

REVIEW ARTICLE

Three immune-mediated disease models induced by Theiler's virus: Multiple sclerosis, seizures and myocarditis

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Abstract

Theiler's murine encephalomyelitis virus (TMEV) infection has been used as a viral model for multiple sclerosis (MS), as TMEV can induce chronic inflammatory demyelinating lesions with viral persistence in the spinal cord of SJL/J mice. In contrast, when C57BL/6 mice are infected with TMEV, the mice can clear the virus from the central nervous system (CNS), without viral persistence or demyelination, but develop seizures and hippocampal sclerosis, which has been used as a viral model for seizures/epilepsy. In the two TMEV-induced CNS disease models, not only viral infection, but also immune responses contribute to the pathogenesis. Interestingly, acquired immunity plays an effector role in the MS model, whereas innate immunity appears to contribute to the development of seizures. Recently, we have established the third TMEV-induced disease model, a mouse model for viral myocarditis, using C3H mice. TMEV-induced myocarditis is a triphasic disease, which mimics human myocarditis; phase I, mediated by viral replication in the heart and innate immunity; phase II, mediated by acquired immunity; and phase III, resulted from cardiac fibrosis. The genetic susceptibility to the aforementioned three models (MS, seizures and myocarditis) differs among mouse strains. We have compared and contrasted the three models induced by one single pathogen, TMEV, particularly in regard to the roles of T helper cells and natural killer T cells, which will give an insight into how interactions between the immune system and the host's genetic background determine the tissue tropism of virus and the development of virus-induced organ-specific immunopathology.

Introduction**Theiler's murine encephalomyelitis virus**

Theiler's murine encephalomyelitis virus (TMEV) infection in mice has been used as a viral model for multiple sclerosis (MS).^{1–4} More recently, TMEV infection has also been used as a mouse model for seizures/epilepsy⁵ and myocarditis.^{6,7} While immune responses and genetic background of hosts have been shown to play key roles in all three models, the immune effector cells/molecules and susceptible mouse strain in each model differ among the models. In the present review, we will compare and contrast the three distinct disease models induced by one single pathogen, TMEV, which will give an insight into how the interactions between the

immune system and the host's genetic background determine the tissue tropism of TMEV, as well as the development of virus-induced, organ-specific, immune-mediated diseases. We also discuss the prevalence, viral involvement, and comorbidities of MS, seizures and myocarditis.

Virology of TMEV

TMEV is a positive-sense, single-stranded (ss) RNA virus that belongs to the genus *Cardiovirus*, family *Picornaviridae*, order *Picornavirales* (Table 1).⁸ The TMEV virion contains a capsid that is composed of four structural proteins: VP1, VP2 and VP3 form the shell, whereas VP4 lies on its inner surface.⁹ TMEV binds to a cell surface receptor (sialic acid is

Table 1 Theiler's murine encephalomyelitis virus

Classification	<i>Theilovirus</i> , the genus <i>Cardiovirus</i> , family <i>Picornaviridae</i> , order <i>Picornavirales</i> Two subgroups: neurovirulent GDVII (GDVII, FA), demyelinating TO (BeAn, DA)
Genome (8.1 kb)	Positive-sense ssRNA with IRES and poly(A) One ORF encoding non-structural proteins and four capsid proteins VP1-4
Replication (8 h)	Receptor binding → uncoating → polypeptide translation and processing → virion assembly → release by cell lysis

GDVII, George's disease 7; IRES, internal ribosome entry site; ORF, open reading frame; ssRNA, single-stranded RNA; TO, Theiler's original.

used, at least, *in vitro*), while the plausible receptor binding site is a depression around the fivefold axis of the virion called the pit,¹⁰ leading to virion uncoating¹¹ and viral RNA genome delivery into the cytoplasm. The viral RNA genome, possessing a single long open reading frame, an internal ribosome entry site and a poly(A) tail, is infectious and has three roles: mRNA, template during replication and genetic material in the virion. The RNA genome is initially translated into a single protein, polyprotein, using an internal ribosome entry site.¹² Then, polyprotein is processed proteolytically to form functional multiple viral proteins; that is, four capsid proteins and non-structural proteins. Newly synthesized virions are released from the cell by lysis (lytic infection).

TMEV is divided into two subgroups, George's disease 7 (GDVII) and Theiler's original (TO) based on different neurovirulence after intracerebral infection.⁹ Highly neurovirulent GDVII and FA strains belong to the GDVII subgroup, which causes acute fatal polioencephalomyelitis with high levels of viral replication in neurons,¹³ neuronal apoptosis¹⁴ and axonal degeneration¹⁵ in the central nervous system (CNS), with neither induction of antiviral immune responses nor demyelination; all infected mice die within 1 week postinfection (p.i.). The TO subgroup viral strains, including less neurovirulent BeAn 8386 (BeAn), Daniels (DA), TO and WW, can induce a biphasic CNS disease.¹⁶ During the acute phase, similar to GDVII virus, the TO subgroup viruses infect neurons and cause polioencephalitis. Although inflammation is severe in the gray matter of the brain with limited spinal cord involvement, infected mice usually have no obvious clinical signs and all mice survive. Then, during the chronic phase, the mice develop spastic paralysis clinically and inflammatory demyelination histologically with viral persistence in the white matter of the spinal cord.¹⁷ The

susceptibility to the chronic demyelinating disease by the TO subgroup viruses depends on major histocompatibility complex (MHC) class I and is induced only in susceptible mouse strains, such as SJL/J mice, but not in resistant mouse strains, such as C57BL/6 (B6) or BALB/c mice, whereas acute neuronal infection in the gray matter of the brain (polioencephalitis) is caused by both GDVII and TO subgroup viruses in any mouse strains.¹⁸

TMEV-induced demyelinating disease

Roles of T helper cell subsets in viral infections and autoimmunity

MS is an inflammatory demyelinating disease of the CNS,^{19,20} affecting more than 2 million people worldwide; the prevalence of MS in North America and Europe is 0.1–0.2% of the population.²¹ Although the precise etiology of MS is unknown, MS is believed to be an immune-mediated disease,²² occurring in genetically susceptible individuals,²³ precipitated by one or more environmental agents,²⁴ particularly viruses, including Epstein-Barr virus (human herpesvirus [HHV]-4),²⁵ HHV-6, measles virus and retroviruses.^{21,26–28} CD4⁺ T helper (Th) cells have been proposed to play a key role in MS pathogenesis.^{29,30} CD4⁺ T cells can be divided into four subsets, Th1, Th2, Th17 and regulatory T cells (Tregs), based on their cytokine production: Th1 cells, interferon (IFN)- γ and interleukin (IL)-2; Th2 cells, IL-4, IL-5 and IL-13; Th17 cells, IL-17, IL-21 and IL-22; and Tregs, transforming growth factor (TGF)- β and IL-10 (Fig. 1).^{31–33} Specific transcription factor activation mediated by the cytokine milieu plays a central role in the differentiation toward distinct Th cell subsets: Th1 cells, T-box expressed in T cells (T-bet);³⁴ Th2 cells, GATA-binding protein 3 (GATA3);³⁵ Th17 cells, retinoic acid receptor-related orphan receptor- γ t (ROR γ t);³⁶ and Tregs, forkhead box P3 (Foxp3).

In viral infections, Th1 cells help antiviral cellular immune responses by activation of macrophages and generation of CD8⁺ cytotoxic T lymphocytes (CTL), whereas Th2 cells help production of antiviral antibodies. The roles of Th17 cells and Tregs for viral infections are largely unknown.³⁷ In autoimmune diseases, including an autoimmune model for MS, experimental autoimmune encephalomyelitis (EAE), Th1 and Th17 cells have been associated with organ-specific and cellular autoimmunities,^{38,39} whereas Th2 cells have been associated with systemic and humoral autoimmunities.⁴⁰ Tregs can suppress such autoreactive Th1 and Th17 immune responses.⁴¹

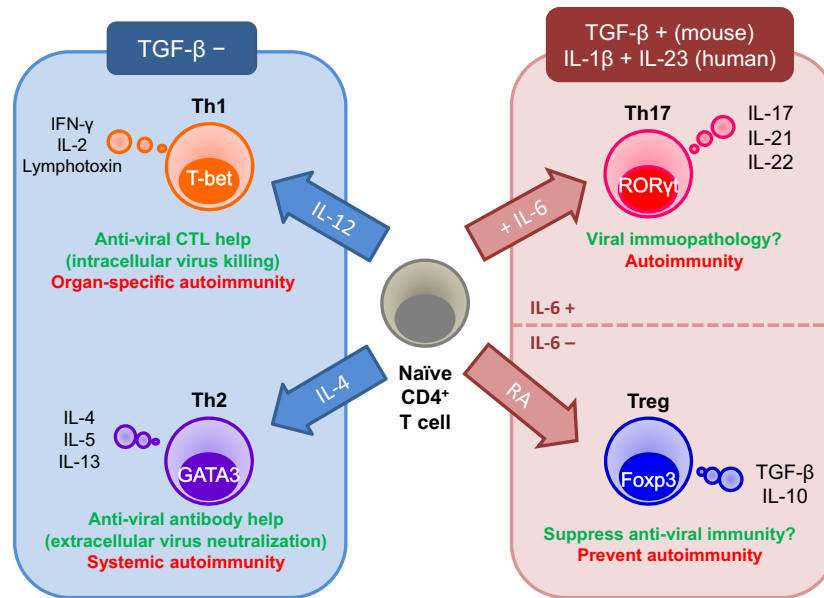


Figure 1 Roles of T helper (Th) cell subsets in viral infections and autoimmunity. Th cell subset differentiation is influenced by the cytokine milieu and master transcription factor. Th1 cells are induced in the presence of interleukin (IL)-12 and the transcription factor, T-box expressed in T cells (T-bet). Th1 cells help induction of antiviral cytotoxic T lymphocytes (CTL) and activation of macrophages that eradicate intracellular virus, while Th1 cells can also be a pathogenic effector in organ-specific autoimmunity. Th2 cells are induced by IL-4 and the transcription factor, GATA-binding protein 3 (GATA3). Th2 cells help production of antiviral antibody that neutralizes extracellular virus, but can be detrimental to promote autoantibody production in systemic autoimmunity. Although Th1 and Th2 differentiation is inhibited by transforming growth factor (TGF)- β , both Th17 cells and regulatory T cells (Tregs) are induced by TGF- β . In addition, Th17 cell differentiation requires IL-6, whereas Treg differentiation requires retinoic acid (RA). Th17 cells express the transcription factor, retinoic acid receptor-related orphan receptor- γ t (ROR γ t), secrete IL-17, 21 and 22, and might be involved in immunopathology in viral infections and autoimmunity. Tregs express the transcription factor, forkhead box P3 (Foxp3), and secrete the anti-inflammatory cytokines, TGF- β and IL-10, which can play a detrimental or protective role by suppressing antiviral immunity or autoimmunity, respectively.

Th1 cells have been shown to suppress Th2 and Th17 cells, and vice versa.⁴²

TMEV-induced demyelinating disease, a viral model for MS

TMEV-induced demyelinating disease (TMEV-IDD) has been most widely used as a viral model for MS.^{43,44} Historically, although TMEV was first isolated and characterized by Max Theiler early in January 1933,^{45,46} TMEV-IDD was discovered by Daniels et al.⁴⁷ in 1952, and started to be used as a viral model for MS after Howard L. Lipton⁴⁸ awakened interest in TMEV-IDD in 1975. Although the precise pathomechanism is unclear, both viral persistence and immune responses play pathogenic roles in TMEV-IDD. Unlike EAE, a pure autoimmune model for MS, adoptive transfer of immune cells from TMEV-infected mice into naïve mice does not induce demyelination; immune cell alone cannot induce TMEV-IDD.⁴⁹

Intracerebral, but not peripheral, injection of TMEV can result in a biphasic disease in the CNS,

depending on mouse strains. During the acute phase, 1 week p.i., the virus predominantly infects neurons in the gray matter of the brain, and induces polioencephalitis with neuronal apoptosis¹⁴ and axonal damage.¹⁵ Antiviral Th1, CTL and antibody responses contribute to viral clearance from the brain; here, resistant mouse strains, such as B6 and BALB/c mice, can eradicate the virus completely. In contrast, some mouse strains, including susceptible SJL/J mice and intermediate susceptible C3H mice, cannot clear the virus from the CNS;^{13,18} during the subclinical phase, 2–3 weeks p.i., although the virus is largely cleared from the brain, the virus is axonally transported to the white matter of the spinal cord,^{50,51} where axonal degeneration and microglia activation increase over time.⁵² Infected mice start to show obvious paralysis approximately 1 month p.i. during the chronic phase, when inflammatory demyelination is histologically present in the white matter of the spinal cord (Fig. 2); it should be noted that, in TMEV-IDD, axonal degeneration precedes demyelination (“Inside-Out model”).^{12,53} During the chronic phase, persistent viral infection can be

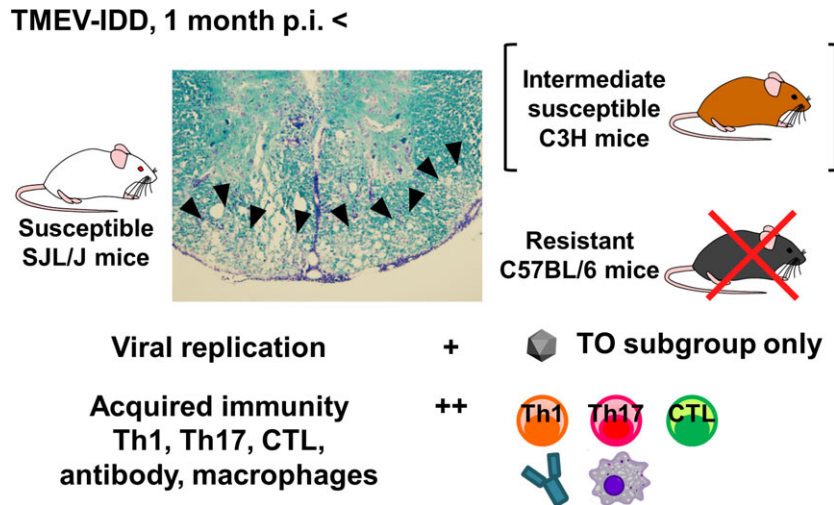


Figure 2 Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD), a viral model for multiple sclerosis. During the chronic phase of TMEV infection, 1 month postinfection (p.i.), susceptible SJL/J mice develop severe inflammatory demyelination (arrowheads; Luxol fast blue stain) in the spinal cord, whereas no lesion is observed in the spinal cord of resistant C57BL/6 mice. As C3H mice develop mild demyelination in the spinal cord, C3H mice are intermediately susceptible to TMEV-IDD. The Theiler's original (TO), but not GDVII, subgroup of TMEV can cause TMEV-IDD. Both viral pathology and immunopathology play key roles in TMEV-IDD. During the chronic phase, TMEV persistently infects glial cells and macrophages in the spinal cord at low levels; viral persistence is necessary for the pathogenesis of TMEV-IDD. Acquired immune responses, including Th1 and Th17 cells, CTL, antibodies and macrophages, also play a pathogenic role in TMEV-IDD.

detected in macrophages and glial cells, including oligodendrocytes,^{13,54,55} whereas TMEV-specific Th1 cells and CTL have been proposed to play pathogenic roles in demyelination.^{56,57} Anti-TMEV antibodies can cross-react with myelin antigens, further contributing to demyelination.⁵⁸ Thereafter, the disease progresses continuously with no remission.

Roles of Th17 cells and Tregs in TMEV-IDD

In EAE, Th17 cells can induce immune-mediated tissue damage (immunopathology) by production of pro-inflammatory cytokines,^{1,59–61} whereas Tregs can play a protective role by suppressing immunopathology; for example, by production of anti-inflammatory cytokines, such as IL-10 and TGF- β .⁶² Here, the roles of Th17 cells and Tregs had mainly been investigated in EAE, whose Th17/Treg responses were decreased by blocking antibodies, small interfering RNA or gene knockout (KO) mice ("loss-of-function" approach).³⁸ This raised questions as to: (i) whether the Th17/Treg responses play the same role in TMEV-IDD as in EAE, as the roles of Th17 cells and Tregs in viral infections are largely unclear; and (ii) whether an increase in Th17/Treg responses affects inflammatory demyelinating diseases; this is a clinically relevant concern, as "gain-of-function" mutations have been shown to alter immune responses, including Th cells, and to

increase the susceptibility to several inflammatory diseases and infections in humans.^{63,64}

We determined the roles of Th17 cells and Tregs in TMEV-IDD.^{65,66} To clarify how increased Th17/Treg immune responses can influence TMEV-IDD, where both viral pathology (viral replication) and immunopathology play key roles, we have established two innovative "gain-of-function" approaches: (i) ROR γ t transgenic (Tg) B6 mice overexpressing ROR γ t, which have increased Th17 cells;^{66,67} and (ii) adoptive transfer of *ex vivo* expanded induced Tregs (iTregs).^{65,68,69}

Although TMEV-infected wild-type B6 littermates, which are known to be resistant to TMEV-IDD,⁷⁰ do not develop chronic demyelination in the CNS, TMEV-infected ROR γ t Tg B6 mice develop severe demyelinating lesions (with relative preservation of axons [see below]) and have viral persistence, higher levels of IL-17, lower levels of IFN- γ and fewer CD8⁺ T cells.⁶⁶ Here, we have proposed three roles of Th17 cells in TMEV-IDD (Fig. 3). First, Th17 cells could impair antiviral CTL, promoting viral replication and persistence, which is consistent with a theory proposed by Hou et al.⁷¹ that IL-17 could inhibit antiviral CTL functions and might be responsible for susceptibility to TMEV-IDD. Second, increased Th17 cells in ROR γ t Tg B6 mice could play a role as a pro-inflammatory effector cells, contributing to inflammatory demyelination (immunopathology). Third, Th17 cells

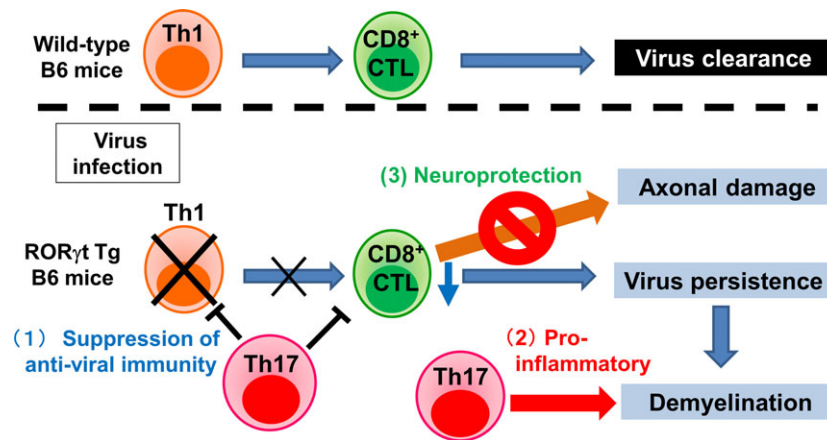


Figure 3 Three roles of Th17 cells in TMEV infection. (Upper) Wild-type C57BL/6 (B6) mice mount an antiviral Th1 response, which helps the generation of CD8⁺ CTL, leading to viral clearance. (Lower) In RORγt transgenic (Tg) B6 mice, increased Th17 cells (1) suppress antiviral Th1 and CTL responses, resulting in viral persistence; and (2) secrete pro-inflammatory cytokines, causing inflammatory demyelination (detrimental role of Th17 cells). In contrast, Th17 cells can (3) suppress anti-axonal (autoreactive) CD8⁺ CTL, preventing axonal damage (neuroprotective role of Th17 cells).

might impair anti-axonal CTL, resulting in suppression of axonal damage (neuroprotective role). In TMEV infection, although CTL are important for viral clearance, CTL have also been proposed to induce axonal damage as effector cells.⁷² Interestingly, although we observed severe inflammatory demyelinating lesions in the spinal cord of TMEV-infected RORγt Tg B6 mice comparable with those of infected SJL/J mice, RORγt Tg B6 mice did not develop paralysis clinically and had only minimum axonal damage histologically. Thus, viral persistence with relative preservation of axons in RORγt Tg B6 mice could be a result of inhibition of effector CTL functions by Th17 cells.

iTregs can be expanded by our method to generate approximately 20 million iTregs from the spleen of one naive mouse.^{68,69} These iTregs express CD4, Foxp3 and CD25, and can suppress the proliferation of other CD4⁺ T cells *in vitro* as well as suppress inflammation in a mouse model of inflammatory bowel disease in B6 mice *in vivo*. We determined the role of iTregs during the time-course of TMEV infection by administering iTregs to TMEV-infected SJL/J mice either at day 0 p.i. (iTreg-early mice) or day 30 p.i. (iTreg-late mice).⁶⁵ During the acute phase, iTreg-early mice showed more severe clinical signs compared with control mice (TMEV infection alone without iTreg injection): iTreg-early mice lost more weight with an impaired righting reflex, and had higher viral replication in the CNS than control mice. As less inflammation was observed in the CNS of the iTreg-early mice compared with control mice, this suggests that Tregs prevent antiviral

immune cells from homing to the CNS, resulting in more active viral replication in iTreg-early mice. In contrast, during the chronic phase, less inflammatory demyelination with higher production of IL-10 was observed in iTreg-late mice, compared with controls, suggesting a protective role of Tregs. In summary, these results showed that: (i) Th17 cells could play a detrimental role in TMEV-IDD, to induce immunopathology with viral persistence, as well as a beneficial role to protect axonal degeneration; and (ii) Tregs could also play a detrimental role to suppress antiviral immune responses, favoring viral replication, as well as a beneficial role to inhibit immunopathology.

TMEV-induced myocarditis

Viral myocarditis

Myocarditis is inflammation of the heart muscle.^{73,74} The discovery of myocarditis in 1–9% of autopsies showed that myocarditis is an underdiagnosed cause of sudden death.^{73–77} For example, studies of military recruits found that 20–42% of sudden deaths were attributable to unsuspected myocarditis.^{78,79} Myocarditis most commonly results from infections with viruses (38–80%), including adenovirus, HHV-6, parvovirus B19, hepatitis C virus and picornaviruses (coxsackievirus B [CVB] and enterovirus).^{80–83}

Viral myocarditis has been proposed to be a triphasic disease:^{37,75,77,81,84} in phase I, the disease is triggered by viral infection in the heart (viral pathology); in phase II, cardiomyocytes are damaged by uncontrolled antiviral immune and/or autoimmune

responses that can be generated by molecular mimicry and determinant (or epitope) spreading (immunopathology);⁴⁰ in phase III, as a result of phases I and II, cardiac fibrosis and remodeling lead to dilated cardiomyopathy (Fig. 4).⁸⁵ Depending on pathogens and the host's genetic background, pathophysiology of viral myocarditis might be different. Some viral infections only cause viral pathology (phase I) with no involvement of acquired immunity (e.g. only phase I in human parvovirus infections and a murine reovirus model, where no immune cell infiltrates in the heart).⁸⁶

In human viral myocarditis, the roles of Th subsets remain unclear, partly because only small numbers of immunological studies have been carried out in humans. Endomyocardial biopsies from patients with acute myocarditis and dilated cardiomyopathy showed the presence of several different cardiotropic viruses, and increased markers of Th1 cells, without changes in Th2 and Th17 markers.⁸⁷ In the

peripheral blood from viral myocarditis, high levels of Th1 cells were associated with early cardiomyocyte damage, whereas Th2 cells increased during the recovery phase.⁸⁸ Although these findings need to be confirmed by analyses of larger cases, they are consistent with what has been observed in other immune-mediated diseases, where Th1 cells are pro-inflammatory, and Th2 cells are anti-inflammatory (Table 2).

TMEV-induced myocarditis, a novel viral myocarditis model

During the acute phase, TMEV has been observed to cause viremia and infect various organs, including the skeletal muscle and the heart.⁸⁹ Historically, although Gómez et al.^{7,90} described TMEV-induced cardiac pathology in 1976, TMEV was not used for myocarditis research until we used it in a mouse model for viral myocarditis. We showed that,

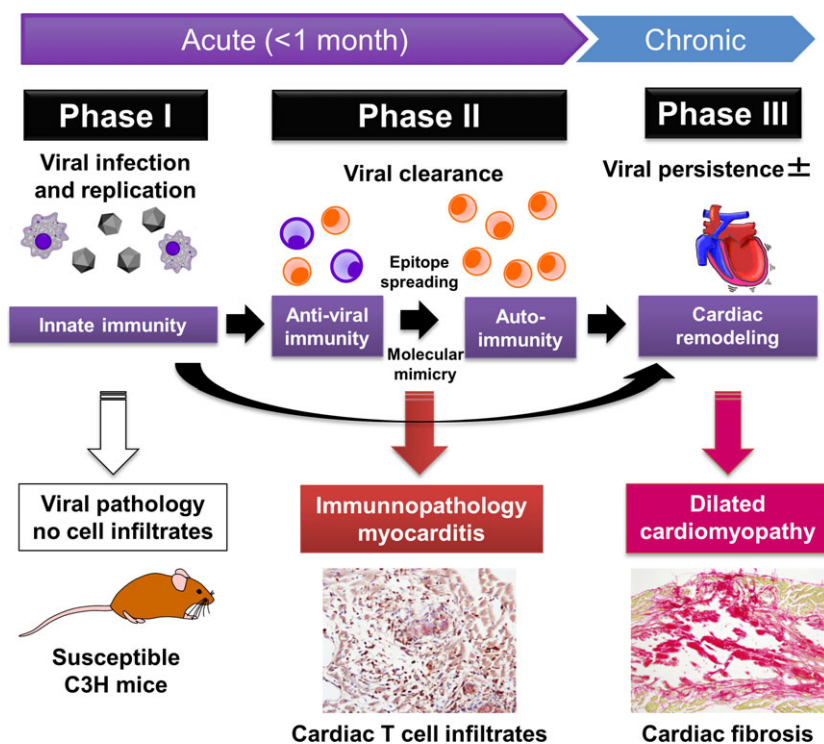


Figure 4 Possible pathomechanisms in the three phases of viral myocarditis. In phase I, viral infection and replication in the heart cause myocardial damage directly (viral pathology). Innate immune responses to the virus play a key role in viral clearance, but might also damage cardiomyocytes. There are no immune cell infiltrates in the heart in phase I. In phase II, antiviral acquired immune responses, including Th1 cell (orange) and Th2 cells (purple), are induced to clear the virus. When antiviral immune responses are uncontrolled, T cells infiltrate in the heart (visualized by immunohistochemistry using anti-CD3 antibody), leading to myocardial damage in a bystander fashion or by generation of heart-specific autoimmunity through determinant (or epitope) spreading from viral antigens to cardiac antigens (immunopathology). In addition, if viral and cardiac antigens share immune epitopes, the "molecular mimicry" also results in immunopathology. When the myocardial damage is severe either in phase I or II, cardiac remodeling and fibrosis (visualized by picrosirius red staining) occur, resulting in dilated cardiomyopathy, regardless of the presence or absence of viral persistence in phase III.

Table 2 Possible roles of T helper cell subsets and natural killer T cells in myocarditis

	Th1	Th2	Th17	NKT
Cytokines	IFN- γ	IL-4, IL-10	IL-17	IL-4, IFN- γ
Human cases	Cardiac damage	<i>Recovery</i>	No role	?
CVB model	<i>Protective</i>	Inflammation	Inflammation	No role or <i>Anti-viral</i>
TMEV model	Detrimental	<i>Protective</i>	No role	<i>Protective</i>
Mutant mice [†]	T-bet Tg	GATA3 Tg	ROR γ t Tg	J α 18 KO

CVB, coxsackievirus B; IFN, interferon; IL, interleukin; TMEV, Theiler's murine encephalomyelitis virus.

[†]Transgenic (Tg) and knockout (KO) mice used for myocarditis research. Protective role in italics. Detrimental role with bold. T-box expressed in T cells (T-bet) Tg, GATA-binding protein 3 (GATA3) Tg and retinoic acid receptor-related orphan receptor- γ t (ROR γ t) Tg mice have increased numbers of T helper (Th)1, Th2, and Th17 cells, respectively, whereas J α 18KO mice are deficient in natural killer T (NKT) cells.

depending on mouse strains, TMEV injection can lead to myocarditis; a high-dose intraperitoneal TMEV injection is the most efficient and consistent route for myocarditis induction, whereas an intracerebral injection of TMEV also induces myocarditis.⁶ The susceptibility to and progression of TMEV-induced myocarditis differ among mouse strains; C3H mice developed all three phases, whereas B6 mice had only mild cell infiltrates (phase II) without cardiac fibrosis. No SJL/J mice had immune cell infiltrates in the heart histologically; SJL/J mice developed only phase I cardiac pathology.

Using C3H mice, we have determined viral replication, cardiac damage (quantified by serum cardiac troponin), immunopathology and biomarkers in each phase of myocarditis, with multivariate data, such as virological and histological data, as well as a principal component analysis of microarray transcriptome data of the infected hearts. In phase I, although the heart appeared normal, we found active viral replication and upregulation of innate immune molecules, such as IFN-induced genes and chemokines, including IP-10/CXCL10 (IFN- γ -induced protein 10/C-X-C motif chemokine ligand 10). In phase II, the viral titer in the heart was lower than in phase I, whereas cardiomyocyte damage quantified by serum cardiac troponin was greater than in phase I. We found CD3⁺ T-cell infiltrates in the heart by immunohistochemistry, as well as upregulation of genes associated with CD3, pro-inflammatory IFN- γ pathway and MHC class II by microarray, suggesting involvement of MHC class II-restricted Th1 cells. In phase III, although we found only a small number of T cells with no viral replication in the heart, progressive cardiac fibrosis was observed by picrosirius red staining and Masson's trichrome staining, which was consistent with upregulation of cardiac remodeling genes, such as matrix metalloproteinase 12.

We have characterized the role of immune cells, using T-bet, GATA3, or ROR γ t Tg mice, and NKT cell-

deficient J α 18 KO mice.⁹¹ Among the Tg and KO mice infected with TMEV, Th1-biased T-bet Tg mice and NKT KO mice developed more severe myocarditis with lower ejection fraction in echocardiography than wild-type mice, whereas Th2-biased GATA3 Tg mice were resistant to TMEV-induced myocarditis.⁹¹ In summary, our studies suggest a pathogenic role of pro-inflammatory Th1 cells, and a protective role of Th2 cells and NKT cells, which were consistent with the findings in human viral myocarditis cases (Table 2). The working hypothesis of TMEV-induced myocarditis is as follows (Fig. 5): in phase I, viral infection and replication in the heart induce innate immune molecules, IFN and chemokines (e.g. IP-10/CXCL10), which recruit Th cell subsets and NKT cells.⁹² The balance between pro-inflammatory versus regulatory cytokines determines the extent of immune-mediated tissue damage (immunopathology) in phase II. Severe immunopathology can lead to cardiac fibrosis and remodeling in phase III.

Unlike TMEV-induced myocarditis model, other current viral and autoimmune myocarditis models have not consistently reproduced the findings in human viral myocarditis. For example, in the CVB model and some other models, Th1 cells seemed to play a protective role, whereas Th2 and Th17 cells play a pathogenic role.^{93,94} This suggests that some of these models mimic human acute fulminant fatal myocarditis (animals succumb to acute myocarditis; thus, these models are inappropriate to study phase III), where viral pathology is the major cause of cardiac damage, whereas inflammation has beneficial effects to complete viral clearance.^{77,95}

Among other immune cells, NKT cells are known as major effector cells that can produce both IFN- γ and IL-4.⁹⁶ In other immune-mediated diseases, NKT cells can play a protective role in Th1/Th17-mediated diseases by production of IL-4.⁹⁷ In CVB infection, however, the findings are not consistent: both wild-type and NKT KO mice developed severe

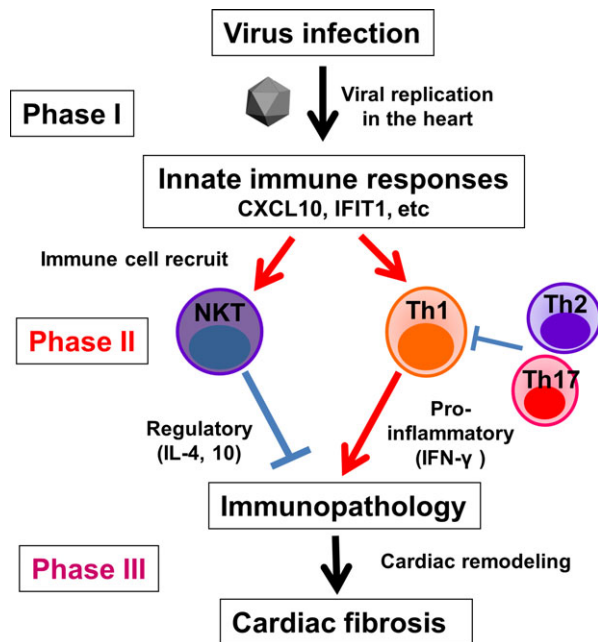


Figure 5 The working hypothesis of the mechanism of viral myocarditis. In phase I, viral infection and replication induce innate immune responses, upregulating chemokine and IFN-related genes (e.g. IFIT1) in the heart. In phase II, chemokines (e.g. CXCL10) recruit Th1 and natural killer T (NKT) cells to the heart. Th1 cells secrete pro-inflammatory IFN- γ , resulting in cardiac inflammation. In contrast, NKT cells produce IL-4 and IL-10, suppressing inflammation, while Th2 and Th17 cells might also suppress Th1 cells. The imbalance between pro-inflammatory versus anti-inflammatory immune responses causes immunopathology, followed by cardiac remodeling and fibrosis in phase III.

myocarditis, suggesting that NKT cells might play no significant role,⁹⁸ whereas a potential antiviral role has been suggested by others.⁹⁹ The inconsistency in immunopathology between human viral myocarditis and current animal model systems can be due to several factors:^{85,100} (i) some viruses are used in their non-natural hosts (e.g. the natural host of CVB is humans, not mice, whereas TMEV is a natural pathogen of mice);¹⁰¹ (ii) the pathophysiology of human viral myocarditis is heterogeneous – one model cannot represent all cases (e.g. the reovirus model develops only phase I,¹⁰² whereas other models represent fulminant fatal myocarditis); (iii) some viral models are only inducible in neonatal animals; and (iv) an autoimmune model, experimental autoimmune myocarditis does not have microbial components.

TMEV-induced seizures/epilepsy

Seizures and epilepsy

A seizure is a clinical sign due to transient dysfunction of the brain, which is caused by an abnormal

electrical discharge of neurons, while epilepsy is a chronic disorder with recurrence of seizures.¹⁰³ Epidemiological studies of epilepsy are interested in active epileptic patients who have had at least one seizure during the past 5 years or are treated with anti-epileptic drugs. The prevalence of active epilepsy per 1000 population ranges from 6.2 to 7.6 in Europe and North America. Globally, a total of 43 million people have active epilepsy: 10 million children, 30 million adults and 3 million older adults.¹⁰⁴

The three main pathologies from epilepsy surgery, 70–80% of which are for the treatment of temporal lobe epilepsy, are hippocampal sclerosis, cortical dysplasia and low-grade tumors. In hippocampal sclerosis (Ammon's horn sclerosis), pyramidal neuronal loss and gliosis are identified mainly in the CA1 subfield and in the hillock region with sparing of the subiculum, and relative resistance of CA2 pyramidal neurons and dentate granule cells¹⁰⁵ (the hippocampus proper has three subdivisions: CA3, CA2 and CA1; CA comes from cornu ammonis¹⁰⁶).

Most cases of epilepsy result from interactions between genetic and non-genetic factors;¹⁰⁷ risk factors for the development of seizures/epilepsy, including head trauma, brain tumors, CNS infection and inflammation, and cerebrovascular disease, vary with age, geographic location and other conditions.¹⁰⁸ Clinically, several different viruses have been suggested to play a role in the development of seizures and epilepsy; HHV-6, herpes simplex virus type 1 (HSV-1 = HHV-1), Japanese encephalitis virus, Nipah virus and human immunodeficiency virus, as well as viruses in the family *Picornaviridae*, such as coxsackievirus, enterovirus and parechovirus, have been linked to seizures and epilepsy.⁵ Experimentally, although viral infections have been used to induce seizures in animals, some of them have not been used as an ideal animal model to study the roles of immune responses during and after infections, as infected animals in these models often died of acute encephalitis. In contrast, TMEV-induced seizures have been used as a unique animal model for seizures, as (i) all mice infected with the DA strain of TMEV survive after infection and (ii) host animals are inbred B6 mice, which have many advantages, such as a well-characterized immune system, and availabilities of KO and Tg mice, as well as immunomodulatory reagents.

TMEV-induced seizures, a viral model for seizures

Historically, TMEV-induced seizures were first observed inadvertently¹⁰⁹ during experiments that

aimed to see a role of axonal degeneration in TMEV infection, using C57BL/Wld^s (C57/OlaHsd-Wld, Wallerian degeneration slow mutant [Wld]) mice.⁷⁰ Wld mice are a substrain of B6 mice, and have prolonged survival of the distal stumps of transected axons. Because TMEV can use axons to disseminate in the CNS, slow axonal degeneration in Wld mice favors viral spread, compared with wild-type B6 mice, suggesting that axonal degeneration in B6 mice could be a beneficial mechanism that limits the viral spread.^{12,50} Unexpectedly, both Wld and B6 mice infected with TMEV developed seizures, 3–6 days p.i. TMEV-induced seizures in mice with a B6 genetic background were not recognized until the present study, as the B6 mice strain is resistant to TMEV-IDD, thus, not usually used in TMEV research.

In the aforementioned initial set of experiments,¹⁰⁹ we found that, in TMEV-induced seizures: (i) both the GDVII and DA strains of TMEV induce seizures – the GDVII strain does not induce a TMEV-specific immune response,¹³ thus, acquired immunity has no role; (ii) unlike B6, other mouse strains resistant to TMEV-IDD, such as BALB/c,⁹⁸ did not develop seizures; (iii) the incidence of seizures is associated with hippocampal CA1 lesions, such as neuronal loss and apoptosis;¹⁴ and (iv) axonal degeneration has no role, as both Wld and wild-type B6 mice develop seizures with similar severity and incidence.

Subsequent studies have further characterized TMEV-induced seizures.⁵ Seizures were observed as early as on day 3, and peaked on day 6 after infection with any TMEV strains examined, including the GDVII strain, less neurovirulent TO subgroup viral strains (DA, BeAn, WW) and mutant strains (H101⁸⁹ and DApBL2M^{110,111}). The seizures lasted for 1–2 min with a Racine scale seizure score 3 (forelimb clonus), 4 (rearing) or 5 (rearing and falling).^{6,112,113} During the chronic phase, 2–7 months p.i., TMEV-infected B6 mice developed spontaneous epileptic seizures, monitored by video electroencephalogram, with hippocampal atrophy histologically.¹¹⁴ Innate immune responses, but not acquired immune responses, seem to contribute to seizures,¹¹⁵ as the number of infiltrating macrophages, but not lymphocytes, were associated with seizures (Fig. 6).¹¹⁶ Among molecules in the innate immune system, IL-6,¹¹⁷ tumor necrosis factor- α and complement component 3¹¹⁸ have been suggested to contribute to the development of seizures, but other factors, including polymorphonuclear cells, natural killer cells,¹¹⁷ NKT cells,⁹¹ IL-1, MyD88¹¹⁵ or toll-like receptor 4,⁶ were

found to not contribute to the development of seizures in B6 mice.

While the TMEV-induced seizure model is a rare viral model for seizures/epilepsy, further studies are required to clarify the pathomechanism. For example, although NBQX, a glutamate receptor antagonist, has been shown to suppress seizures in other animal models, it exacerbated TMEV-induced seizures.¹¹⁹ Furthermore, plausible contributing factors to seizures in B6 mice, such as macrophage infiltration and pro-inflammatory cytokine upregulation, have also been seen in the brain from TMEV-infected SJL/J mice that are resistant to TMEV-induced seizures.¹²⁰

Prevalence, virus infections, and comorbidities in MS, myocarditis and seizures

In TMEV infection, C3H mice develop all three immune-mediated diseases: (i) inflammatory demyelination in the spinal cord; (ii) myocarditis in the heart; and (iii) seizures triggered by the brain lesions, whereas SJL/J mice or C57BL/6 mice develop only demyelination or seizures, respectively (Fig. 7). This shows that one single virus can induce any three organ-specific immune-mediated diseases depending on the host's genetic background.

Although MS, myocarditis, and seizures/epilepsy are common diseases with their prevalence of 0.1–0.2, 1–9 and 0.5–1.0%, respectively,¹⁰⁴ the comorbidities among the three diseases have not been investigated, except between MS and epilepsy.¹²¹ The prevalence of epilepsy is 0.5–1.0% in the general population.^{104,122} Several MS cohort- and population-based studies reported that the prevalence of epilepsy in the MS population was two to three-fold higher (0.5–8.3%) than the general age-matched population.^{121,123–125} Marrie et al.¹²³ showed that the comorbidity of epilepsy in the male MS population was higher than the female MS population, although the prevalence of epilepsy was not different between male and female matched populations. Although it has not been proven whether inflammatory demyelination in the brain can explain the increased frequency of seizures in MS,¹²⁶ Calabrese et al.¹²⁷ reported that relapsing–remitting MS patients with epilepsy have more extensive cortical inflammation than relapsing–remitting MS patients without epilepsy.

The brain and the heart have several histological similarities; for example, their major cell types, including neurons, glial cells and cardiomyocytes, are post-mitotic, and both organs are relatively

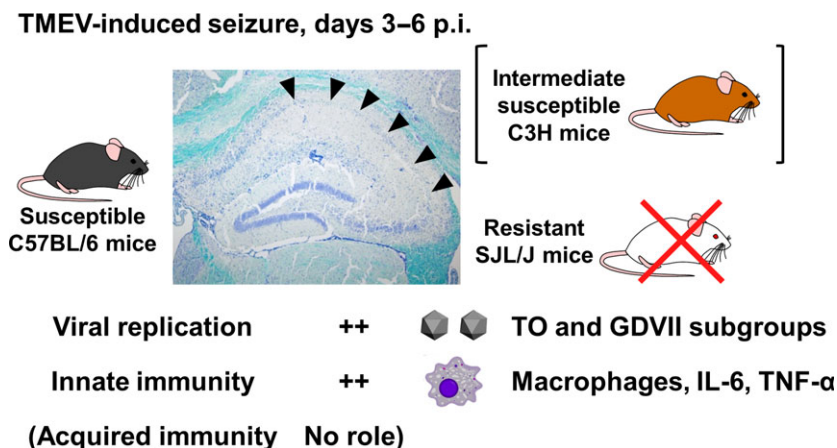


Figure 6 TMEV-induced seizures. During the early acute phase of TMEV infection, 3–6 days p.i., susceptible B6 mice develop seizures with severe neuronal loss in the pyramidal cell layer of the hippocampus (arrowheads), whereas no resistant SJL/J mice develop seizures. A small percentage of infected C3H mice also develop less severe seizures, compared with B6 mice; C3H mice are intermediately susceptible. High levels of viral replication in the brain have been detected in the brain of seized mice infected with both TO and GDVII subgroup viruses of TMEV. Innate immune responses, including macrophages, IL-6 and tumor necrosis factor- α , have been proposed to contribute to the development of TMEV-induced seizures, while acquired immunity has no role.




		Innate immunity	Acquired immunity				
Mouse strain	Organ (Disease)	Time postinfection					
		3 days	6 days	7 days	2-3 weeks	1-2 months	
	CNS	Polioencephalitis (brain)				Demyelination (spinal cord)	
	Heart (Myocarditis)	(phase I)					
	CNS	Seizure	Polioencephalitis (brain)			Demyelination (spinal cord)	
	Heart (Myocarditis)	(phase I)	Inflammation (phase II)		Fibrosis (phase III)		
	CNS	Seizure	Polioencephalitis (brain)				
	Heart (Myocarditis)	(phase I)	Inflammation (phase II)				

Figure 7 Susceptibility to three TMEV-induced immune-mediated disease models for multiple sclerosis (demyelination, green), myocarditis, (phases II and III, red and red-violet, respectively) and seizures (blue) among three mouse strains. In the central nervous system (CNS), 3–6 days p.i., TMEV induces innate immune responses, which cause severe seizures in B6 and mild seizures in C3H mice, but no clinical signs in SJL/J mice. Approximately 1 week p.i., TMEV infects neurons in the gray matter of the brain and causes inflammation (polioencephalitis) in all three mouse strains histologically without causing obvious clinical signs. Approximately 1 month p.i. (chronic phase), TMEV persistently infects the white matter of the spinal cord of SJL/J mice and C3H mice, inducing severe and mild inflammatory demyelination, respectively, whereas B6 mice eradicate the virus completely with no lesions in the spinal cord. In contrast, in the heart, although TMEV can infect the heart in all three mouse strains (phase I), only C3H mice develop substantial inflammation in phase II, 1–3 weeks p.i., leading to cardiac fibrosis in phase III, 1–2 months p.i. SJL/J mice develop no cardiac inflammation, whereas only a few B6 mice develop mild inflammation in phase II.

immune-privileged sites. Several research groups have attempted to link some brain diseases, including epilepsy, with cardiac dysfunction through: (i) the autonomic nervous system; (ii) “neurocardiac” gene expression;¹²⁸ or (iii) hemodynamics.^{129,130} However, no epidemiological studies seem to have

been carried out to investigate the comorbidity between myocarditis and seizures/epilepsy. Based on the data from TMEV infection, here, we hypothesize that one single viral infection or virally induced-immune responses might lead to both myocarditis and epilepsy. This is consistent with clinical findings

in humans: (i) some viral infections, such as herpesviruses and picornaviruses (e.g. coxsackievirus and enterovirus) can infect both the brain and the heart; and (ii) myocarditis and CNS involvement have been observed in several autoimmune diseases, including systemic lupus erythematosus.¹³¹

Although MS lesions are, in general, confined only in the CNS, increased comorbidities with other diseases in general organs, such as thyroiditis, have been reported. However, there are only a few reports that describe cardiac abnormalities in MS. Although most reports did not address the pathophysiological mechanism of cardiac dysfunction in MS, Akgül et al.¹³² speculated that a single microbial agent might induce myocarditis and CNS immunopathology in some MS patients. In MS, there is a case report describing one MS patient who died of acute eosinophilic myocarditis with cardiac histological findings.¹³³ Although the authors speculated that myocarditis was likely drug-induced, there are several concerns on this case report; for example: (i) virological examination was incomplete;¹³⁴ (ii) the drug responsible for hypersensitivity was unclear;^{135,136} and (iii) the association with MS was unclear (the patient had anti-nuclear antibodies, immunomodulatory treatment, and active CD4⁺ T-cell infiltration in the brain and the heart). Cosgrove et al.¹³⁷ reported a case with neuromyelitis optica spectrum disorder with involvement of cardiac and skeletal muscle with sepsis. Although the authors speculated that this case represents a new spectrum disorder with myocarditis, the authors neither

carried out histological examination (the only evidence for “myocarditis” was increased cardiac troponin) nor identified the microbe responsible for sepsis, while it is unclear which virological examination was carried out in that case.

In Table 3, we summarize the prevalence, associated viruses, and comorbidities between MS, myocarditis and seizures/epilepsy. Based on the high prevalence and common associated viruses, as well as data from TMEV studies, we propose that one single virus can induce or exacerbate any one of the aforementioned three immune-mediated conditions depending on the host’s genetic background. Clinical, epidemiological, and virological studies on the comorbidities between MS, myocarditis and seizures/epilepsy might lead to the discovery of a new spectrum of virally-mediated disorders, whose specific diagnosis and treatment can be beneficial for a subset of patients. In addition, experimental comparative studies among mouse strains to clarify the mechanism of different susceptibility to three different TMEV-induced organ-specific immune-mediated disease models (demyelinating disease, myocarditis and seizures) are useful not only to clarify the mechanism of human MS, viral myocarditis and seizures, but also to understand the organ-specific viral tropism and immunopathology, in general.⁶

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Table 3 Prevalence, associated viruses, and comorbidity of multiple sclerosis, myocarditis and seizures

	Prevalence	Associated viruses	Comorbidity		
			Multiple sclerosis	Myocarditis	Seizure
Multiple sclerosis	0.1–0.2% USA and Europe	EBV HHV-6 MeV retroviruses	NA	± case report	+
Myocarditis	1–9% autopsy	Picornaviruses (CVB, enterovirus) parvovirus HHV-6 adenovirus, HCV	± case report	NA	ND
Seizures/epilepsy	0.5–1.0% active epilepsy	HHV-6, HSV-1 JEV, HIV, picornaviruses (CVA, CVB, enterovirus, parechovirus)	+	ND	NA

CVA, coxsackievirus A; CVB, coxsackievirus B; EBV, Epstein-Barr virus; HCV, hepatitis C virus; HHV-6, human herpesvirus type 6; HIV, human immunodeficiency virus; HSV-1, herpes simplex virus (type)-1; JEV, Japanese encephalitis virus; MeV, measles virus; NA, not applicable; ND, not determined.

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Conflict of interest

None declared.

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