ NKT cells are resistant to experimental hepatitis but are partially susceptible to generalised Shwartzman reaction. *Clin Exp Med* **7**: 30–38.
- Huber S, Sartini D, Exley M (2003). Role of CD1d in coxsackievirus B3-induced myocarditis. *J Immunol* **170**: 3147–3153.
- Illés Z, Kondo T, Newcombe J, Oka N, Tabira T, Yamamura T (2000). Differential expression of NK T cell $V_{\alpha}24J_{\alpha}Q$ invariant TCR chain in the lesions of multiple sclerosis and chronic inflammatory demyelinating polyneuropathy. *J Immunol* **164**: 4375–4381.
- Ito T, Ishibashi K, Imai K, Koseki H, Ra C, Fernandez E, Kantake M, Saito T, Taniguchi M (1991). Monoclonal antibody against murine T cell receptor $V_{\alpha}14$ cross-reacts with human CD3 ϵ and detects disulfide-linked dimeric form. *Int Immunol* **3**: 991–995.
- Jahng AW, Maricic I, Pedersen B, Burdin N, Naidenko O, Kronenberg M, Koezuka Y, Kumar V (2001). Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* **194**: 1789–1799.
- Kaminsky SG, Nakamura I, Cudkovicz G (1983). Selective defect of natural killer and killer cell activity against lymphomas in SJL mice: Low responsiveness to interferon inducers. *J Immunol* **130**: 1980–1984.
- Kaminsky SG, Nakamura I, Cudkovicz G (1985). Genetic control of the natural killer cell activity in SJL and other strains of mice. *J Immunol* **135**: 665–671.
- Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M (1997). CD1d-restricted and TCR-mediated activation of $V_{\alpha}14$ NKT cells by glycosylceramides. *Science* **278**: 1626–1629.
- Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Sato H, Kondo E, Harada M, Koseki H, Nakayama T, Tanaka Y, Taniguchi M (1998). Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated $V_{\alpha}14$ NKT cells. *Proc Natl Acad Sci U S A* **95**: 5690–5693.
- Kim BS, Fuller AC, Koh C-S (2005). Cytokines, chemokines and adhesion molecules in TMEV-IDD. In: *Experimental models of multiple sclerosis*. Lavi E, Constantinescu CS (eds). New York: Springer Science + Business Media, pp 659–671.
- Levy O, Orange JS, Hibberd P, Steinberg S, LaRussa P, Weinberg A, Wilson SB, Shaulov A, Fleisher G, Geha RS, Bonilla FA, Exley M (2003). Disseminated varicella infection due to the vaccine strain of varicella-zoster virus, in a patient with a novel deficiency in natural killer T cells. *J Infect Dis* **188**: 948–953.
- Makino Y, Kanno R, Ito T, Higashino K, Taniguchi M (1995). Predominant expression of invariant $V_{\alpha}14^{+}$ TCR α chain in $NK1.1^{+}$ T cell populations. *Int Immunol* **7**: 1157–1161.
- Mars LT, Laloux V, Goude K, Desbois S, Saoudi A, Van Kaer L, Lassmann H, Herbelin A, Lehuen A, Liblau RS (2002). $V_{\alpha}14$ - $J_{\alpha}281$ NKT cells naturally regulate experimental autoimmune encephalomyelitis in nonobese diabetic mice. *J Immunol* **168**: 6007–6011.
- McCright IJ, Tsunoda I, Whitby FG, Fujinami RS (1999). Theiler's viruses with mutations in loop I of VP1 lead to altered tropism and pathogenesis. *J Virol* **73**: 2814–2824.
- Mieza MA, Itoh T, Cui JQ, Makino Y, Kawano T, Tsuchida K, Koike T, Shirai T, Yagita H, Matsuzawa A, Koseki H, Taniguchi M (1996). Selective reduction of $V_{\alpha}14^{+}$ NK T cells associated with disease development in autoimmune-prone mice. *J Immunol* **156**: 4035–4040.
- Miyake S, Yamamura T (2005). Therapeutic potential of glycolipid ligands for natural killer (NK) T cells in the suppression of autoimmune diseases. *Curr Drug Targets- Immune Endocr Metab Disord* **5**: 315–322.
- Miyamoto K, Miyake S, Yamamura T (2001). A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* **413**: 531–534.
- Nakagawa R, Nagafune I, Tazunoki Y, Ehara H, Tomura H, Iijima R, Motoki K, Kamishohara M, Seki S (2001). Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by α -galactosylceramide in mice. *J Immunol* **166**: 6578–6584.
- Paya CV, Patick AK, Leibson PJ, Rodriguez M (1989). Role of natural killer cells as immune effectors in encephalitis and demyelination induced by Theiler's virus. *J Immunol* **143**: 95–102.
- Pirko I, Lucchinetti CF, Sriram S, Bakshi R (2007). Gray matter involvement in multiple sclerosis. *Neurology* **68**: 634–642.
- Potvin DM, Metzger DW, Lee WT, Collins DN, Ramsingh AI (2003). Exogenous interleukin-12 protects against lethal infection with coxsackievirus B4. *J Virol* **77**: 8272–8279.
- Rigaud S, Fondanèche M-C, Lambert N, Pasquier B, Mateo V, Soulas P, Galicier L, Le Deist F, Rieux-Laucat F, Revy

- P, Fischer A, de Saint Basile G, Latour S (2006). XIAP deficiency in humans causes an X-linked lymphoproliferative syndrome. *Nature* **444**: 110–114.
- Rose JW, Watt HE, White AT, Carlson NG (2004). Treatment of multiple sclerosis with an anti-interleukin-2 receptor monoclonal antibody. *Ann Neurol* **56**: 864–867.
- Schofield L, McConville MJ, Hansen D, Campbell AS, Fraser-Reid B, Grusby MJ, Tachado SD (1999). CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. *Science* **283**: 225–229.
- Serizawa I, Koezuka Y, Amao H, Saito TR, Takahashi KW (2000). Functional natural killer T cells in experimental mouse strains, including NK1.1⁻ strains. *Exp Anim* **49**: 171–180.
- Sharif S, Arreaza GA, Zucker P, Mi Q-S, Delovitch TL (2002). Regulation of autoimmune disease by natural killer T cells. *J Mol Med* **80**: 290–300.
- Singh AK, Wilson MT, Hong S, Olivares-Villagómez D, Du C, Stanic AK, Joyce S, Sriram S, Koezuka Y, Van Kaer L (2001). Natural killer T cell activation protects mice against experimental autoimmune encephalomyelitis. *J Exp Med* **194**: 1801–1811.
- Singh AK, Yang J-Q, Parekh VV, Wei J, Wang C-R, Joyce S, Singh RR, Van Kaer L (2005). The natural killer T cell ligand α -galactosylceramide prevents or promotes pristane-induced lupus in mice. *Eur J Immunol* **35**: 1143–1154.
- Slifka MK, Pagarigan RR, Whitton JL (2000). NK markers are expressed on a high percentage of virus-specific CD8⁺ and CD4⁺ T cells. *J Immunol* **164**: 2009–2015.
- Taniguchi M, Makino Y, Cui J, Masuda K, Kawano T, Sato H, Kondo E, Koseki H (1996). V α 14⁺ NK T cells: A novel lymphoid cell lineage with regulatory function. *J Allergy Clin Immunol* **98**: S263–S269.
- Taniguchi M, Seino K, Nakayama T (2003). The NKT cell system: bridging innate and acquired immunity. *Nat Immunol* **4**: 1164–1165.
- Teige A, Teige I, Lavasani S, Bockermann R, Mondoc E, Holmdahl R, Issazadeh-Navikas S (2004). CD1-dependent regulation of chronic central nervous system inflammation in experimental autoimmune encephalomyelitis. *J Immunol* **172**: 186–194.
- Trambley J, Bingaman AW, Lin A, Elwood ET, Waitze S-Y, Ha J, Durham MM, Corbascio M, Cowan SR, Pearson TC, Larsen CP (1999). Asialo GM1⁺ CD8⁺ T cells play a critical role in costimulation blockade-resistant allograft rejection. *J Clin Invest* **104**: 1715–1722.
- Tsunoda I, Fujinami RS (1999). Theiler's murine encephalomyelitis virus. In *Persistent viral infections*. Ahmed R, Chen, ISY (eds). Chichester, West Sussex, England: John Wiley & Sons, pp. 517–536.
- Tsunoda I, Kuang L-Q, Theil DJ, Fujinami RS (2000). Antibody association with a novel model for primary progressive multiple sclerosis: Induction of relapsing-remitting and progressive forms of EAE in H2^s mouse strains. *Brain Pathol* **10**: 402–418.
- Tsunoda I, Libbey JE, Kuang L-Q, Terry EJ, Fujinami RS (2005). Massive apoptosis in lymphoid organs in animal models for primary and secondary progressive multiple sclerosis. *Am J Pathol* **167**: 1631–1646.
- Tsunoda I, Tanaka T, Saijoh Y, Fujinami RS (2007a). Targeting inflammatory demyelinating lesions to sites of Wallerian degeneration. *Am J Pathol* **171**: 1563–1575.
- Tsunoda I, Tanaka T, Terry EJ, Fujinami RS (2007b). Contrasting roles for axonal degeneration in an autoimmune versus viral model for multiple sclerosis: When can axonal injury be beneficial? *Am J Pathol* **170**: 214–226.
- Tsunoda I, Wada Y, Libbey JE, Cannon TS, Whitby FG, Fujinami RS (2001). Prolonged gray matter disease without demyelination caused by Theiler's murine encephalomyelitis virus with a mutation in VP2 puff B. *J Virol* **75**: 7494–7505.
- Tsunoda I, Tanaka T, Fujinami RS. (2008). Regulatory role of CD1d in neurotropic virus infection. *J Virol*, **82**: 10279–10289.
- van der Vliet HJJ, von Blomberg BME, Nishi N, Reijm M, Voskuyl AE, van Bodegraven AA, Polman CH, Rustemeyer T, Lips P, van den Eertwegh AJM, Giaccone G, Scheper RJ, Pinedo HM (2001). Circulating V α 24⁺ V β 11⁺ NKT cell numbers are decreased in a wide variety of diseases that are characterized by autoreactive tissue damage. *Clin Immunol* **100**: 144–148.
- Van Kaer L (2007). NKT cells: T lymphocytes with innate effector functions. *Curr Opin Immunol* **19**: 354–364.
- Yamamura T, Sakuishi K, Illés Z, Miyake S (2007). Understanding the behavior of invariant NKT cells in autoimmune diseases. *J Neuroimmunol* **191**: 8–15.
- Yoshimoto T, Bendelac A, Hu-Li J, Paul WE (1995). Defective IgE production by SJL mice is linked to the absence of CD4⁺, NK1.1⁺ T cells that promptly produce interleukin 4. *Proc Natl Acad Sci U S A* **92**: 11931–11934.