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Role of $\gamma\delta$ T cells in antibody production and recovery from SFV demyelinating disease

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Abstract

Semliki Forest Virus (SFV) encephalomyelitis has been used to study the pathogenesis of virus-induced demyelination and serves as a model for multiple sclerosis. SFV-infection of mice invariably leads to clinical weakness accompanied by CNS inflammation, viral clearance and primary demyelination by day 21 postinfection (pi), followed by recovery and remyelination by day 35 pi. We have applied this model to examination the effects of $\gamma\delta$ T cells in antibody production and the pathogenesis of demyelinating lesions. SFV-infection of $\gamma\delta$ T cell KO mice resulted in more severe clinical signs than in wild type (WT) B6 mice. SFV-infected WT and $\gamma\delta$ KO mice both cleared virus by day 10 pi and inflammation was comparable. Demyelination also appeared to be similar in both groups except that KO mice did not exhibit extensive remyelination which was seen in WT mice by day 21. SFV-infected WT mice showed widespread remyelination by day 35 pi, whereas KO mice still displayed some demyelination through day 42 pi. Both WT and KO mice developed serum antibodies to SFV. However, the reactivity of WT sera with the SFV epitope, E2 T_h peptide₂, was significantly higher than in KO sera. Immunization with E2 T_h peptide₂ resulted in elevated antibody production to this peptide ($p < 0.05$) and earlier remyelination (day 28 pi) in KO mice. Thus, our study has shown for the first time that immunization of SFV-infected $\gamma\delta$ T cell KO mice with a viral peptide, E2 T_h peptide₂, led to enhanced recovery and repair of the CNS.

Keywords

Semliki Forest virus; T cell receptor $\gamma\delta$; brain mononuclear cells; knock-out; wild-type; E2 T_h peptide₂; remyelination; antibody; immunization; inflammation

1-Introduction

Multiple sclerosis (MS) is a debilitating disease and the most common inflammatory demyelinating disease in the central nervous system (CNS) (Frohman, Racke and Raine, 2006). Viral infections have been implicated in the pathogenesis of this disease and several

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animal models have been described (Fazakerley et al., 1997). One model is CNS infection with the non-lethal A7 (74) strain of Semliki Forest virus (SFV). SFV causes paralysis in mice and leads to myelin damage and demyelination after clearance of virus, followed by extensive remyelination. The major surface protein of SFV which evokes antibody responses to this virus is E2 glycoprotein. Identification of linear epitopes of SFV E2 has shown that the T helper cell epitopes (E2 T_h peptides) of the virus elicits a strong antibody response (Boere et al, 1983). Studies have shown that viral clearance and demyelination are mediated by both CD8⁺ T cells (Subak-Sharpe et al., 1993), and an antibody response to SFV-infection (Smith-Norowitz et al., 2000). We have found that molecular mimicry plays a role in the development of primary demyelination, which occurs after viral clearance (Mokhtarian et al., 1999). The mechanisms involved in remyelination, however, have not been studied.

$\gamma\delta$ T cells comprise a minor component of the T cell population (up to 10%) in human peripheral blood (Lanier et al., 1987). In mice, $\gamma\delta$ T cells are prominently found in the skin and these cells are more abundant in intraepithelial layers of the small intestine than in spleen or lymph nodes—20–50% in the former versus 1–5% of the latter, of total T cells (Pardoll et al., 1987; Chien YH et al., 2007). $\gamma\delta$ T cells responded to lipid and non-protein as well as to protein antigens, including heat shock protein (hsp) expressed in damaged brain in MS (Selmaj et al., 1991).

A prominent role for human peripheral blood $\gamma\delta$ T cells in humoral immunity and B cell help for antibody production has been found (Pao et al., 1996; Zheng et al., 2003). Studies have suggested that $\gamma\delta$ T cells regulate the immune response after a variety of immune stimuli, including autoimmunity (Clark et al., 1998). It has also been found that $\gamma\delta$ T cells are involved in the immune response against viruses such as herpes simplex (Sciammas et al., 1994), Coxsackie B (Huber et al., 2001), vesicular stomatitis virus (Maloy et al., 1998), and mouse hepatitis virus (Dandekar et al., 2002). Some studies of $\gamma\delta$ T cells in viral infections (Carding et al., 1990; Hou et al., 1992; Selin et al., 2001; Wang et al., 2003) and autoimmunity (Kobayashi et al., 1997; Rajan et al, 2000, Blink et al 2009), have suggested that these cells may play a role in recovery from CNS damage. $\gamma\delta$ T cells were also shown to accumulate and respond preferentially to hsp expressed in damaged brain (Selmaj et al, 1991), and in the CSF of MS patients and although their function is not entirely understood, it is clear that these cells may have roles in regulating autoimmune inflammation in the CNS (Blink et al., 2009).

Here, we have studied the role of $\gamma\delta$ T cells in the pathogenesis of SFV-infection in $\gamma\delta$ KO and WT mice and in the production of antibody to viral epitopes and to non-protein components of the CNS during SFV-infection. Our results suggest that $\gamma\delta$ T cells are involved in the production of an anti SFV E2 peptide antibody and immunization of SFV-infected KO and WT mice with this peptide led to higher anti-E2 peptide antibody production and improved recovery of $\gamma\delta$ KO mice from SFV infection.

2-Materials and Methods

Infection of Mice

Female, 5 to 6-week-old C57BL/6 (WT) and B6.129P2-Tcrd^{tm1Mom} congenic mice with a disrupted TCR C δ region ($\gamma\delta$ KO) (Jackson Laboratory, Bar Harbor, ME), were used in all experiments. Notably, adult $\gamma\delta$ KO mice have been shown to lack TCR $\gamma\delta$ cells in the thymus, intestine and peripheral blood but maintain normal numbers of TCR $\alpha\beta$ cells (Itohara et al., 1993). All mice were inoculated ip with 1×10^7 Tissue Culture Infective Dose 50 (TCID₅₀) units (containing 1×10^4 PFU) of the avirulent SFV strain A7 (74), (Mokhtarian

and Swoveland, 1987). Mice were observed daily for clinical manifestations of disease and scored on scale of 0–6 as shown below.

0=no abnormality; 1=mild hind limb weakness (some difficulty righting when turned on their back); 2=moderate hind limb weakness, sometimes associated with floppy tail; 3=paresis of hind limbs accompanied by some forelimb weakness, sometimes more marked on one side, but not complete paralysis; 4=hind limb paralysis accompanied by moderate forelimb weakness; 5=paralysis of hind limbs, associated with forelimb paralysis, still able to move; and 6=quadriplegia, moribund. For all experiments, animals were randomly selected and anesthetized with Metofane (Schering-Plough Animal Health, Union, NJ), prior to sacrifice.

Infectivity assay: Tissue Culture Infective dose 50 (TCID₅₀)

To measure clearance of SFV virus from brains of infected animals, a TCID₅₀ assay was performed, as previously described (Mokhtarian et al., 1999; Mahy, 1985). Briefly, on days 0, 3, 5, 7, 10, 14 and 21 pi, brain tissue from WT and $\gamma\delta$ KO mice was homogenized and titrated in 96-well tissue culture plates (Costar, Cambridge, MA). Plates were incubated at 37°C, in 5% CO₂, for 36 to 48 hours. Stock SFV A7 (74) virus was used as a positive control and culture medium was used as a negative control. Finally, viral titers were calculated as the lowest dilution of the virus that caused cytopathic effect (CPE) in 50% of the cultured wells and was expressed as $-\log_{10}$ TCID₅₀ units in 0.1 ml.

Immunohistochemistry of viral antigens

Presence of residual viral antigens was tested in paraffin sections (10 μ m) from brains of SFV infected WT, KO, and immunized WT and KO mice. Sections were prepared for immunohistochemistry and DAB labeling, with some modification (Soilu-Hänninen et al, 1994, Mokhtarian et al, 2003). After deparaffinization, intrinsic peroxidase activity was blocked by incubation with 5% H₂O₂ in phosphate-buffered saline (PBS) for 10 minutes. Briefly, the sections were further blocked with 5% goat serum for one hour and incubated with mouse serum containing anti-SFV antibody overnight at 4°C. Biotinylated anti-mouse secondary antibody (1:200), ABC and DAB were each applied for one hour consecutively. Slides were mounted with Vectashield media (Vector, Burlingame, CA) and visualized by light microscope.

Isolation of mononuclear cells and flow cytometry

Brain mononuclear cells (BMNC) were purified from the brains of KO and WT SFV-infected and noninfected mice on designated days, by digestion and Ficoll-Paque gradient centrifugation, as previously described (Irani and Griffin, 1991). Immunofluorescent staining and flow cytometric analysis was performed, as previously described (Mokhtarian et al, 2003). FACS analysis of TCR $\gamma\delta^+$ cells in SFV-infected WT and $\gamma\delta$ KO mice confirmed scarcity of CD3⁺TCR $\gamma\delta^+$ T cells in KO mice.

Anti-Semliki Forest Virus (anti-SFV) IgG antibodies and normal IgG

The total IgG fraction was commercially prepared (Strategic Biosolutions, Ramona, CA) from pooled sera of 3 rabbits immunized once with UV-inactivated SFV A7 (74) strain emulsified in CFA and boosted 3 times with antigen in IFA at weeks 3, 5, and 7. The IgG fraction of the pooled antisera was purified by ion-exchange chromatography and contained 8.7 mg IgG/ml of PBS. Antibody activities to SFV and peptides of SFV, to E2, and to recombinant myelin oligodendrocyte glycoprotein (rMOG) and its peptides were confirmed by ELISA. Normal rabbit IgG, isolated from pooled normal rabbit sera by ion-exchange chromatography (Sigma Chemical Co., St. Louis, MO), was dissolved in saline. Protein

concentration was determined by the Bradford assay (Sigma), using normal rabbit IgG as the standard. The IgG solutions were diluted to 3 mg protein/ml in saline and sterilized by passage through a 0.22 μ m filter (Millipore Corp., Bedford, MA).

Synthesis of peptides

The SFV epitopes, E2 T_h peptide₁ and E2 T_h peptide₂ were synthesized (Dept. of Biophysics, JHU, School of Hygiene and Public health, Baltimore, MD), as previously described (Mokhtarian et al., 1994) and used to determine antibody responses in different groups of mice (Smith-Norowitz et al, 2000). UV-irradiated SFV A7 (74) virus was used as a positive control, and Purified Protein Derivative (PPD) (Connaught, Swiftwater, PA), was used as a negative control.

Histopathology of SFV-infected mice

On days 0, 7, 14, 21, and 35 pi, WT and $\gamma\delta$ KO mice, were sampled and perfused by intracardiac puncture with Trump's fixative, containing 4% paraformaldehyde and 1% glutaraldehyde. Brains were removed and postfixed in Trump's fixative. Alternating sections from each brain were stained with hematoxylin and eosin (H&E) to assess inflammation and demyelination (Smith-Norowitz et al., 2000).

Brain Pathology Scoring of SFV-infected mice

Each brain was sectioned coronally into three pieces through the infundibulum and optic chiasm. The pieces were then dehydrated and embedded in paraffin. Sections from each block were mounted on slides and stained with H&E to identify pathology in the following brain regions: cortex, corpus callosum, hippocampus, brainstem, striatum and cerebellum. Pathologic scores were assigned under blinded conditions. Slides stained with H&E were quantified in a blinded fashion for numbers of inflammatory foci (each aggregate containing ≥ 10 mononuclear cells) for the entire brain (Smith-Norowitz et al, 2000).

Preparation of thin sections

To detect de- and remyelination, CNS tissues were obtained for histopathology from three WT and one to two KO mice on 0, 7, 14, 21, 28, 35 and 42 days pi. For this, mice were anesthetized with ether and perfused through the heart with cold Trump's fixative. Optic nerve, brain, spinal cord and spinal nerve roots were dissected. Slices were taken across the cerebral hemispheres through the basal ganglia/hippocampus and through the cerebellum/IVth ventricle/brainstem. Samples were post-fixed in cold 1% osmic acid and embedded in Epon. One micron epoxy sections were cut from all levels, stained with 1% toluidine blue and read by observers blinded to the code (Raine et al., 1984).

Enzyme-Linked Immunosorbent Assay (ELISA) for antibody response to SFV and peptides

To measure antibody response in both WT and $\gamma\delta$ KO mice against SFV and two of the peptides of the SFV envelope protein, E2, an indirect ELISA was performed. In each experiment, sera were collected from 2–3 SFV-infected WT and 1–2 KO mice on days 0, 7, 14, 21 and 35 pi. Briefly, Immunolon 2HB 96 well microtiter plates (VWR, Bridge Port, NJ), were coated with 1 μ g/well of each antigen in 100 μ l/well carbonate buffer overnight at room temperature. After washing, plates were blocked and serum samples added. Initial experiments with serial 2-fold dilutions (1:20 through 1:160) of sera against SFV and the two T_h E2 peptides, showed the optimal dilution of the sera to be 1:80. After washing, biotinylated goat anti-mouse Igs (BD Pharmingen, Torreyana, CA), Streptavidin-conjugated to horse radish peroxidase (Vector Laboratories, Burlingame, CA), and OPD substrate (Sigma, St. Louis, MO), were used. The absorbance at 450 nm was measured using a Vmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA), and results expressed as

optical density units [OD]. Values were considered positive when they were > 0.3 OD units and/or exceeded the mean value plus three standard deviations of antibody response on day 0 pi of mice of the same strain (Mokhtarian et al, 1999).

E2 T_h peptide₂ immunization during SFV disease

Five-week old C57Bl6 (B6) WT and KO mice (Jackson Laboratories, Bar Harbor, MA) were all infected with SFV and divided into: unimmunized and immunized WT, and unimmunized and immunized KO groups. Immunizations were performed subcutaneously with E2 T_h peptide₂ (0.5 mg/injection or 25 mg/kg, based on the average weight of 20 g/mouse) in IFA. Inoculations of E2 T_h peptide₂/IFA were carried out on days 5, 15, and 20 after SFV-infection. Due to the unavailability of a large number of $\gamma\delta$ KO mice to purchase at one time point, two separate experiments were done. Total numbers of unimmunized and immunized WT mice were 14 and 11 and total numbers of unimmunized and immunized KO mice were 13 and 9, respectively.

Statistics

Histopathology, clinical results and SFV epitope antibody responses (ELISA) were analyzed using a two-tailed Student's *t*-test, by InStat v.2.01. KS statistics were performed for flow cytometry data on Cell Quest v.3.5.

3- Results

More severe clinical disease in SFV-infected $\gamma\delta$ KO than in WT

SFV-infection of B6 and $\gamma\delta$ KO mice produced three types of clinical outcomes consisting of weakness, ruffled fur, and weight loss, from which the mice recovered or progressed to permanent paralysis or death (Smith-Norowitz et al., 2000). Mice were observed daily for clinical manifestations of SFV and were scored on a scale of 0–6 as described in Materials and Methods.

During SFV-infection, B6 mice usually showed acute signs of disease on days 6–8 pi, and began to recover thereafter. It should be noted that grades 1–3 were weakness primarily due to systemic viral effects from which mice recover, and grades 4–6 were permanent and led to fatal paralysis due to CNS immunopathology. In different experiments, only about 10–20% of SFV infected WT mice showed visible clinical signs of grade 3 or higher. The remainder exhibited grades 1 and 2 or were asymptomatic. All mice, including the latter groups, however, showed inflammation and demyelination in the CNS after histological examination, as previously reported (Mokhtarian et al, 1994; 1999; 2003). Consequently, the average clinical score on different days is low.

In three separate experiments using a total of 54 WT and 34 KO mice, the KO group showed a significantly higher average clinical score on day 7 pi, $p < 0.005$ (Figure 1). Mice in both groups recovered (either partially or completely) following day 7. However, the clinical scores of WT and KO mice remained significantly different on subsequent days pi ($p < 0.01$ on day 14, $p < 0.05$ on day 21 and $p < 0.04$ on day 35 pi). Thus, $\gamma\delta$ KO mice showed more severe clinical disease than WT mice at all times.

Significantly lower antibody response to SFV epitope, E2 T_h peptide₂ in $\gamma\delta$ KO than WT mice

Sera obtained from SFV-infected WT mice were used at a dilution determined from serial dilution curves of sera (normally 1:80) against SFV and the two E2 epitopes, in ELISA assays. In three separate experiments, on day 7 pi sera reacted strongly with SFV and with E2 T_h peptide₂, increasing in titer on days 14, 21 and 35 pi (Table I). Although sera from

SFV-infected $\gamma\delta$ KO mice reacted vigorously with SFV, they reacted minimally with E2 T_h peptide₂. The reactivity of KO sera with this epitope was less than that with WT sera on all days pi (Table I), and at significantly lower levels during the peaks of antibody response and remyelination on day 21 ($p < 0.05$), and day 35 pi ($p < 0.01$).

Antibody responses to E2 T_h peptide₁ in SFV-infected WT mice remained significantly lower than the antibody response to E2 T_h peptide₂ on all days pi. The antibody response of SFV-infected KO mice to E2 T_h peptide₁ fluctuated on different days pi. These differences frequently occurred because individual mice responded differently to this peptide. Reactivity of sera from SFV-infected KO mice with E2 T_h peptide₁ was lower, but not significantly different than WT on all days pi. Sera from neither group showed reactivity with control protein- Purified Protein Derivative (PPD) (Table I).

Similar antibody responses to components of brain in WT and KO

Both WT and KO mice developed antibody responses to rMOG and galactocerebroside (GalC) antigens, after SFV-infection. However, in contrast to anti-viral antibody responses, no significant differences between sera from WT and $\gamma\delta$ KO mice in anti-rMOG and anti-GalC antibody responses was detected at any time point after infection.

No significant differences between SFV-induced inflammation and demyelination in KO and WT

Inflammation in brains of SFV-infected mice was noted on day 7 pi in both groups. At this time, perivascular and parenchymal inflammation was widespread, characterized by focal lymphocytic infiltrates around endothelial cells, and occasional vacuolation of the parenchyma. This eventually led to gray and white matter pathology throughout the cerebrum and cerebellum (Mokhtarian et al., 2003; Smith-Norowitz et al., 2000; Atkins et al., 1999). For both groups, inflammatory changes peaked on day 7 pi. Inflammatory foci were spread throughout most areas of the brain, namely, cerebral hemispheres, cerebellum and brainstem (average number of inflammatory foci from three experiments was 20.6 in WT and 24.0 in KO mice). Numbers of focal inflammatory lesions at day 7 pi in $\gamma\delta$ KO mice, though higher, were not significantly different from WT mice. The inflammatory response was reduced on days 14 and 21 pi in both groups (average number of inflammatory foci was 1.6 in WT and 2.0 in KO mice on day 35 pi), and then diminished on day 42 pi (Table II).

Delayed and less extensive remyelination in SFV-infected $\gamma\delta$ KO, compared to WT

In order to be able to analyze de- and remyelination accurately in SFV-infected WT and KO mice, we studied one micron sections of brain tissue on days 7, 14, 21, 35 and 42 pi. Inflammation was present on day 7 pi in WT and KO mice. Inflammation and demyelination were also seen on days 14 and 21 pi in the brains of both groups. CNS white matter of WT mice was extensively remyelinated by day 35 pi. Unlike WT, sections from brains of KO mice, exhibited many fibers that were either demyelinated or were at very early stages of remyelination, on days 35 (data not shown). Sections of corpus callosum from SFV-infected WT mice were extensively remyelinated by day 42 pi (Figure 2A). The myelin repair was associated with an increased number of density-staining reactive oligodendrocytes. Brains of KO mice were still not fully remyelinated at 42 days pi, where areas of very early remyelination, lightly stained because of thin myelin and demyelinated regions, alternated with normally myelinated areas. This was particularly evident in corpus callosum where fibers were longitudinally oriented (Figure 2B). In these areas, oligodendrocytes were less conspicuous.

E2 T_h peptide₂ immunization increased remyelination in SFV-infected $\gamma\delta$ KO mice after immunization with E2 T_h peptide₂

Peptide immunization of KO mice resulted in extensive early remyelination and abundant reactive oligodendrocytes visible at day 28 pi (Figure 2C), compared to non-immunized KO mice. Normally myelinated tissue is shown for comparison (Figure 2D). In this tissue, myelin sheaths were uniformly thicker and oligodendrocytes non-reactive and less prominent. Thus, remyelination occurred earlier in E2 T_h peptide₂-immunized SFV-infected $\gamma\delta$ KO mice compared to non-immunized mice.

E2 T_h peptide₂ immunization increased the anti E2 T_h peptide₂ antibody response of KO mice during SFV infection

It has been shown that in some conditions, the functions of $\gamma\delta$ T cells can be substituted by $\gamma\delta$ T cells (Itoharu et al, 1993). Therefore, we attempted to increase the titer of anti-E2 T_h peptide₂ antibody by immunization of $\gamma\delta$ KO mice with this peptide. WT and KO mice were immunized subcutaneously with E2 T_h peptide₂ on days 5, 15 and 20 pi, as discussed in Materials and Methods. Mice were sampled on days 7, 14, 21, and 35 pi, and sera were tested by ELISA for reactivity with SFV and its peptides (Figure 3). In nonimmunized SFV-infected KO (black bars) and WT (gray bars), the reactivity of KO sera with this epitope was significantly less than with WT sera on days 21 ($p < 0.04$) and 35 pi ($p < 0.02$) (Figure 3). After immunization with E2 T_h peptide₂, sera from KO mice showed increased levels of antibody to this peptide, which had reached the comparable levels in WT mice (gray bars), on all days pi. Thus, immunization with E2 T_h peptide₂ increased the antibody response of KO mice to this peptide.

Clinical Scores of SFV-infected $\gamma\delta$ KO mice improved after immunization with E2 T_h peptide₂

In two experiments (Figure 4), clinical signs of SFV-infected WT mice (hatched bars) were seen to peak on day 7 pi and to decrease thereafter. In non-immunized SFV-infected KO mice (gray bars), the severity of disease was also high on day 7 pi, but unlike WT, the severity increased further and remained high on subsequent days pi and were higher than WT mice on days 14 ($p < 0.01$), 21 ($p = 0.01$) and 35 pi ($p = 0.01$). Severity of clinical signs in SFV-infected $\gamma\delta$ KO mice decreased significantly after immunization with E2-T_h peptide₂ (black bars) (Figure 4; $p = 0.01$ on day 14, $p = 0.02$ on day 21 and $p = 0.03$ on day 35, non-immunized vs. immunized, KO mice). Thus, in SFV-infected, E2 T_h peptide₂-immunized KO mice, clinical scores were significantly reduced compared to non-immunized KO mice.

SFV-infected WT and $\gamma\delta$ KO mice both cleared virus by day 10 pi

Viral infectivity was verified and viral titers were determined in three experiments. Brains from non-infected, day 0, WT and KO mice had no detectable virus by TCID₅₀ assay. Viral replication was detected in the brains of both groups on day 3 post SFV infection and reached an average peak titer of 1×10^4 TCID₅₀ units/ml on day 5 pi (Table III). WT and $\gamma\delta$ KO mice both cleared SFV by day 10 pi (Table III).

SFV-infected E2 T_h peptide₂ immunized WT and $\gamma\delta$ KO mice both cleared viral antigens prior to day 15 pi

Sections of brains from WT, KO, immunized WT and immunized KO mice were stained for viral antigens with mouse anti SFV antibody sera. On day 0, WT mice brain showed no staining for viral antigens and was used as a negative control (Figure 5A). Viral antigen residues were present in the brains of all infected groups on day 7 pi (Figure 5B, C & D), and all groups had cleared residual viral antigens by day 15 pi (Figure 5E & F). There were no differences between viral antigen residues in WT, KO, immunized KO and immunized

WT mice, indicating that immunization with this peptide did not result in an earlier viral clearance.

4- Discussion

Semliki Forest Virus (SFV) is one of the CNS neurotropic viruses that has been used to study the pathogenesis of demyelinating diseases such as MS (Fazakerley et al., 1997). SFV-infection causes paralysis in mice and leads to myelin injury after clearance of virus, followed by remyelination (Mokhtarian and Swoveland., 1987). In the present study, we have investigated the role of $\gamma\delta$ T cells in the pathogenesis of the SFV model from the standpoint of clinical disease, inflammation, antibody response, viral clearance after infection with SFV, and de- and remyelination.

Previous Studies of influenza (Carding et al., 1990), and Sendai (Allan et al., 1992), viruses have indicated that though necessary for viral clearance, $\gamma\delta$ T cells alone are not sufficient for reduction/elimination of viral load. However, the physiologic role of $\gamma\delta$ T cells in mediating protection in the Sendai virus model is not clear (Hou et al., 1992). In Vaccinia virus infection of $\gamma\delta$ T cell-deficient mice higher viral titers than normal mice were shown (Selin et al., 2001). In summary, $\gamma\delta$ T cells appear to play an accessory role to enhance the immune response and recovery from a viral infection. During typical SFV-infection of WT B6 mice, viral replication peaks on days 5–6 and virus is cleared when inflammation peaks by day 10 pi. Inflammation greatly subsides after viral clearance and is completely resolved by day 21 pi (Smith-Norowitz et al 2000). In the present study, SFV-infected $\gamma\delta$ KO mice cleared the virus by day 10 pi and inflammation was not significantly more intense than WT on any post infection day. In the absence of $\gamma\delta$ T cells, other mechanisms such as $\alpha\beta$ T cells were able to clear the virus equally well in KO mice (Dandekar et al., 2002). Earlier studies indicated that virus did not persist in CNS tissue (Mokhtarian and Swoveland, 1987 Smith-Norowitz et al., 2000; Mokhtarian et al., 1994). Although SFV-infected $\gamma\delta$ KO mice displayed a higher clinical score than WT, this may be attributed to other regulatory mechanisms such as slow and inefficient remyelination. Our results from SFV-infected $\gamma\delta$ KO mice appear to confirm the accessory role of $\gamma\delta$ T cells in the immune response to viral infections in that the remyelinating response appeared greater.

While some studies of $\gamma\delta$ T cells in EAE have found a pathogenic role for these cells (Rajan et al., 1998; Sphan et al., 1999; Rajan et al., 2000), others have suggested a suppressive effect (Kobayashi et al., 1997), or no effect at all (Clark et al., 1998; Matsumoto et al., 1998). Results obtained in the collagen-induced arthritis model suggest both inflammation-promoting and suppressing roles for $\gamma\delta$ T cells, depending on the stage of the disease process (Peterman et al., 1993). FACS analysis of mononuclear cells of brains from EAE mice (Rajan et al., 1996), have shown that the percentage of CD3⁺TCR $\gamma\delta$ cells significantly increases during the height of the acute phase, and again at the time of relapse in EAE, indicating their role in pathogenesis of this autoimmune disease and further confirming the possibility of different roles that these cells might play in CNS pathology. $\gamma\delta$ T cells were also shown to accumulate and respond preferentially to heat shock protein (hsp) expressed in damaged brain (Selmaj et al,1991) and in the CSF of MS patients and although their function is not entirely understood, it is clear that these cells may have roles in regulating autoimmune inflammation in the CNS (Blink et al., 2009).

In SFV-infected brains, pathology was seen on days 7–10 pi, in the form of inflammation and destruction in both gray and white matter (Fazakerley, 2004). Our previous (Mokhtarian et al., 2003) and present studies using thin sections have shown areas of primary demyelination in several lesions of white matter in SFV-infected WT B6 mice on days 14 – 21 pi. This primary demyelination contained small numbers of inflammatory cells. These

regions soon became mixed with thinly remyelinated fibers which were extensively remyelinated by day 35 pi (Mokhtarian et al., 2003). Brains of SFV-infected $\gamma\delta$ KO mice, however, unlike WT, did not efficiently remyelinate after viral clearance by day 35 pi. In comparison, KO and WT showed a visible difference in remyelination on day 35 pi. CNS tissues of KO mice remained only partially remyelinated, even on day 42 pi. There were no significant differences in inflammation and demyelination of SFV-infected KO and WT mice, therefore changes in these parameters cannot have accounted for the differences in remyelination between the two groups. The latter differences may be in part attributed to the mechanisms related to low antibody response to E2 T_h peptide₂ in KO mice, which is discussed below.

It has been previously reported that unlike the Theiler's model of MS in which persistence of virus leads to demyelination (Tsunoda et al., 2009), demyelination in the SFV model occurs after viral clearance and is immune-mediated (Mokhtarian et al., 1987). Similarly, SFV-infection of T cell-deficient, nude mice (Fazakerley et al., 2004), and B-cell deficient mice (Smith-Norowitz et al., 2000), did not lead to demyelination but resulted in longer virus replication.

As we and others have previously reported (Smith-Norowitz et al., 2000; Snijders et al., 1991), mice inoculated with SFV develop antibodies to SFV and to E2 peptides. The lack of $\gamma\delta$ T cells in the present model, however, appeared only to affect antibody production to one of the E2 peptides, E2 T_h peptide₂, whereas the levels of anti-SFV antibody were similar in both groups. Anti-E2 T_h peptide₂ antibody in SFV-infected WT mice reached significant levels on day 14 pi and was significantly higher than levels of this antibody in SFV-infected KO mice. Immunization of KO mice with E2 T_h peptide₂ was performed at peak of virus replication (day 5 pi), and it significantly increased the antibody to this peptide by day 21 pi. Therefore, immunization with the peptide appeared not to affect viral clearance in immunized KO mice. Immunological studies of these peptides have shown that the T cell epitopes for both proliferation and antibody production are located in E2 peptides (Snijders et al., 1989 and 1992). Our studies have confirmed and further shown that two of these peptides; E2 T_h peptide₁ and E2 T_h peptide₂ are involved in T cell responses and antibody production. However, the titer of anti-E2 T_h peptide₁ antibodies has been invariably lower than that of anti-E2 T_h peptide₂ antibodies (Smith-Norowitz et al., 2000). In this study, reactivity of WT sera with E2 T_h peptide₂ began earlier and was significantly higher than KO sera. Reactivity of sera from SFV-infected WT and KO to E2 T_h peptide₁, to SFV and to control peptide, however, was not significantly different.

The most striking differences in the pathogenesis of SFV-infected WT and KO mice were in clinical disease and antibody production against E2 T_h peptide₂ at the time of remyelination. With most viral infections, immunization at an early time point may abort or neutralize infection. With SFV, it has been reported that antibody to one of the peptides of E2, which can induce T cell proliferation, neutralized virus-infected cells in vitro, whereas the antibody to the helper T cell epitopes (E2 T_h peptides ₁ and ₂), did not (Snijders et al., 1991, Fernandez et al, 1998). In the SFV model, $\gamma\delta$ T cells appeared to play a role as helper cells in antibody production to E2-T_h peptide₂, which in turn promoted recovery and regulated remyelinating mechanisms. Collectively, our findings are consistent with $\gamma\delta$ T cells providing a role in humoral immunity during remyelination. Moreover, anti-SFV antibody staining showed no significant differences in amount of viral antigen residues on day 7 and day 15 pi between all groups, and peptide immunization was not able to clear viral antigens in immunized mice earlier than in non-immunized groups. Thus, persistence of virus probably did not account for the delay in remyelination in KO mice. The above findings support the conclusion that increase of anti-E2 T_h peptide₂ antibody in SFV-infected KO

mice may have enhanced recovery and remyelination, albeit through activation of some as yet unknown regulatory mechanism(s).

It has been shown that the recombinant form of a human IgM autoantibody and a mouse IgM mAb against spinal cord homogenate, show remyelinating capabilities when tested in the Theiler's virus model (Mitsunaga et al., 2002; Warrington et al., 2000; Rodriguez et al., 1996), and in an EAE model (Miller et al., 1997). The latter studies suggested that the binding of certain autoantibodies to oligodendrocytes may play a part in the regulation of oligodendrocyte function and remyelination (Teitelbaum et al., 1991; Ure et al., 2002; Paz Soldán et al., 2003; Rodriguez et al., 2009). Some studies of the remyelinating mechanisms for these antibodies indicated that they appear to work directly at the level of the glial cell and were not dependent on immune modulation (Ciric et al., 2004). Interestingly, our findings indicate that the differences in remyelination between WT and KO mice were also reflected by differences in the reactivity of oligodendrocytes (see Figure 2), which are well known for their ability to become reactive, differentiate and proliferate during remyelination in MS (Raine et al., 1981) and chronic relapsing EAE (Raine, 1984).

The mechanisms involved in promoting remyelination after E2T_h peptide₂ immunization in the SFV model has been hampered by difficulty in quantitation of small lesions of de- and remyelination in the CNS. Moreover, this evaluation is complicated by spontaneous remyelination in the brains of SFV-infected mice. To investigate whether this immunization plays a role in promoting remyelination, further experiments in the EAE model of MS may be helpful, where remyelination does not occur as readily, and where it can be quantitated in the spinal cords of EAE mice. Such experiments are presently underway in our laboratory (manuscript in preparation). It should be mentioned, however, that SFV and EAE are different demyelinating models and differ in terms of immunopathogenesis as well as in the roles of T cells and antibody responses.

Given the complex and unknown etiology of MS, elucidation of the mechanism by which remyelination-promoting antibodies exert their beneficial effect is a worthwhile avenue in the development of targeted therapies for MS, especially with regard to heterogeneity in the etiologies of demyelination and patterns of remyelination at different stages of MS (Mateo Paz Soldan et al., 2002; Barnett et al., 2004; Ludwin et al., 2008).

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Figure 1.

Comparison of average clinical scores of SFV infected WT and KO mice are shown on different days post infection. Total of 54 WT and 34 KO mice were used in three separate experiments. All mice were inoculated intraperitoneally with 10^4 PFU of SFV A7 (74) on day 0. Following the injection, animals were observed daily for clinical manifestations of disease and were scored on a scale of 0 to 6 (see methods). Since mice were sampled weekly, clinical scores of sacrificed and dead animals have been considered in average calculations until the end of the experiments. KO mice showed significantly higher clinical scores on days 7 pi ($p < 0.005$), 14 pi ($p < 0.01$), 21 pi ($p < 0.05$) and 35 pi ($p < 0.04$) compared to WT mice.

Figure 2. Inflammation, demyelination and remyelination in SFV-infected WT B6 and immunized and nonimmunized $\gamma\delta$ KO B6 mice. All figures come from one micron epoxy sections stained with toluidine blue and photographed by light microscopy

A. Cerebral hemisphere; corpus callosum; SFV-infected WT day 42 pi. In the upper half of this field, the white matter layer displays widespread thin remyelination. Note several reactive oligodendrocytes bearing processes typical of myelinating cells (arrows). Ventricle below X750

B. Cerebral hemisphere; corpus callosum; SFV-infected $\gamma\delta$ KO mice, day 42 pi. Demyelinated fibers at the top left of this field are seen in longitudinal section and in cross section in the lower right quadrant. Some of the longitudinally-oriented callosal fibers elsewhere display thin myelin sheaths, reminiscent of very early remyelination. Oligodendrocytes are less conspicuous and smaller in this less remyelinated tissue (arrows). Ventricle below X 750.

C. Cerebral hemisphere; corpus callosum; E2 T_h peptide₂ immunized, SFV-infected $\gamma\delta$ KO mice, day 28 pi. The fibers display uniform remyelination characterized by thin myelin sheaths in longitudinal section and prominent dense reactive oligodendrocytes, some with processes (arrows). Ventricle below, note ependymal cells. X 750.

D. Cerebral hemisphere; corpus callosum; Normal thickly myelinated white matter and normal oligodendrocytes which are often difficult to distinguish (arrows) are shown for comparison with figures A–C. Note the well-myelinated nerve fibers in longitudinal section. Ventricle below, note ependymal cells. X 750.

Figure 3.

Comparison of anti E2-T_h peptide₂ antibody response ($\pm$ SEM) in immunized KO, KO and WT mice. Average of two experiments including 14 WT mice, 13 KO mice, 11 immunized WT and 9 immunized KO, is shown. Amount of antibody is shown as mean optical density (OD) of sera from two experiments + standard error of the mean (SEM). The background OD is subtracted from each time point. * KO mice produced significantly less anti E2-T_h peptide₂ antibody compared with WT mice $p < 0.04$ on day 21 and $p < 0.02$ on day 35 pi. E2-T_h peptide₂ antibody in immunized KO mice was comparable to WT mice on day 21 and 35 pi ($p = \text{NS}$).

Figure 4.

Comparison of clinical scores in WT, E2_{Th} peptide₂ immunized KO and nonimmunized KO mice are shown. Two separate experiments were performed and in each experiment mice were divided in four WT, KO, immunized KO and immunized WT groups. Total numbers of mice in two experiments include 14 WT mice, 13 KO mice, 11 immunized WT and 10 immunized KO. There is a significant difference between average clinical scores of SFV infected WT and KO mice on days 14 ($p < 0.01$), 21 ($p = 0.01$) and 35 pi ($p = 0.01$).

Comparison of average clinical scores ($\pm$ SEM) of KO and E2- T_h peptide₂ immunized KO mice showed significant differences on days 14 ($p = 0.01$), 21 ($p = 0.02$) and 35 ($p = 0.03$) pi. Immunized WT data is not shown (NS).

Figure 5.

Presence of viral antigens in the brains of SFV infected mice. WT mice brain on day 0 was used as a negative control in this experiment (A). Viral antigens are seen in the brains of WT, KO and immunized KO mice on day 7 pi (B, C, D, x40 and x100). KO and immunized KO mice show no residual viral antigens on day 15 pi (E, F x 40 and x100). Samples from all groups, WT, KO and immunized WT mice, had cleared viral antigens by day 15 pi (not shown).

Table 1

Antibody responses to SFV and E2 T_H peptides in the sera of SFV-infected WT and $\gamma\delta$ KO mice *

Antigen	Average net changes in the levels of antibody ($\Delta OD \pm SD$) ^{**} in post-infection days									
	Day 0		Day 7		Day 14		Day 21 ^a		Day 35 ^a	
	WT	KO	WT	KO	WT	KO	WT	KO	WT	KO
SFV	0	0	1.18 $\pm$ 0.21	0.87 $\pm$ 0.64	1.79 $\pm$ 0.22	1.62 $\pm$ 0.47	1.89 $\pm$ 0.27	1.64 $\pm$ 0.58	2.10 $\pm$ 0.16	1.86 $\pm$ 0.47
E2 T _H peptide2	0	0	0.84 $\pm$ 0.39	0.55 $\pm$ 0.37	1.83 $\pm$ 0.47	0.51 $\pm$ 0.22	2.19$\pm$0.76	0.63$\pm$0.19	2.08$\pm$0.67	0.57$\pm$0.40
E2 T _H peptide1	0	0	0.82 $\pm$ 0.40	0.79 $\pm$ 0.50	0.91 $\pm$ 0.14	0.48 $\pm$ 0.32	0.95 $\pm$ 0.22	0.71 $\pm$ 0.23	0.85 $\pm$ 0.07	0.41 $\pm$ 0.27
PPD	0	0	0.24 $\pm$ 0.06	0.54 $\pm$ 0.07	0.42 $\pm$ 0.15	0.41 $\pm$ 0.02	0.46 $\pm$ 0.25	0.24 $\pm$ 0.08	0.41 $\pm$ 0.21	0.41 $\pm$ 0.15

* Pooled serum samples from 3–6 WT and 2–3 KO mice per experiment in three separate experiments were assayed. Indirect ELISA was used on diluted sera against SFV and E2 T_H peptides, as described in Materials and Methods. Results are an average of three experiments $\pm$ SD.

** In each assay, the net increase in the levels of antibody (ΔOD) at each time point was calculated by subtracting optical density of day 0 pi from the other post-infection days; Dx OD – D0 OD = ΔOD , where x is any day pi.

^a KO mice sera showed significantly lower reactivity with E2 T_H peptide2 than WT mice on days 21 pi (p < 0.05) and on day 35 pi (p < 0.01).

Table IIInflammation in the brains of SFV-infected WT and $\gamma\delta$ KO mice on days postinfection*

Day post-infection	WT	KO
0	0, 0, 0 (0.0)	0, 0, 0 (0.0)
7	25, 16, 21 (20.6 $\pm$ 2.6)	23, 19, 30 (24.0 $\pm$ 3.2)
14	4, 7, 8 (6.3 $\pm$ 1.2)	3, 9, 9 (7.0 $\pm$ 2)
21	3, 3, 6 (4.0 $\pm$ 1)	3, 6, 5 (4.7 $\pm$ 0.8)
35	1, 3, 1 (1.6 $\pm$ 0.6)	2, 2, 2 (2.0)
42	0, 0, 0 (0.0)	0, 0, 0 (0.0)

* Each number represents an average of number of inflammatory foci $\pm$ SEM from three separate experiments + SEM. In each experiment, three H&E stained brain sections of 1 WT and 1 KO mice on day 0,7,14,21,35 and 42 pi were evaluated and the average number of inflammatory foci is shown. Each inflammatory focus is an aggregate of 10 or more mononuclear cells.

Table III

Viral titration of brains from SFV-infected WT and $\gamma\delta$ KO mice on days post infection

Viral titers ($-\log_{10}$ TCID ₅₀ units in 0.1ml)* on days pi								
	Day 0	Day 3	Day 5	Day 7	Day 10	Day 14	Day 21	
WT	0.00	2.75	4.00	2.00	**N	N	N	
KO	0.00	3.00	4.00	3	N	N	N	

Brain samples were obtained from a pool of brains from 2–3 WT and 1–2 $\gamma\delta$ KO of mice, at each indicated time point, post SFV-infection. One representative of three experiments is shown. TCID₅₀ assay was carried out as described in Materials and Methods.

* Limit of detection in this assay is 2.

** N= Negative.