Published in final edited form as:

Brain Behav Immun. 2015 January ; 0: 86–97. doi:10.1016/j.bbi.2014.07.008.

Th17-biased ROR γ t Transgenic Mice Become Susceptible to a Viral Model for Multiple Sclerosis

Nicholas E. Martinez, Ph.D.¹, Fumitaka Sato, Ph.D.¹, Eiichiro Kawai, M.D.¹, Seiichi Omura, Ph.D.¹, Keigyou Yoh, M.D., Ph.D.², Satoru Takahashi, M.D., Ph.D.³, and Ikuo Tsunoda, M.D., Ph.D.^{1,*}

¹Department of Microbiology and Immunology, Center for Molecular and Tumor Virology, Louisiana State University Health Sciences Center, Shreveport, LA 71130, USA

²Department of Nephrology, Division of Clinical Medicine, Faculty of Medicine, University of Tsukuba

³Department of Anatomy and Embryology, Faculty of Medicine, International Institute for Integrative Sleep Medicine (WPI-IIS), Life Science Center of Tsukuba Advanced Research Alliance (TARA), Laboratory Animal Resource Center (LARC), University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305, Japan

Abstract

In a viral model for multiple sclerosis (MS), Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD), both immune-mediated tissue damage (immunopathology) and virus persistence have been shown to cause pathology. T helper (Th) 17 cells are a Th cell subset, whose differentiation requires the transcription factor retinoic acid-related orphan receptor (ROR) γ t, secrete pro-inflammatory cytokines, including IL-17, and can antagonize Th1 cells. Although Th17 cells have been shown to play a pathogenic role in immune-mediated diseases or a protective role in bacterial and fungal infections, their role in viral infections is unclear. Using newly established Th17-biased ROR γ t Tg mice, we tested whether Th17 cells could play a pathogenic or protective role in TMEV-IDD by contributing to immunopathology and/or by modulating anti-viral Th1 immune responses. While TMEV-infected wild-type littermate C57BL/6 mice are resistant to TMEV-IDD, ROR γ t Tg mice developed inflammatory

© 2014 Elsevier Inc. All rights reserved.

*Corresponding author: Ikuo Tsunoda, M.D., Ph.D., Department of Microbiology and Immunology, Center for Molecular and Tumor Virology, Louisiana State University Health Sciences Center, School of Medicine in Shreveport, 1501 Kings Highway, Shreveport, LA 71130, USA, Tel: 318-675-5757, Fax: 318-675-5764, itsuno@lsuhsc.edu.

The authors declare no conflicts of interest.

Author Contributions

Nicholas E. Martinez: Performed animal and immunological experiments, designed experiments and prepared the manuscript.

Fumitaka Sato: Performed animal experiments, helped to design the immunological experiments, and aided in manuscript preparation.

Seiichi Omura and Eiichiro Kawai: Performed immunological experiments and aided in manuscript preparation.

Satoru Takahashi and Keigyou Yoh: Established ROR γ t Tg mice and aided in manuscript preparation.

Ikuo Tsunoda: Designed experiments and aided in manuscript preparation.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

demyelinating lesions with virus persistence in the spinal cord. TMEV-infected ROR γ t Tg mice had higher levels of IL-17, lower levels of interferon- γ , and fewer CD8 $^{+}$ T cells, without alteration in overall levels of anti-viral lymphoproliferative and antibody responses, compared with TMEV-infected wild-type mice. This suggests that a Th17-biased “gain-of-function” mutation could increase susceptibility to virus-mediated demyelinating diseases.

Keywords

Picornaviridae; CNS Disease; Demyelinating disease; Viral CNS infections; CD8-positive lymphocytes; Disease susceptibility; Inflammation

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) (1, 2). The inflammatory demyelination found in MS is accompanied by neuronal and/or axonal degeneration, which leads to permanent neurological impairment (3–5). Although, the precise etiology of MS remains unclear, several factors have been linked to susceptibility to the disease, including genetic predispositions, autoimmunity, and environmental factors, particularly virus infections (6–8). In the viral theory of MS, the initial demyelinating attack could be caused by 1) immune responses to viral antigen in the CNS resulting in myelin damage in a bystander fashion and 2) direct killing of oligodendrocytes, the myelin forming cells, by the virus (9). Clinically, the viral etiology of MS has been supported by detection of virus itself or anti-viral immune responses from MS patients. While a particular virus has never been confirmed as the causative factor of MS, several viruses have been linked to the prevalence of MS. This includes Epstein-Barr virus (EBV), where up to 99% of MS patients are seropositive but only 85% of the general population is (10). In addition, several other viruses have been linked to MS, including human herpes virus (HHV)-6, herpes simplex virus (HSV)-1, measles virus, canine distemper virus, and multiple sclerosis-associated retrovirus (MSRV) (11, 12).

Experimentally, Theiler’s murine encephalomyelitis virus (TMEV) has been used to induce a demyelinating disease in mice that has pathological features similar to what is found in MS patients. TMEV is a non-enveloped single-stranded positive-sense RNA virus that belongs to the family *Picornaviridae* (13). In susceptible mouse strains, such as SJL/J mice, TMEV induces a biphasic disease (14, 15). Around 1 week post infection (p.i.), during the acute phase, which affects all mouse strains, TMEV predominately infects neurons in the brain and causes inflammation and neuronal loss in the gray matter, histologically, with or without the induction of seizures (16). The neuropathology caused during the acute phase is primarily associated with viral replication (viral pathology). Although resistant mouse strains, such as C57BL/6 and BALB/c mice can eradicate virus from the CNS, susceptible strains, such as SJL/J mice cannot clear TMEV from the CNS. The resistance to TMEV has been associated with MHC class I-restricted CD8 $^{+}$ T cells, while CD4 $^{+}$ T cells and antibody have also been shown to contribute to viral clearance (17–20). The chronic (demyelinating) phase begins around 3 weeks to 1 month p.i. in susceptible mice, where TMEV infects macrophages and glial cells, including oligodendrocytes, leading to chronic progressive

paralysis clinically, and inflammatory demyelinating lesions with axonal degeneration in the spinal cord. Unlike an autoimmune model for MS, EAE, TMEV-IDD pathogenesis requires both virus persistence and immune effector cells. The damage caused during the chronic phase of disease requires both virus persistence and immune-mediated pathology (immunopathology) (21, 22). For example, adoptive transfer of effector T cells into naïve animals can induce demyelinating disease in EAE, while T cell transfer alone has not been shown to cause disease in the TMEV model. Although the precise effector mechanism of the immunopathology is unknown, multiple immune components have been shown to play key roles. For example, CD4⁺ T helper (Th)1 cells have been associated with inflammatory demyelination, CD8⁺ T cells could play an effector role in axonal degeneration, and anti-viral antibody can cross react with myelin antigen (20, 23–25) (only TMEV-specific antibody can play a pathogenic role in TMEV-IDD; no other pathogenic antibodies have been reported in TMEV-IDD). This chronic TMEV-induced demyelinating disease (TMEV-IDD) resembles MS both clinically and histologically.

Expression of the transcription factor retinoic acid related orphan receptor (ROR) γ t is required for the differentiation of Th17 cells. T helper (Th) 17 cells secrete proinflammatory cytokines, such as interleukin (IL)-17 (26). In mice, naïve CD4⁺ T cells are differentiated into Th17 cells by priming in the presence of transforming growth factor (TGF)- β and IL-6, which induces their hallmark transcription factor ROR γ t (27). The cytokines released by Th17 cells can inhibit Th1 cells, while Th1- and Th2-associated cytokines, such as IL-2, IL-4, IL-12 and interferon (IFN)- γ , have been shown to inhibit the differentiation of Th17 cells (26). Since the IL-17 receptor is present on a broad range of cell types, Th17 cells can promote a widespread reaction, including the production of IL-6 and other inflammatory cytokines. The release of inflammatory cytokines from Th17 cells can cause severe immunopathology; dysregulation of Th17 cells has been implicated in many immune-mediated diseases ranging from MS to inflammatory bowel disease (IBD) (28).

Although Th17 cells have been shown to play protective roles in some bacterial and fungal infections, the physiological role of Th17 cells is unclear in viral infections (14). In some viral infections, Th17 cells have been shown to be detrimental to the host due to induction of immunopathology (29, 30). Since the production of IL-17 can prevent the differentiation of Th1 cells, Th17 cells can inhibit the production of IL-2 and IFN- γ , which have cytotoxic T lymphocyte (CTL) induction and anti-viral functions, respectively (31). Thus, the inhibition of anti-viral Th1 cells by Th17 cells could lead to viral persistence (viral pathology).

Although Th17 cells have been suggested to play a pathogenic role in MS and its autoimmune model, experimental autoimmune encephalomyelitis (EAE), their role in virus-induced demyelination is largely unknown (32). Theoretically, Th17 cells can play two contrasting roles: 1) Th17 cells may inhibit Th1 immune response facilitating viral replication or 2) Th17 cells may induce immunopathology, attacking myelin sheaths in the CNS, either directly or indirectly through interactions with other immune effector cells and molecules (14).

In TMEV infection, it has been proposed that Th17 cells can contribute to susceptibility to TMEV-IDD (14). Hou et al. found that susceptible SJL/J mice had higher levels of Th17 cells compared with resistant C57BL/6 mice and that administering a bacterial endotoxin,

lipopolysaccharide (LPS), which promoted Th17 development, prior to TMEV infection, could render C57BL/6 mice susceptible to a persistent infection (33). They also found that enhanced Th17 immune responses were associated with decreased anti-viral CTL responses and administration of IL-17 neutralizing antibody restored CTL function. These studies suggest that genetic or environmental factors that predispose or influence the Th17 immune response may affect susceptibility to persistent virus infection. Genetic factors such as inherited genes or “gain-of-function” mutations have been shown to influence susceptibility to infections and immune-mediated diseases in humans (34, 35). Although genes influence susceptibility, other factors may be necessary to trigger the disease such as environmental factors (36). Thus, it is possible that genetic factors could predispose individuals to MS, but an environmental trigger is needed to initiate disease.

To determine whether a preexisting bias (gain-of-function mutation) toward Th17 immune responses would influence the susceptibility to a demyelinating disease, we have established transgenic (Tg) mice on the resistant C57BL/6 mouse background that overexpress ROR γ t in T cells and are biased toward a Th17 immune response (37). Since C57BL/6 mice have poor Th17 induction (33), these mice will allow us to investigate the role of Th17 cells in TMEV infection without the use of adjuvants. We tested whether the ROR γ t Tg mice would become susceptible to TMEV-IDD by monitoring ROR γ t Tg mice and their wild-type C57BL/6 littermates infected with TMEV for development of TMEV-IDD. The ROR γ t Tg mice became susceptible to TMEV-IDD, while none of the wild-type C57BL/6 mice developed TMEV-IDD. The susceptibility was associated with higher Th17 immune responses without alterations of overall anti-TMEV immune responses during the chronic phase of disease. During the acute phase of disease, in response to TMEV, the ROR γ t Tg mice had higher Th17 immune responses and reduced numbers of CD8⁺ T cells. To our knowledge, this is the first report of susceptibility to a chronic viral infection due to a genetic bias toward a Th17 immune response.

Materials and Methods

Mice

To generate ROR γ t Tg mice, a full-length cDNA encoding the murine ROR γ t protein was inserted into a VA CD2 transgene cassette that contained the upstream gene regulatory region and locus control region of the human CD2 gene (37). The ROR γ t Tg mice were maintained as heterozygotes for the transgene by breeding them with wild-type C57BL/6 mice. In vitro, in both polarizing and non-polarizing conditions, the ROR γ t Tg mice have higher percentages of cells that convert to Th17 cells and lower percentages of Th1 and Th2 cells, compared with wild-type mice; there are similar numbers of Tregs present in both mouse strains. Additionally, while plasmacytosis, splenomegaly and enlargement of the lymph nodes has been observed in BALB/c F1 ROR γ t Tg mice, we did not observe plasmacytosis or changes in the spleen or lymph nodes in the C57BL/6 ROR γ t Tg mice in the current experiment. Both wild-type and ROR γ t Tg mice had similar levels spleen cells and plasma cell infiltration was not observed in the CNS of C57BL/6 ROR γ t Tg mice regardless of infection.

Female 4-week-old SJL/J and C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Harlan Laboratories, Inc. (Indianapolis, IN), respectively. Animals were maintained on 12/12-hour (h) light/dark cycles in standard animal cages with filter tops under specific pathogen-free conditions in our animal care facility at Louisiana State University Health Sciences Center (LSUHSC)-Shreveport and given standard laboratory rodent chow and water ad libitum. All experimental procedures involving the use of animals were reviewed and approved by the Institutional Animal Care and Use Committee of LSUHSC and performed according to the criteria outlined by the National Institutes of Health.

TMEV infection

Four to six-week-old wild-type C57BL/6, ROR γ t Tg C57BL/6, and SJL/J mice were infected intracerebrally (i.c.) with 2×10^5 plaque forming units (PFUs) of the Daniels (DA) strain of TMEV (38). Mice were weighed and observed daily for up to 3 months for comparison to other TMEV manuscripts. During the first 2 weeks, mice were observed for seizures for 1 hour/day for seizures, which were scored using the Racine 5-stage seizure scale (Stage 1, chewing and drooling; Stage 2, head nodding; Stage 3, unilateral forelimb clonus; Stage 4, rearing with bilateral forelimb clonus; and Stage 5, rearing and falling) (39). Clinical signs of TMEV-IDD were evaluated by examining impairment of righting reflex: the proximal end of the mouse's tail was grasped and twisted to the right and then to the left (0, a healthy mouse resists being turn over; 1, the mouse is flipped onto its back but immediately rights itself on one side; 1.5, the mouse is flipped onto its back but immediately rights itself on both sides; 2, the mouse rights itself in 1 to 5 seconds; 3, righting takes more than 5 seconds; 4, the mouse cannot right itself) (40). For neutralization of IL-17 *in vivo*, mice were intraperitoneally injected with 100 μ g anti-mouse IL-17A or isotype control antibody (clone eBioMM17F3; eBioscience) in 200 μ l PBS at days 0, 7, and 14 p.i.

Neuropathology

Mice were perfused with phosphate-buffered saline (PBS) followed by a 4% paraformaldehyde solution (Sigma-Aldrich) in PBS. The CNS tissues were harvested and fixed with 4% paraformaldehyde. The spinal cord and brain were divided into 10 to 12 transversal segments and five coronal slabs, respectively, and embedded in paraffin. Four- μ m-thick sections were stained with Luxol fast blue (Solvent blue 38; Sigma-Aldrich) for myelin visualization. Histological scoring of the CNS was performed as described previously (40, 41). For scoring of spinal cord pathology, each spinal cord segment (10–12 per mouse) was divided into four quadrants: the ventral funiculus, the dorsal funiculus, and each lateral funiculus. Any quadrant containing demyelination, meningitis, or perivascular cuffing was given a score of 1 in that pathological class. The total number of positive quadrants for each pathological class was determined and then divided by the total number of quadrants present on the slide and multiplied by 100 to give the percent involvement for each pathological class. An overall pathology score was also determined by giving a positive score if any pathology was present in the quadrant, and presented as the percent involvement. Brain pathology scores were evaluated as follows: meningitis (0, no meningitis; 1, mild cellular infiltration; 2, moderate cellular infiltration; 3, severe cellular infiltration), perivascular cuffing (0, no cuffing; 1, 1 to 10 lesions; 2, 11 to 20 lesions; 3, 21

to 30 lesions; 4, 31 to 40 lesions; 5, over 40 lesions), and demyelination (0, no demyelination; 1, mild demyelination; 2, moderate demyelination; 3, severe demyelination). Each score from the brain was combined for a maximum score of 11 per mouse.

CD3⁺ T cells were visualized by antigen retrieval using citrate-based Vector[®] Antigen Unmasking Solutions (Vector Laboratories, Burlingame, CA), followed by immunohistochemistry with anti-CD3 antibody (Dako, Carpinteria, CA), using the avidin-biotin-peroxidase complex (ABC) technique (Vector Laboratories) (42). The numbers of CD3⁺ cells were quantified under a light microscope using 10 to 12 transverse spinal cord segments per mouse as previously described (42). Damaged axons and viral antigen-positive cells were visualized by immunohistochemistry with SMI311 (Sternberger Monoclonal, Inc., Baltimore, MD), a cocktail of monoclonal antibodies (SMI32, 33, 37, 38, and 39) against nonphosphorylated neurofilament (43), and hyperimmune serum against TMEV (16), respectively. For SMI311 staining, tissue sections were pretreated in double distilled (dd) H₂O by the Digital Decloaking Chamber I (Biocare Medical, Concord, CA) for 15 minutes at 120° C for antigen retrieval. The number of SMI311 positive axons and viral antigen positive cells in the CNS was counted under a light microscope using 10 to 12 transverse spinal cord segments per mouse as previously described (41).

Lymphoproliferative assay

Spleens were harvested from TMEV-infected mice during the acute or chronic phase of disease, mononuclear cells (MNCs) were isolated using Histopaque[®] 1083 (Sigma-Aldrich) (40). A volume of 100 µl of 2×10^5 MNCs in RPMI 1640 supplemented with 1% glutamine, 1% antibiotics, 50 µM 2-mercaptoethanol, and 10% fetal bovine serum (FBS) was added to each well of 96-well plates. This was incubated with 100 l of solution containing 2×10^5 TMEV-infected antigen-presenting cells (TMEV-APCs) or 2×10^5 sham-infected APCs (Sham-APCs) (44). TMEV-APCs were made from whole spleen cells infected *in vitro* with DA virus at a multiplicity of infection (MOI) of 1 and irradiated with 2,000 rads, while Sham-APCs were prepared from sham-infected spleen cells. The cells were cultured for 4 days, after which time each well was pulsed with 1 µCi of [³H]thymidine. Then, 18 to 24 hours later, the cells were harvested on Reeves Angel 934AH filters (Brandel, Gaithersburg, MD) using a PHD[™] Harvester (Brandel). The incorporated radioactivity was measured by Wallac 1409 Liquid Scintillation Counter (PerkinElmer). Lymphoproliferation assays were performed in triplicate in 96-well plates. The CPM was calculated using the following formula: [counts per minute (cpm) of MNCs incubated with TMEV-APCs or TMEV] - (cpm of MNCs incubated with Sham-APCs or unstimulated MNCs).

Cytokine assay

MNCs isolated from spleens of mice infected with TMEV were cultured with 2×10^6 cells/well in 6-well plates (Corning Inc., Corning, NY) and stimulated with 5 µg/ml of concanavalin A (ConA) or 1 MOI of TMEV for 48 hours. After 2 days the supernatants were collected and analyzed by enzyme-linked immunosorbent assay (ELISA) (42). The levels of IL-4, IL-6, IL-10, and IFN-γ concentrations were detected with BD OptEIA kits (BD Biosciences, San Diego, CA) and IL-17A was detected with a Mouse IL-17A ELISA

MAX[™] kit (Biolegend, Inc., San Diego, CA) according to the manufacturer's instructions (45). ELISAs were performed in duplicate in 96-well plates.

Serum TMEV antibody assay

When the mice were killed, blood was collected from the heart of TMEV-infected mice. The levels of serum TMEV antibody were assessed by ELISA as described previously (46). Ninety-six-well flat-bottom Nunc-Immuno plates, MaxiSorp surface (Thermo Fisher Scientific Inc., Rochester, NY) were coated with purified TMEV proteins overnight. After blocking with 10% FBS, serial dilutions of sera were added to the plates and incubated for 90 minutes. Following washing, a peroxidase-conjugated anti-mouse IgG (H + L), IgG1, IgG2b, IgG2c, or IgG3 (Life Technologies, Gaithersburg, MD) was added to the plates for 90 minutes. Immunoreactive complexes were detected with *o*-phenyldiamine dihydrochloride (Sigma-Aldrich) and were read at 492 nm on a Multiskan MCC/340 Microplate Reader (Thermo Fisher Scientific Inc.). The anti-TMEV antibody titer was determined as the highest reciprocal of the dilution that had an absorbance higher than the average plus two standard deviations of five naïve serum samples at a dilution of 2⁶ (41).

Real-time reverse transcription polymerase chain reaction (PCR)

Mice were killed and perfused with sterile PBS and then the tissues were harvested and flash frozen in liquid nitrogen. The tissue was homogenized in TRI Reagent® (Molecular Research Center Inc, Cincinnati, OH) and the total RNA was isolated from the homogenate, using a RNeasy® Mini kit (Qiagen Inc, Valencia, CA) (47). The reverse transcription reaction was performed with 1 µg of total RNA, using an ImProm-II[™] Reverse transcription system (Promega, Madison, WI). Sequential reaction conditions were annealing at 25°C for 5 minutes and extension at 42°C for 1 hour. Real-time PCR was performed in iCycler iQ[™] 96-well PCR plates (BioRad, Hercules, CA) containing 12.5 µl RT² Fast SYBR® Green qPCR Master Mix (SA Biosciences, Valencia, CA), 10.5 µl dd H₂O, 1.0 µl template cDNA (50 ng), and 1.0 µl gene-specific 10 µM PCR primer pair stock in a MyiQ2 Two Color Real-time PCR Detection System (BioRad). Sequential reaction conditions for real-time PCR were activation of HotStart Taq DNA polymerase at 95°C for 5 minutes, and then 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The primers were purchased from RealTimePrimers.com (Elkins Park, PA). The primer pair sequences for viral protein 2 (VP2), a virus capsid protein, were forward (5'-TGGTCTGACTCTGTGGTTACG-3') and reverse (5'-GCCGGTCTTGCAAAGATAGT-3') (48). The primer pair sequences for granzyme B were forward (5'-TGGCCTTACTTTTCGATCA AG-3') and reverse (5'-CAGCATGATGTCATTGGAGA-3'). The primer pair sequences for IFN-γ were forward (5'-CAAAAGGATGGTGACATGAA-3') and reverse (5'-TTGGCAATACTCATGAATGC-3'). The primer pair sequences for IL-17 were forward (5'-TCCCTCTGTGATCTGGGAAG-3') and reverse (5'-TCCCTCTGTGATCTGGGAAG-3'). The primer pair sequences for CD3 were forward (5'-ATATCTCATTGCGGGACAGG-3') and reverse (5'-TATCTCATTGCGGGACAGG-3'). Phosphoglycerate kinase (PGK) 1 and glyceraldehyde-3-phosphate dehydrogenase (GAPD) were used as an internal reference to normalize the results of the general organs and the CNS, respectively (49, 50). The PGK1 primers were forward (5'-GCAGATTGTTTGAATGG TC-3') and reverse (5'-TGCTCACATGGCTGACTTTA-3').

The GAPD primers were forward (5'-CTGGAGAAACC TGCCAAGTA-3') and reverse (5'-TGTTGCTGTAGCCGTATTCA-3'). Cycle threshold (Ct) was calculated by subtracting the internal reference_{Ct} from the TMEV_{Ct}.

Flow cytometry

Fc receptors of cells were blocked with anti-CD16/32 (Biolegend, San Diego, CA). MNCs were stained with antibodies against CD3 (Biolegend), CD4 (Biolegend), CD8 (Biolegend), CD11c (Biolegend) a dendritic cell marker, B220 (Biolegend) a B cell marker (51), F4/80 (Biolegend) a macrophage marker, FOXP3 (eBioscience, San Diego, CA), IFN- γ (Biolegend), IL-4 (Biolegend), IL-10 (Biolegend) and IL-17A (Biolegend). Cells were permeabilized and fixed using the BD Cytotfix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences, San Jose, CA). The flow cytometry data was acquired on a FACSCalibur (BD Biosciences) and analyzed using Cellquest Pro (BD Biosciences). For intracellular cytokine staining, cells were incubated with TMEV at a MOI of 1 for 48 hours, and 500 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 25 ng/ml ionomycin (Sigma-Aldrich), and 1 μ l/ml of brefeldin A (GolgiPlug™, BD Biosciences) for 6 hours before staining.

Results

ROR γ t Tg mice develop a CNS disease similar to wild-type mice during the acute phase

To test whether Th17 immune responses could influence susceptibility to TMEV-IDD, we infected ROR γ t Tg mice that are biased toward a Th17 immune response and their wild-type littermates on the normally resistant C57BL/6 mouse background (control) with TMEV. Clinically, both mouse strains showed no difference in weight change or righting reflex (data not shown). During the acute phase of TMEV infection, we also evaluated TMEV-induced seizures using the Racine scale (39), since C57BL/6 mice are susceptible to seizures, in which hippocampal pathology and innate immunity have been suggested to play key roles (52, 53). We found that there was no difference in the incidence of seizures between wild-type (20%, 7/35) and ROR γ t Tg (27%, 9/33) mice ($P = 0.54$, χ^2), and that the seizures occurred during a similar period of time, from days 3 to 8 p.i. Histologically, both mouse strains had similar amounts of inflammation and neuronal death in the CNS (Supplemental Figure 1A), where the majority of lesions were found in the gray matter, particularly the hippocampus (Supplemental Figures 1B and C) and few spinal cord segments had lesions, which is typical during the acute phase of TMEV-infection (21). Demyelination was not detected in the CNS in wild-type or ROR γ t Tg mice during the acute phase of disease.

ROR γ t Tg mice become susceptible to TMEV-IDD

We next compared the neuropathology between the wild-type and ROR γ t Tg mice, 2–3 months p.i. As we expected, we found neither inflammation nor demyelination in the control wild-type TMEV-infected C57BL/6 mice (Figure 1A, 2A). On the other hand, the TMEV-infected ROR γ t Tg mice developed TMEV-IDD with severe inflammatory demyelinating lesions in the spinal cord (Figures 1B). The lesions were primarily in the lateral and ventral funiculi, with little involvement of the dorsal funiculus in the spinal cord, while no lesions

were found in the brain. Inflammatory cells were primarily composed of MNCs. The severity and distribution of lesions found in the ROR γ t Tg mice was similar to what was observed in susceptible SJL/J mice with TMEV-IDD (Figures 1C and 2A). Using immunohistochemistry against CD3 (a T cell marker), we found that a majority of the inflammatory cells in the spinal cord of ROR γ t Tg mice were CD3 positive. While only a few T cells were sporadically seen in the meninges of TMEV-infected wild-type C57BL/6 mice, we detected a large number of CD3⁺ T cells in the meninges, perivascular cuffing (inflammation), and parenchyma of the spinal cord of ROR γ t Tg mice, which was comparable to lesions in SJL/J mice (Figures 2B and 3D). Around demyelinating lesions in ROR γ t Tg mice, we found viral antigen positive cells by immunohistochemistry, while no viral antigen positive cells were detectable in wild-type C57BL/6 mice (Figure 3A and 3B). Using real time reverse transcription PCR on spinal cord samples from TMEV-infected mice 2 months p.i, we detected significantly higher levels of CD3 expression with significantly higher levels of IL-17 and IFN- γ in ROR γ t Tg mice than in wild-type mice (*, $P < 0.05$, Figure 3E), while viral RNA was only detected in the spinal cord of ROR γ t Tg mice, but not in wild-type mice. Thus, inflammatory demyelination in ROR γ t Tg mice was associated with T cells infiltration, pro-inflammatory cytokines and viral persistence, as demonstrated in SJL/J mice previously (54). We observed naïve ROR γ t Tg mice for up to 2 years and found no neuropathology at any time point.

Interestingly, although the ROR γ t Tg mice developed inflammatory demyelination histologically, they did not show any clinical signs of disease, such as weight loss, waddling gait, or an impaired righting reflex during the 2–3 month observation period (data not shown). This was in contrast to clinical signs of SJL/J mice with TMEV-IDD, which develop spastic paralysis, waddling gait, and impaired righting reflex. Thus, we determined whether the difference in clinical signs among the different mouse strains infected with TMEV could be associated with a difference in axonal damage, by using antibody against non-phosphorylated neurofilaments (damaged axons). We found that there were significantly less damaged axons per spinal cord segment in the ROR γ t Tg mice compared with SJL/J mice (mean number of damaged axons per spinal cord segment $\pm$ SEM 2 months p.i.: ROR γ t Tg, 11.9 ± 3.2 ; SJL/J, 57.6 ± 4.5 ; $P < 0.05$, t -test) (Figures 1E, F and 2C). Additionally, in the spinal cords of ROR γ t Tg mice there was no difference in the amount of axonal damage 1 month or 3 months p.i. Thus, the discrepancy in clinical signs of TMEV-IDD between ROR γ t Tg and SJL/J mice could be explained by the level of axonal damage, but not inflammatory demyelination.

ROR γ t Tg mice have enhanced TMEV-specific Th17 immune responses and reduced Th1 immune responses during the chronic phase of TMEV infection

To test whether the levels of TMEV-specific cellular immune responses correlated with neuropathology, we isolated MNCs from TMEV-infected wild-type and ROR γ t Tg mice 2 months p.i. and conducted lymphoproliferative assays using TMEV to stimulate cells. However, we found that the amount of lymphoproliferative responses to TMEV was similar between wild-type and ROR γ t Tg mice (cpm at 1 MOI $\pm$ SEM 2 months p.i.: wild-type, 732 ± 642 ; ROR γ t Tg, 652 ± 418 ; $P = 0.86$, t -test), demonstrating that overall TMEV-specific cellular (T cell) immune responses were similar between the two groups

(Supplemental Figure 2A). We also compared the function of APCs from wild-type and ROR γ t Tg mice with lymphoproliferative assays, where responder MNCs from TMEV-infected mice were stimulated with TMEV-APCs or Sham-APCs that were generated *in vitro* from naïve wild-type or ROR γ t Tg mice. We found that both groups of TMEV-APCs were able to stimulate responder MNCs equally, regardless of whether the MNCs originated from TMEV-infected wild-type or ROR γ t Tg mice (Supplemental Figure 2B).

Then, we hypothesized that ROR γ t overexpression could alter cytokine profiles, without changing overall anti-viral immune responses. We determined the cytokine profiles by stimulating MNCs with TMEV or a T cell mitogen, ConA. After stimulation with TMEV, MNCs from the ROR γ t Tg mice produced significantly more IL-17 (mean pg/ml after 1 MOI TMEV stimulation $\pm$ SEM: wild-type, undetectable; ROR γ t Tg, 28 ± 16 ; $P < 0.05$, *t*-test) and tended to produce less IFN- γ (Th1) and IL-10 than those from wild-type mice, while IL-4 was undetectable (Figures 4A–D). There were no significant differences in cytokine production induced by ConA between the mouse strains; cytokine production from unstimulated MNCs was not detectable in wild-type or ROR γ t Tg mice (data not shown).

Since we found a change in the IL-17 and IFN- γ responses in the ROR γ t Tg mice, we determined which cell types were contributing to the cytokine profiles. Flow cytometry on the MNCs from TMEV-infected mice demonstrated that there were similar numbers of CD4 $^{+}$ T cells and significantly less CD8 $^{+}$ T cells [cell number ($/10^6$) $\pm$ SEM: wild-type, 1.2 ± 0.12 ; ROR γ t Tg, 0.57 ± 0.15 ; $P < 0.05$, *t*-test] in the ROR γ t Tg mice compared with the wild-type mice (Figure 5A). Among CD4 $^{+}$ T cells, we found significantly more IL-17 $^{+}$ cells [cell number ($/10^5$) $\pm$ SEM: wild-type, 0.1 ± 0.07 ; ROR γ t Tg, 1.9 ± 0.08 ; $P < 0.05$, *t*-test] in the ROR γ t Tg mice, while there were similar amounts of IFN- γ , FOXP3, IL-4, and IL-10 positive cells (Figure 5B and data not shown). Among CD8 $^{+}$ T cells, the ROR γ t Tg mice had significantly fewer IFN- γ $^{+}$ cells [cell number ($/10^5$) $\pm$ SEM: wild-type, 1.5 ± 0.1 ; ROR γ t Tg, 0.1 ± 0.005 ; $P < 0.05$, *t*-test] compared with the wild-type mice. There were no significant differences in the number of IL-17/IFN- γ double-positive CD4 $^{+}$ or CD8 $^{+}$ T cells. Thus the increased IL-17 and reduced IFN- γ response to TMEV in the ROR γ t Tg mice can be explained by the increase in the number of IL-17 secreting CD4 $^{+}$ T cells and the decrease of IFN- γ secreting CD8 $^{+}$ T cells, respectively (Figure 5B).

We also compared humoral immune responses to TMEV between wild-type and ROR γ t Tg mice 2 months p.i. using ELISA; we found slightly less anti-TMEV IgG (heavy and light chain) responses in the ROR γ t Tg mice compared with the wild-type mice. Since Th subsets have been associated with immunoglobulin isotype class switching, we determined anti-TMEV IgG isotype responses. Consistent with the cytokine profile by ELISAs the ROR γ t Tg mice had significantly less anti-TMEV IgG2c (mean Ab titer $\pm$ SEM: wild-type, 13.3 ± 0.25 ; ROR γ t Tg, 11.3 ± 0.3 ; $P < 0.05$, *t*-test) and less IgG3 (mean Ab titer $\pm$ SEM: wild-type, 10.25 ± 1.0 ; ROR γ t Tg, 8.0 ± 0.6 ; $P = 0.15$, *t*-test) in their serum, both of these isotypes have been shown to be induced by IFN- γ (55), while other isotype levels were similar between the wild-type and ROR γ t Tg mice (Figure 5C).

ROR γ t Tg mice have reduced CD8⁺ T cell numbers and enhanced IL-17 production during the acute phase

Since events during the acute phase could affect the susceptibility to TMEV-IDD, we examined TMEV-infected ROR γ t Tg and wild-type mice for immunological differences during the acute phase, 1 week p.i. Immunologically, we compared the overall cellular and humoral immune responses to TMEV between the two groups. We found that the wild-type and ROR γ t Tg mice had similar levels of lymphoproliferation in response to TMEV (cpm at 1 MOI $\pm$ SEM 1 week p.i.: wild-type, 418 ± 129 ; ROR γ t Tg, 341 ± 155 ; $P = 0.72$, t -test) and anti-TMEV antibody responses (mean Ab titer $\pm$ SEM: wild-type, 11.7 ± 0.33 ; ROR γ t Tg, 11.7 ± 0.33 ; $P = 1.00$, t -test) (Supplemental Figures 3A & B). We also characterized the cytokine responses of the mice to TMEV during the acute phase. In response to TMEV, spleen MNCs from ROR γ t Tg mice secreted significantly more IL-17 (mean pg/ml after 1 MOI TMEV stimulation $\pm$ SEM: wild-type, undetectable; ROR γ t Tg, 50 ± 15 ; $P < 0.05$, t -test) and IFN- γ (mean ng/ml after 1 MOI TMEV stimulation $\pm$ SEM: wild-type, 0.86 ± 0.04 ; ROR γ t Tg, 1.4 ± 0.02 ; $P < 0.05$, t -test) compared with wild-type mice, while there were no significant differences in the amounts of IL-4 or IL-6 (Figures 6A–D). Flow cytometry on the spleen MNCs of the TMEV-infected mice demonstrated that the ROR γ t Tg mice had a significantly lower number of CD8⁺ T cells [number of CD8⁺ cells ($/10^6$) $\pm$ SEM: wild-type, 0.87 ± 0.11 ; ROR γ t Tg, 0.26 ± 0.02 ; $P < 0.05$, t -test] and similar numbers of CD4⁺ T cells and B cells (B220⁺ cells) compared with wild-type mice (Figures 7A, B and C). The wild-type mice and ROR γ t Tg mice had similar numbers of total MNCs per spleen (Supplemental Figure 3C). Among CD4⁺ T cells, while the ROR γ t Tg mice had similar percentages of IFN- γ positive cells compared with wild-type mice, the percentage of IL-17 positive cells was significantly higher (percentage of IL-17⁺ cells after gating on CD4⁺ cells $\pm$ SEM: wild-type, 0.25 ± 0.05 ; ROR γ t Tg, 9.3 ± 0.05 ; $P < 0.05$, t -test), and only the ROR γ t Tg mice had substantial numbers of IFN- γ /IL-17 double-positive cells (Figures 7D, E, and F). The wild-type and ROR γ t mice had similar numbers of FOXP3⁺ and IL-4⁺ CD4⁺ cells (data not shown).

ROR γ t Tg mice have more viral RNA present and enhanced Th17 immune responses in the CNS

To determine if the altered immune response to TMEV affected the replication and distribution of TMEV, we quantified viral RNA using real-time reverse transcription PCR on the tissues from the CNS and general organs of the mice 1 week p.i. We found that substantial viral RNA was present in the brain, spinal cord, heart and muscles from both wild-type and ROR γ t Tg mice, although viral RNA was not detectable in other organs, such as the liver and kidney (Figure 8A). While there were similar amounts of viral RNA in the heart and skeletal muscle, the ROR γ t Tg mice tended to have larger amounts of viral RNA in the CNS. To assess the immune responses in the brain during the acute stage of infection, we performed real-time reverse transcription PCR and found that the ROR γ t Tg mice expressed significantly more IL-17 and significantly less granzyme B, while expressing similar amounts of CD3 and IFN- γ (Figure 8B).

Discussion

In this report, we have demonstrated that a genetic bias toward a Th17 immune response could render a normally TMEV-resistant mouse strain susceptible to TMEV-IDD. A potential detrimental role of Th17 cells in TMEV-IDD has previously been reported by Hou et al, where simultaneous administration of LPS and TMEV can render resistant mice susceptible to a chronic TMEV infection, which was accompanied by induction of Th17 immune responses (33). Although LPS is not a pure Th17 immune response inducer (56), the authors suggested that the induction of Th17 immune responses by LPS cells may be impairing the function of anti-viral CTLs and preventing apoptosis of virus infected cells, facilitating viral persistence.

In the ROR γ t Tg mice, although lymphoproliferation in response to TMEV was similar to wild-type mice, we found a higher ratio of Th17 to Th1 immune responses compared with the wild-type mice, suggesting that the skewed Th balance could be responsible for the susceptibility. We also demonstrated higher levels of IL-17 mRNA in the spinal cord of TMEV-infected ROR γ t Tg mice than wild-type mice. Although IL-17 mRNA has been shown to be expressed in astrocytes and oligodendrocytes (57), it is likely that the increase in IL-17 mRNA is largely due to the presence of IL-17 producing T cells, since ROR γ t is being overexpressed in the T cells of the ROR γ t Tg mice. (37). Here, a more Th17 skewed immune response, which has been shown to be effective against extracellular pathogens but can inhibit CTL activity, would be inefficient at clearing a virus; this is in contrast to an anti-viral Th1 immune response, which can be effective against intracellular pathogens by promoting apoptosis of infected cells and CTL activity. In addition, we found that the ROR γ t Tg mice had significantly reduced expression of granzyme B in the brain, during the acute phase of infection, this supports a potential role of Th17 cells in suppression of CTLs, since granzyme B is a key effector molecule of CTLs (55). We also observed reduced levels of IgG2c and IgG3 anti-TMEV antibodies in the sera of ROR γ t Tg mice compared with wild-type mice; both of these antibody isotypes are associated with Th1 immune responses (55). The other antibody isotypes were present at similar levels in both mouse strains. Currently it is not completely understood which antibody isotype profile is associated with Th17 type immune responses, but our current results in the ROR γ t Tg mice will be useful to clarify it (58–60).

In contrast to the IL-17A blocking administration experiments in TMEV-IDD by Hou et al (33), where administration of IL-17A blocking antibody prevented susceptibility to TMEV-IDD, we did not see significant differences in TMEV-IDD in ROR γ t Tg mice receiving IL-17A blocking antibody or control antibody (Supplemental Figure 4). The discrepancy between Hou et al and our results is likely due to differences in the two experimental systems, 1) genetic differences between SJL/J and ROR γ t Tg (C57BL/6 background) mice, 2) differences in immunomodulation by LPS versus ROR γ t overexpression (LPS is not a pure IL-17 inducer), and that 3) differences in the disease severities and methods of evaluation of TMEV-IDD; where Hou et al demonstrated all SJL/J and LPS-treated C57BL/6 mice developed clinical signs, while no ROR γ t Tg (C57BL/6 background) mice developed clinical signs. Hou et al assessed the efficacy of the treatment by the incidence of mice with clinical signs, while we assessed the disease histologically. These factors may

alter the efficacy of IL-17A antibody treatment in TMEV-IDD. Additionally, IL-17A is not the only effector mechanism that contributes to pathogenesis by Th17 cells; Th17 cells can play an effector role themselves and secrete other effector cytokines that may contribute to pathogenesis in our model, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-17F, IL-21, and IL-22 (61–63).

Overall, the chronic CNS lesions in the TMEV-infected ROR γ t Tg mice were similar to what has been observed in SJL/J mice, where large amounts of demyelination were associated with CD3⁺ T cell infiltration and virus persistence. Inflammatory demyelination was observed in the spinal cord, but not the brain in ROR γ t Tg mice, similar to what occurs in TMEV-IDD in SJL/J mice. We found that areas of demyelination were similar between SJL/J and ROR γ t Tg mice; there was no data suggesting differences in remyelination between the two strains (64). In EAE, Th17 cells have been shown to infiltrate the cerebellum via the choroid plexus, leading to ataxic EAE (65–67). However, in our TMEV model, we did not observe lesions in the cerebellum or choroid plexus and no TMEV-infected mice developed ataxia.

However, there were significantly fewer damaged axons present in lesions compared with SJL/J mice, which may be due to inhibition of CD8⁺ CTLs by IL-17 and may explain the lack of clinical signs. This is consistent with the findings of Rivera-Quinones et al. (68), where TMEV infection in major histocompatibility complex (MHC) class I knock-out mice (CD8 deficient mice) resulted in preservation of motor function, despite a similar extent and distribution of demyelinating lesions to TMEV-infected SJL/J mice that had impaired motor function. In addition, they found that the amount of axonal preservation in the TMEV-infected CD8 deficient mice was greater than that in the TMEV-infected SJL/J mice and attributed the lack of clinical signs in the CD8 deficient mice to the axonal preservation. The authors suggested that MHC class I-restricted CD8⁺ T cells could contribute to axonal degeneration. Since ROR γ t Tg mice have lower numbers of CD8⁺ T cells, (discussed later) the lack of axonal damage and motor function impairment within inflammatory demyelinating lesions in our model and others support the hypothesis that CTLs are primarily responsible for axonal damage in TMEV-IDD.

To determine additional factors contributed to the susceptibility to TMEV-IDD in normally resistant C57BL/6 mice, we examined the differences during the acute phase of TMEV infection between the wild-type C57BL/6 and the ROR γ t Tg mice. Due to the alterations of the Th subset balance of the ROR γ t Tg mice, we initially suspected that the ROR γ t Tg mice may not mount anti-viral immune responses comparable to wild-type mice, leading to an altered distribution of virus in the general organs. However, we found no differences in lymphoproliferation to TMEV or anti-TMEV antibody responses between the strains, suggesting that there was no impairment of the overall immune response to TMEV. Additionally, a regular peripheral antigen administration induced similar lymphoproliferative responses between ROR γ t Tg and wild type mice [using purified protein derivative (Martinez et al, manuscript in preparation)]. We also found no additional pathology in the general organs of the mice, and the virus could only be found in the heart and muscle (in addition to the CNS) of the mice at similar amounts between the strains.

Thus, the susceptibility to TMEV-IDD of ROR γ t Tg mice does not seem to be due to immunodeficiency or the ability of the virus to infect additional organs.

Interestingly, during the acute phase, the ROR γ t Tg mice secreted significantly not only more IL-17 but also more IFN- γ in response to TMEV, compared with wild-type mice. Flow cytometry showed that the percentage of CD4⁺ T cells secreting IFN- γ alone was similar between the two groups, and that only the ROR γ t Tg mice had IL-17/IFN- γ double-positive cells. Since there was no increase in IFN- γ secreting CD8⁺ T cells in the ROR γ t Tg mice, the increase in IFN- γ production could be solely due to the double-positive cell population. Clinically and experimentally, it has been reported that IL-17/IFN- γ double-positive cells were present in inflamed tissue or the blood of individuals with chronic inflammatory conditions, including MS, and that they were short-lived and phenotypically closer to Th17 cells than Th1 cells (69–71). In these studies, based on the higher amounts of IL-17 and lower amounts of IFN- γ , the authors suggested that these double-positive cells exhibited a Th17 phenotype and eventually converted to IL-17⁺IFN- γ [−] cells. This is consistent with our finding during the chronic phase of TMEV infection, where few IL-17/IFN- γ double-positive cells were found. Since the ROR γ t Tg mice became susceptible to a chronic TMEV infection, the cytokine milieu in the ROR γ t Tg mice seemed not to support efficient viral clearance and is more aligned with a Th17 type milieu rather than a Th17/Th1 milieu.

We also found that there was a significant reduction in the number of CD8⁺ T cells in the ROR γ t Tg mice compared with the wild-type mice. This data along with the increased amount of IL-17 production in response to TMEV, the low amount of axonal damage, and decreased granzyme B expression in TMEV-infected ROR γ t Tg mice is consistent with previous findings by Hou et al that impaired or reduced CD8⁺ T cell activity plays a role in susceptibility to TMEV-IDD. CD8⁺ T cells appear to be a double-edged sword, where they can contribute to not only viral clearance but also axonal destruction. Here, to inhibit immunopathology caused by encephalitogenic CD8⁺ T cells, the host may boost Th17 immune responses, which would suppress CD8⁺ T cell activity and prevent motor dysfunction, but in turn increase susceptibility to a persistent viral infection.

The imbalance of an immune response shown here demonstrates how genetic factors could influence the susceptibility and severity of viral infections. Th17 cells can be detrimental to the host if they suppress anti-viral Th1 immune responses, leaving viral replication uncontrolled. This could result in persistent viral infections from viruses that are normally nonpathogenic in the general population, as we demonstrated in the current manuscript. These infections could in turn result in the release of antigens from immune-privileged tissue and cause autoimmunity by epitope (or determinant) spreading, which could lead to a chronic inflammatory disease such as MS. “Gain-of-function” mutations have been shown to cause auto-inflammatory diseases and change Th immune responses and susceptibility to microbial infections (33, 36), although they have not been shown to cause susceptibility to chronic virus infections in humans. Here we demonstrated how a gain-of-function mutation can cause susceptibility to a viral model of MS and possible trigger immunopathology. Translational application of information from our findings will be clinically useful in the future, for example, where if cytokine profiles are found that are biased toward Th17

immune responses in an individual whose family members with MS, the individual may be advised to take prophylactic medicine to block Th17 immune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant numbers and sources of support: Supported by the fellowships (F. Sato and S. Omura) from the Malcolm Feist Cardiovascular Research Endowment, Louisiana State University Health Sciences Center, and grants from the National Institute of Neurological Disorders and Stroke of the NIH (R21NS059724, I. Tsunoda), and the National Institute of General Medical Sciences COBRE Grant (8P20GM103433 and P30-GM110703, I. Tsunoda)

This work was supported by fellowships (F Sato and S Omura) from the Malcolm Feist Cardiovascular Research Endowment, LSU Health Sciences Center-Shreveport, and grants from the National Institute of General Medical Sciences COBRE Grant (8P20GM103433 and P30-GM110703). We thank Elaine A. Cliburn and Christi L. Eugene for excellent technical assistance.

References

1. Sato, F.; Omura, S.; Martinez, NE.; Tsunoda, I. Animal Models of Multiple Sclerosis. In: Minagar, A., editor. *Neuroinflammation*. Boston, MA: Elsevier; 2010. p. 55-66.
2. Martinez NE, Sato F, Omura S, Minagar A, Alexander JS, Tsunoda I. Immunopathological patterns from EAE and Theiler's virus infection: Is multiple sclerosis a homogenous 1-stage or heterogenous 2-stage disease? *Pathophysiology*. 2013; 20:71–84. [PubMed: 22633747]
3. Ferguson B, Matyszak MK, Esiri MM, Perry VH. Axonal damage in acute multiple sclerosis lesions. *Brain: a journal of neurology*. 1997; 120 (Pt 3):393–9. [PubMed: 9126051]
4. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bo L. Axonal transection in the lesions of multiple sclerosis. *The New England journal of medicine*. 1998; 338:278–85. [PubMed: 9445407]
5. Pirko I, Lucchinetti CF, Sriram S, Bakshi R. Gray matter involvement in multiple sclerosis. *Neurology*. 2007; 68:634–42. [PubMed: 17325269]
6. Sato F, Martinez NE, Omura S, Tsunoda I. Heterogeneity versus homogeneity of multiple sclerosis. *Expert Rev Clin Immunol*. 2011; 7(2):165–167. [PubMed: 21426254]
7. Owens GP, Gilden D, Burgoon MP, Yu X, Bennett JL. Viruses and multiple sclerosis. *The Neuroscientist: a review journal bringing neurobiology, neurology and psychiatry*. 2011; 17:659–76.
8. Koch MW, Metz LM, Agrawal SM, Yong VW. Environmental factors and their regulation of immunity in multiple sclerosis. *Journal of the neurological sciences*. 2013; 324:10–6. [PubMed: 23154080]
9. McCarthy DP, Richards MH, Miller SD. Mouse models of multiple sclerosis: experimental autoimmune encephalomyelitis and Theiler's virus-induced demyelinating disease. *Methods in molecular biology*. 2012; 900:381–401. [PubMed: 22933080]
10. Pender MP, Greer JM. Immunology of multiple sclerosis. *Current allergy and asthma reports*. 2007; 7:285–92. [PubMed: 17547851]
11. Donati, DSJ. Viruses and Multiple Sclerosis. In: KAB; JMG, editors. *Polymicrobial Diseases*. ASM Press; Washington, DC: 2002.
12. Tselis A. Evidence for viral etiology of multiple sclerosis. *Seminars in neurology*. 2011; 31:307–16. [PubMed: 21964847]
13. Daniels JB, Pappenheimer AM, Richardson S. Observations on encephalomyelitis of mice (DA strain). *The Journal of experimental medicine*. 1952; 96:517–30. [PubMed: 13022847]
14. Martinez NE, Sato F, Kawai E, Omura S, Chervenak RP, Tsunoda I. Regulatory T cells and Th17 cells in viral infections: implications for multiple sclerosis and myocarditis. *Future virology*. 2012; 7:593–608. [PubMed: 23024699]

15. Lipton HL. Theiler's virus infection in mice: an unusual biphasic disease process leading to demyelination. *Infection and immunity*. 1975; 11:1147–55. [PubMed: 164412]
16. Sato F, Martinez NE, Shahid M, Rose JW, Carlson NG, Tsunoda I. Resveratrol exacerbates both autoimmune and viral models of multiple sclerosis. *The American journal of pathology*. 2013; 183:1390–6. [PubMed: 24091251]
17. Lyman MA, Myoung J, Mohindru M, Kim BS. Quantitative, not qualitative, differences in CD8(+) T cell responses to Theiler's murine encephalomyelitis virus between resistant C57BL/6 and susceptible SJL/J mice. *European journal of immunology*. 2004; 34:2730–9. [PubMed: 15368289]
18. Murray PD, Pavelko KD, Leibowitz J, Lin X, Rodriguez M. CD4(+) and CD8(+) T cells make discrete contributions to demyelination and neurologic disease in a viral model of multiple sclerosis. *Journal of virology*. 1998; 72:7320–9. [PubMed: 9696828]
19. Peterson JD, Waltenbaugh C, Miller SD. IgG subclass responses to Theiler's murine encephalomyelitis virus infection and immunization suggest a dominant role for Th1 cells in susceptible mouse strains. *Immunology*. 1992; 75:652–8. [PubMed: 1350571]
20. Yamada M, Zurbriggen A, Fujinami RS. Monoclonal antibody to Theiler's murine encephalomyelitis virus defines a determinant on myelin and oligodendrocytes, and augments demyelination in experimental allergic encephalomyelitis. *The Journal of experimental medicine*. 1990; 171:1893–907. [PubMed: 1693653]
21. Tsunoda I, Fujinami RS. Neuropathogenesis of Theiler's murine encephalomyelitis virus infection, an animal model for multiple sclerosis. *Journal of neuroimmune pharmacology: the official journal of the Society on NeuroImmune Pharmacology*. 2010; 5:355–69. [PubMed: 19894121]
22. Welsh CJ, Steelman AJ, Mi W, Young CR, Dean DD, Storts R, Welsh TH Jr, Meagher MW. Effects of stress on the immune response to Theiler's virus--implications for virus-induced autoimmunity. *Neuroimmunomodulation*. 2010; 17:169–72. [PubMed: 20134194]
23. Gerety SJ, Rundell MK, Dal Canto MC, Miller SD. Class II-restricted T cell responses in Theiler's murine encephalomyelitis virus-induced demyelinating disease. VI. Potentiation of demyelination with and characterization of an immunopathologic CD4+ T cell line specific for an immunodominant VP2 epitope. *Journal of immunology*. 1994; 152:919–29.
24. Howe CL, Ure D, Adelson JD, LaFrance-Corey R, Johnson A, Rodriguez M. CD8+ T cells directed against a viral peptide contribute to loss of motor function by disrupting axonal transport in a viral model of fulminant demyelination. *Journal of neuroimmunology*. 2007; 188:13–21. [PubMed: 17493690]
25. Myoung J, Kang HS, Hou W, Meng L, Dal Canto MC, Kim BS. Epitope-specific CD8+ T cells play a differential pathogenic role in the development of a viral disease model for multiple sclerosis. *Journal of virology*. 2012; 86:13717–28. [PubMed: 23055563]
26. Harrington LE, Mangan PR, Weaver CT. Expanding the effector CD4 T-cell repertoire: the Th17 lineage. *Current opinion in immunology*. 2006; 18:349–56. [PubMed: 16616472]
27. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature*. 2006; 441:235–8. [PubMed: 16648838]
28. Hunderfean G, Neurath MF, Mudter J. Functional relevance of T helper 17 (Th17) cells and the IL-17 cytokine family in inflammatory bowel disease. *Inflammatory bowel diseases*. 2012; 18:180–6. [PubMed: 21381156]
29. Suryawanshi A, Veiga-Parga T, Rajasagi NK, Reddy PB, Sehrawat S, Sharma S, Rouse BT. Role of IL-17 and Th17 cells in herpes simplex virus-induced corneal immunopathology. *Journal of immunology*. 2011; 187:1919–30.
30. Yang W, Ding X, Deng J, Lu Y, Matsuda Z, Thiel A, Chen J, Deng H, Qin Z. Interferon-gamma negatively regulates Th17-mediated immunopathology during mouse hepatitis virus infection. *Journal of molecular medicine*. 2011; 89:399–409. [PubMed: 21191565]
31. Nakae S, Iwakura Y, Suto H, Galli SJ. Phenotypic differences between Th1 and Th17 cells and negative regulation of Th1 cell differentiation by IL-17. *Journal of leukocyte biology*. 2007; 81:1258–68. [PubMed: 17307864]

32. Matusevicius D, Kivisakk P, He B, Kostulas N, Ozenci V, Fredrikson S, Link H. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Multiple sclerosis*. 1999; 5:101–4. [PubMed: 10335518]
33. Hou W, Kang HS, Kim BS. Th17 cells enhance viral persistence and inhibit T cell cytotoxicity in a model of chronic virus infection. *The Journal of experimental medicine*. 2009; 206:313–28. [PubMed: 19204109]
34. Shoham NG, Centola M, Mansfield E, Hull KM, Wood G, Wise CA, Kastner DL. Pyrin binds the PSTPIP1/CD2BP1 protein, defining familial Mediterranean fever and PAPA syndrome as disorders in the same pathway. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:13501–6. [PubMed: 14595024]
35. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nature genetics*. 2001; 29:301–5. [PubMed: 11687797]
36. Masters SL, Simon A, Aksentjevich I, Kastner DL. Horror autinflammaticus: the molecular pathophysiology of autoinflammatory disease. *Annual review of immunology*. 2009; 27:621–68.
37. Yoh K, Morito N, Ojima M, Shibuya K, Yamashita Y, Morishima Y, Ishii Y, Kusakabe M, Nishikii H, Fujita A, Matsunaga E, Okamura M, Hamada M, Suto A, Nakajima H, Shibuya A, Yamagata K, Takahashi S. Overexpression of RORgammat under control of the CD2 promoter induces polyclonal plasmacytosis and autoantibody production in transgenic mice. *European journal of immunology*. 2012; 42:1999–2009. [PubMed: 22623033]
38. Martinez NE, Karlsson F, Sato F, Kawai E, Omura S, Minagar A, Grisham MB, Tsunoda I. Protective and Detrimental Roles for Regulatory T Cells in a Viral Model for Multiple Sclerosis. *Brain pathology*. 2014
39. Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalography and clinical neurophysiology*. 1972; 32:281–94.
40. Tsunoda I, Tanaka T, Fujinami RS. Regulatory role of CD1d in neurotropic virus infection. *Journal of virology*. 2008; 82:10279–89. [PubMed: 18684818]
41. Tsunoda I, Wada Y, Libbey JE, Cannon TS, Whitby FG, Fujinami RS. Prolonged gray matter disease without demyelination caused by Theiler's murine encephalomyelitis virus with a mutation in VP2 puff B. *Journal of virology*. 2001; 75:7494–505. [PubMed: 11462022]
42. Tsunoda I, Libbey JE, Fujinami RS. TGF-beta1 suppresses T cell infiltration and VP2 puff B mutation enhances apoptosis in acute polioencephalitis induced by Theiler's virus. *J Neuroimmunol*. 2007; 190:80–9. [PubMed: 17804084]
43. Tsunoda I, Kuang LQ, Libbey JE, Fujinami RS. Axonal injury heralds virus-induced demyelination. *The American journal of pathology*. 2003; 162:1259–69. [PubMed: 12651618]
44. Tsunoda I, Tanaka T, Terry EJ, Fujinami RS. Contrasting roles for axonal degeneration in an autoimmune versus viral model of multiple sclerosis: When can axonal injury be beneficial? *The American journal of pathology*. 2007; 170:214–26. [PubMed: 17200195]
45. Tsunoda I, Libbey JE, Kuang LQ, Terry EJ, Fujinami RS. Massive apoptosis in lymphoid organs in animal models for primary and secondary progressive multiple sclerosis. *The American journal of pathology*. 2005; 167:1631–46. [PubMed: 16314476]
46. Tsunoda I, Kuang LQ, Theil DJ, Fujinami RS. Antibody association with a novel model for primary progressive multiple sclerosis: induction of relapsing-remitting and progressive forms of EAE in H2s mouse strains. *Brain pathology*. 2000; 10:402–18. [PubMed: 10885659]
47. Jin YH, Kaneyama T, Kang MH, Kang HS, Koh CS, Kim BS. TLR3 signaling is either protective or pathogenic for the development of Theiler's virus-induced demyelinating disease depending on the time of viral infection. *Journal of neuroinflammation*. 2011; 8:178. [PubMed: 22189096]
48. Deb C, Lafrance-Corey RG, Zoecklein L, Papke L, Rodriguez M, Howe CL. Demyelinated axons and motor function are protected by genetic deletion of perforin in a mouse model of multiple sclerosis. *Journal of neuropathology and experimental neurology*. 2009; 68:1037–48. [PubMed: 19680139]
49. Seol D, Choe H, Zheng H, Jang K, Ramakrishnan PS, Lim TH, Martin JA. Selection of reference genes for normalization of quantitative real-time PCR in organ culture of the rat and rabbit intervertebral disc. *BMC research notes*. 2011; 4:162. [PubMed: 21615931]

50. Boda E, Pini A, Hoxha E, Parolisi R, Tempia F. Selection of reference genes for quantitative real-time RT-PCR studies in mouse brain. *Journal of molecular neuroscience*: MN. 2009; 37:238–53. [PubMed: 18607772]
51. Fukuda T, Yoshida T, Okada S, Hatano M, Miki T, Ishibashi K, Okabe S, Koseki H, Hirose S, Taniguchi M, Miyasaka N, Tokuhisa T. Disruption of the Bcl6 gene results in an impaired germinal center formation. *The Journal of experimental medicine*. 1997; 186:439–48. [PubMed: 9236196]
52. Libbey JE, Kirkman NJ, Smith MC, Tanaka T, Wilcox KS, White HS, Fujinami RS. Seizures following picornavirus infection. *Epilepsia*. 2008; 49:1066–74. [PubMed: 18325012]
53. Kirkman NJ, Libbey JE, Wilcox KS, White HS, Fujinami RS. Innate but not adaptive immune responses contribute to behavioral seizures following viral infection. *Epilepsia*. 2010; 51:454–64. [PubMed: 19845729]
54. Jin YH, Mohindru M, Kang MH, Fuller AC, Kang B, Gallo D, Kim BS. Differential virus replication, cytokine production, and antigen-presenting function by microglia from susceptible and resistant mice infected with Theiler's virus. *Journal of virology*. 2007; 81:11690–702. [PubMed: 17715222]
55. Murphy, K.; Travers, P.; Walport, M.; Janeway, C. *Janeway's immunobiology*. 8. New York: Garland Science; 2012.
56. Chang J, Kim BM, Chang CH. Co-stimulation of TLR4 and Dectin-1 Induces the Production of Inflammatory Cytokines but not TGF-beta for Th17 Cell Differentiation. *Immune network*. 2014; 14:30–7. [PubMed: 24605078]
57. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, Fugger L. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *The American journal of pathology*. 2008; 172:146–55. [PubMed: 18156204]
58. Mitsdoerffer M, Lee Y, Jager A, Kim HJ, Korn T, Kolls JK, Cantor H, Bettelli E, Kuchroo VK. Proinflammatory T helper type 17 cells are effective B-cell helpers. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:14292–7. [PubMed: 20660725]
59. Patakas A, Benson RA, Withers DR, Conigliaro P, McInnes IB, Brewer JM, Garside P. Th17 effector cells support B cell responses outside of germinal centres. *PloS one*. 2012; 7:e49715. [PubMed: 23166752]
60. Shibui A, Shimura E, Nambu A, Yamaguchi S, Leonard WJ, Okumura K, Sugano S, Sudo K, Nakae S. Th17 cell-derived IL-17 is dispensable for B cell antibody production. *Cytokine*. 2012; 59:108–14. [PubMed: 22503615]
61. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of T(H)17 cells. *Nature*. 2008; 453:1051–7. [PubMed: 18563156]
62. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annual review of immunology*. 2009; 27:485–517.
63. Jager A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *Journal of immunology*. 2009; 183:7169–77.
64. Dal Canto MC, Barbano RL. Remyelination during remission in Theiler's virus infection. *The American journal of pathology*. 1984; 116:30–45. [PubMed: 6742106]
65. Reboldi A, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, Lira S, Uccelli A, Lanzavecchia A, Engelhardt B, Sallusto F. C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nature immunology*. 2009; 10:514–23. [PubMed: 19305396]
66. Domingues HS, Mues M, Lassmann H, Wekerle H, Krishnamoorthy G. Functional and pathogenic differences of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *PloS one*. 2010; 5:e15531. [PubMed: 21209700]
67. Rothhammer V, Heink S, Petermann F, Srivastava R, Claussen MC, Hemmer B, Korn T. Th17 lymphocytes traffic to the central nervous system independently of alpha4 integrin expression during EAE. *The Journal of experimental medicine*. 2011; 208:2465–76. [PubMed: 22025301]

68. Rivera-Quinones C, McGavern D, Schmelzer JD, Hunter SF, Low PA, Rodriguez M. Absence of neurological deficits following extensive demyelination in a class I-deficient murine model of multiple sclerosis. *Nature medicine*. 1998; 4:187–93.
69. Kebir H, Ifergan I, Alvarez JI, Bernard M, Poirier J, Arbour N, Duquette P, Prat A. Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Annals of neurology*. 2009; 66:390–402. [PubMed: 19810097]
70. Momcilovic M, Miljkovic Z, Popadic D, Miljkovic D, Mostarica-Stojkovic M. Kinetics of IFN-gamma and IL-17 expression and production in active experimental autoimmune encephalomyelitis in Dark Agouti rats. *Neuroscience letters*. 2008; 447:148–52. [PubMed: 18852028]
71. Suryani S, Sutton I. An interferon-gamma-producing Th1 subset is the major source of IL-17 in experimental autoimmune encephalitis. *Journal of neuroimmunology*. 2007; 183:96–103. [PubMed: 17240458]
72. Syken J, Shatz CJ. Expression of T cell receptor beta locus in central nervous system neurons. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100:13048–53. [PubMed: 14569018]
73. Tsunoda I, Libbey JE, Fujinami RS. Sequential polymicrobial infections lead to CNS inflammatory disease: possible involvement of bystander activation in heterologous immunity. *Journal of neuroimmunology*. 2007; 188:22–33. [PubMed: 17604850]

Figure 1.

Spinal cord pathology during the chronic phase, 2 months post infection (p.i.), of Theiler's murine encephalomyelitis virus (TMEV) infection. Wild-type C57BL/6 mice did not develop inflammatory demyelination (**A**), while ROR γ t transgenic (Tg) C57BL/6 mice became susceptible to inflammatory demyelination (wide arrow heads: demyelination; narrow arrowheads: perivascular cuffing; arrows: meningoencephalitis) (**B**), that was similar to what occurred in susceptible wild-type SJL/J mice (**C**). Wild-type C57BL/6 mice did not have axonal damage (**D**); interestingly, the ROR γ t Tg C57BL/6 mice (**E**) had less axonal damage (arrowheads are representative SM311⁺ axons) than SJL/J mice (**F**), despite the comparable levels of demyelination between the two strains. **A, B** and **C**. Luxol fast blue staining. **C, D** and **E**. Immunohistochemistry against non-phosphorylated neurofilament with antibody SM311, a marker for damaged axons. These are representative spinal cord segments from three independent experiments consisting of 7 to 22 mice per experiment, for a total of 26 wild-type and 24 ROR γ t Tg mice. Magnifications: **A–C**, $\times 38$; **D–F**, $\times 38$, insets $\times 95$.

Figure 2.

Quantification of spinal cord pathology, T cells, and damaged axons, in TMEV-infected wild-type C57BL/6, ROR γ t Tg, and SJL/J mice 2 months p.i. The ROR γ t Tg (open bars) and the SJL/J mice (cross-hatched bars) had similar levels of meningitis, demyelination, and perivascular cuffing (**A**) and numbers of infiltrating CD3⁺ T cells (**B**) in the spinal cords, however the ROR γ t Tg mice had significantly fewer damaged axons than the SJL/J mice (*, $P < 0.05$) (**C**). The wild-type C57BL/6 mice had no inflammation or CD3⁺ T cell infiltration after TMEV infection. **A.** Quantification of inflammation by Luxol fast blue staining of the spinal cord of TMEV-infected mice 2 months p.i. Spinal cord segments (10–12 per mouse) were divided into four quadrants consisting of the ventral funiculus, the dorsal funiculus and each lateral funiculus. The total number of positive quadrants for each pathologic class was determined, then divided by the total number of quadrants present on the slide and multiplied by 100 to give the percent involvement for each pathologic class. The overall pathology was determined by counting the number of quadrants containing any lesions. **B.** Quantification of T cell infiltration using immunohistochemistry against CD3. The number of CD3⁺ cells was counted per spinal cord segment and divided by the total number of spinal cord segments for that mouse, and then the number of CD3⁺ cells per segment was averaged among the group. **C.** Quantification of damaged axons detected by immunohistochemistry using antibody SM311 against non-phosphorylated axons. The number of SM311⁺ cells was counted per spinal cord segment and divided by the total number of spinal cord segments for that mouse, and then the number of SM311⁺ cells per segment was averaged among five mice per group. These are representative results from three independent experiments consisting of 7–22 mice each, for a total of 26 wild-type and 24 ROR γ t Tg mice. #, $P < 0.05$ compared with ROR γ t Tg and SJL/J mice. ND: not detected.

Figure 3.

TMEV-infected RORγt Tg mice had virus persistence and chronic T cell infiltration, 2 months p.i. Wild-type mice did not have virus persistence or T cell infiltration in the white matter (WM) or grey matter (GM) (A and C), while RORγt transgenic (Tg) mice did in the WM (B and D). A few CD3⁺ cells were also detectable in the meninges in C57BL/6 mice, which was comparable to uninfected mice. Viral RNA was present in the spinal cords of RORγt Tg (open bars) but not wild-type (closed bars) mice (E). RORγt Tg mice expressed significantly more CD3, IL-17, and IFN- γ in the spinal cord compared with wild-type mice (E). A and B. Immunohistochemistry against TMEV. C and D. Immunohistochemistry against CD3. Note: CD3⁺ cells in the grey matter were morphologically neurons, but not T cells, neurons have been reported to be CD3⁺ by immunohistochemistry (72, 73). E. Ct values of TMEV, CD3, IL-17, and IFN- γ in the spinal cord were compared with PGK1. Ct was calculated by subtracting the PGK1_{Ct} from the TMEV_{Ct}. These are representative spinal

cord segments from three independent experiments consisting of 7 to 22 mice per experiment, for a total of 26 wild-type and 24 ROR γ t Tg mice. The real-time reverse transcription PCR data is from 4 wild-type and 5 ROR γ t Tg mice. Magnifications: **A** and **B**, $\times 64$, insets $\times 81$; **C** and **D**, $\times 76$, insets, $\times 216$.

Figure 4.

Cytokine responses to TMEV during the chronic phase of infection. The ROR γ t Tg mice (open bars) produced more interleukin (IL)-17 (A) (*, $P < 0.05$), tended to produce slightly less interferon (IFN)- γ (B) and IL-10 (C), and similar amounts of IL-4 (D) and IL-10 in response to TMEV and concanavalin A (ConA) compared with wild-type mice (closed bars). A–D. Mice were killed during the chronic phase 2–3 months p.i. and the splenic mononuclear cells (MNCs) were isolated and stimulated with ConA or TMEV for 48 hours. The levels of cytokine production in the culture supernatant were measured by enzyme-linked immunosorbent assay (ELISA). These are representative results from three independent experiments consisting of 7 to 22 mice per experiment, for a total of 26 wild-type and 24 ROR γ t Tg mice. ND: not detected.

Figure 5.

Flow cytometric analysis and antibody responses of TMEV-infected mice during the chronic phase of infection, 2 months p.i. ROR γ t Tg mice had similar numbers of CD4 $^+$ cells per spleen, but fewer CD8 $^+$ T cells (*, $P < 0.05$) compared with wild-type mice (**A**). After gating on CD4 $^+$ cells, we found that the ROR γ t Tg mice had a significantly higher number of IL-17 single positive and similar number of IFN- γ single positive cells compared with wild-type mice (**B**). After gating on CD8 $^+$ cells, we found that the ROR γ t Tg mice had significantly fewer IFN- γ positive cells compared with wild-type mice (**B**). The ROR γ t Tg mice (open box plots) produced slightly less IgG (H+L) than wild-type mice (shaded box plots) (**C**); ROR γ t Tg mice produced less IgG2c and IgG3 compared with wild-type mice, both of these isotypes are associated with IFN- γ and Th1 immune responses. **A and B.** Spleen MNCs were stimulated with TMEV for 48 hours and phorbol myristate acetate (PMA), ionomycin and brefeldin A for the last 6 hours and stained for the cell surface markers CD4, CD8, and cytokines IL-17 and IFN- γ . The results are mean $\pm$ standard error of mean (SEM); these results are representative of two independent experiments consisting of 10 mice each, for a total of 9 wild-type and 11 ROR γ t Tg mice. **C.** The levels of total IgG (heavy and light chains) and IgG isotypes against TMEV in sera were titrated by ELISA. These are representative results of three independent experiments consisting of 7–22 mice each, for a total of 26 wild-type and 24 ROR γ t Tg mice. Box plots: the closed triangle represents the median, the box represents the first and third quartiles, and the whiskers represent the minimum and maximum values.

Figure 6.

Cytokine responses to TMEV during the acute phase of infection, 1 week p.i. The ROR γ t Tg mice (hatched bars) produced significantly more IL-17 (A) and IFN- γ (B) (*, $P < 0.05$), but not significantly different levels of IL-4 (C) or IL-6 (D) in response to TMEV compared with wild-type mice (closed bars). A–D. MNCs were isolated from the spleens and stimulated with ConA or TMEV for 48 hours. The levels of cytokine production in the culture supernatant were measured by ELISA. Results are mean + standard error of mean (SEM). These results are representative of two independent experiments consisting of 8–11, for a total of 9 wild-type and 10 ROR γ t Tg mice. ND: not detected.

Figure 7.

Flow cytometric analysis of TMEV-infected mice during the acute phase of infection, 1 week p.i. RORγt Tg mice had similar numbers of CD4⁺ T cells and B cells (B220⁺ cells), but significantly fewer CD8⁺ cells compared with wild-type mice (*, $P < 0.05$) (**A**, **B**, and **C**). After gating on CD4⁺ cells, the RORγt Tg mice had a significantly higher percentage of IL-17 single positive and IL-17/IFN-γ double-positive cells, but no significant difference in the percentage of IFN-γ single positive cells compared with wild-type mice (**D**, **E**, and **F**). **A** and **D**. Spleen MNCs were stimulated with TMEV, PMA, ionomycin and brefeldin A and stained for the cell surface markers CD4, CD8, and B220 and cytokines IFN-γ and IL-17. **B**, **C**, **E** and **F**. Representative dot plots for **A** and **D**. Results are mean + standard error of mean (SEM). These results are representative of two independent experiments consisting of 8 or 11 mice, for a total of 9 wild-type and 10 RORγt Tg mice.

Figure 8.

Distribution of TMEV and immune responses in the brain during the acute phase of TMEV infection, 1 week p.i. We detected higher levels of viral RNA in the brains and spinal cords of ROR γ t Tg mice (hatched bars) compared with control mice (closed bars), and similar amounts in the general organs (**A**). In the brain, the ROR γ t Tg mice expressed significantly more IL-17 (*, $P < 0.05$) and significantly less granzyme B RNA compared with wild-type, while they expressed similar amounts of CD3 and IFN- γ (**B**). **A.** Ct values of viral protein 2 (VP2) compared with cellular levels of the house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPD) (heart, muscle, kidney, liver, and whole blood) or phosphoglycerate kinase (PGK) 1 (brain and spinal cord), by real-time PCR. **B.** Ct values of CD3, IL-17, IFN-g, and granzyme B in the brain compared with PGK1. Ct was calculated by subtracting the GAPD_{Ct} or PGK1_{Ct} from the TMEV_{Ct}. Results are mean + standard error of mean (SEM); these results are from 5 wild-type and 5 ROR γ t Tg mice. ND: not detected.