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Immunization with structural and non-structural proteins of Theiler's murine encephalomyelitis virus alters demyelinating disease

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Abstract Theiler's murine encephalomyelitis virus (TMEV) causes a demyelinating disease similar to multiple sclerosis in the central nervous system (CNS) of susceptible SJL/J mice. Immune responses to TMEV contribute to viral clearance as well as to demyelination. We constructed recombinant vaccinia viruses (VV) that encode each or all of the capsid proteins (VV_{VP1}, VV_{VP2}, VV_{VP3}, VV_{VP4}, and VV_{all}) or non-structural proteins (VV_{P2}, VV_{P2P3}, and $VV_{3'P3}$) of the Daniels strain of TMEV. To determine the role of each of the coding regions of TMEV in vivo, we immunized SJL/J mice with each recombinant VV, with or without subsequent TMEV infection. The groups of mice were compared clinically, immunologically, and histologically. No mice immunized with any recombinant VV without subsequent TMEV infection developed demyelination. However, antibody responses to TMEV were detected in mice immunized with VV_{all}. In addition, in some mice, VV_{P2} immunization induced mild meningitis. VV_{VP3} or VV_{VP4} immunization of mice prior to TMEV infection ameliorated TMEV-induced pathology or clinical signs of disease. The beneficial effect of VP4 immunization was also seen through DNA immunization with a plasmid encoding VP4 and leader prior to TMEV infection. Therefore, vaccination against not only surface capsid proteins (VV_{VP3} and VV_{all}) but also non-surface capsid protein (VV_{VP4}), and non-structural proteins (VV_{P2}) can elicit immune responses to virus or modulate subsequent viral-induced CNS disease.

Keywords Autoimmunity · CNS demyelinating diseases · DNA immunization · Picornavirus infection · Vaccination · Viral capsid protein

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Introduction

Theiler's murine encephalomyelitis virus (TMEV) is a naturally occurring enteric picornavirus of mice (Theiler and Gard 1940). Infection of the central nervous system (CNS) of susceptible strains of mice with viral strains from the less neurovirulent subgroup of TMEV results in flaccid paralysis of the hind limbs (Theiler 1934, 1937) and demyelination (Daniels et al. 1952). Further study demonstrated that the strains of the less neurovirulent subgroup of TMEV cause a biphasic disease consisting of acute encephalomyelitis 1–2 weeks postinfection followed by CNS demyelination with viral persistence approximately 1 month postinfection (Lipton 1975). Based on this observation, intracerebral (i.c.) infection of susceptible strains of mice with the less neurovirulent strains of TMEV [Daniels (DA) and BeAn 8386 (BeAn)] has become a favored experimental animal model for multiple sclerosis, the most common demyelinating disease in humans (Dal Canto and Lipton 1977).

The TMEV virion is non-enveloped and contains a positive-sense, single-stranded RNA genome of approximately 8,100 nucleotides [(Ohara et al. 1988; Pevear et al. 1987) reviewed in (Racaniello 2007)]. This RNA genome is

translated into a single large precursor polyprotein prior to posttranslational cleavage by a virally encoded protease into the leader protein (non-structural), the structural capsid proteins, VP1-4, and at least six other non-structural proteins (Fig. 1). The exposed surface of the mature virion consists of the VP1-3 capsid proteins, while the VP4 capsid protein is buried within the interior of the virion (Racaniello 2007).

Host immune responses, $CD8^+$ and $CD4^+$ T cells and antibodies, to TMEV infection of the CNS likely contribute to both viral clearance and protection in resistant strains of mice (C57BL/6) and pathogenesis of virus-induced demyelination in susceptible stains of mice (SJL/J) [reviewed in Libbey and Fujinami (2003) and Tsunoda and Fujinami (2010)]. Four TMEV (DA strain) capsid protein epitopes that are recognized by T cells have been identified in SJL/J mice. $CD4^+$ T cells from SJL/J mice recognized the $VP1_{233–250}$, $VP2_{74–86}$, and $VP3_{24–37}$ epitopes of DA, while $CD8^+$ T cells from SJL/J mice recognized the $VP1_{11–20}$ epitope of DA [Gerety et al. (1994) and Kang et al. (2002a,b) reviewed in Tsunoda and Fujinami (2010)]. No VP4 protein epitopes or nonstructural protein epitopes of DA have been identified in SJL/J mice. However, one nonstructural epitope of BeAn, which falls within the RNA polymerase protein ($3D_{21–36}$) and is recognized by $CD4^+$ T cells, has been identified in SJL/J and C57BL/6 mice (Jin et al. 2009).

Vaccination as a means of protecting SJL/J mice from the development of demyelinating disease has been examined previously. Administration of live wild-type or attenuated DA virus (Kurtz et al. 1995b) or inactivated BeAn virus (Crane et al. 1993) provided protection from the development of demyelinating disease following subsequent TMEV infection. However, these strategies provide no details about the viral capsid proteins and/or epitopes, which are involved in either protection from disease or immune-mediated pathogenesis leading to myelin destruction in susceptible mice.

Previous work by our group explored the utility of intramuscular (i.m.) DNA immunization for the protection of mice against subsequent TMEV infection (Tolley et al. 1999). SJL/J mice immunized with a single injection of DNA encoding VP1 showed exacerbation of demyelinating disease induced with DA, which was not seen with two or three injections. Alternately, SJL/J mice immunized with DNA encoding VP2 or VP3 showed a dose-dependent protection against demyelinating disease induced with DA (Tolley et al. 1999). Thus, the virus-induced demyelinating disease that results from DA infection of SJL/J mice can be modified through prior DNA immunization against surface capsid proteins.

In the current study, we explored the utility of intravenous (i.v.) recombinant vaccinia virus (VV) immunization for the protection of mice against subsequent TMEV infection. SJL/J mice immunized with recombinant VV encoding the 3′ portion of VP3 (VV_{VP3}) or VP4 (VV_{VP4}) capsid proteins showed protection against demyelinating disease induced with DA. The beneficial effect of VP4 was confirmed through DNA immunization. In addition, antibody responses to TMEV were detected in SJL/J mice immunized with VV_{all}, which encodes VP1-4. In addition, in some mice, immunization with VV_{P2}, which encodes most of the P2 region of DA (Fig. 1), induced mild meningitis.

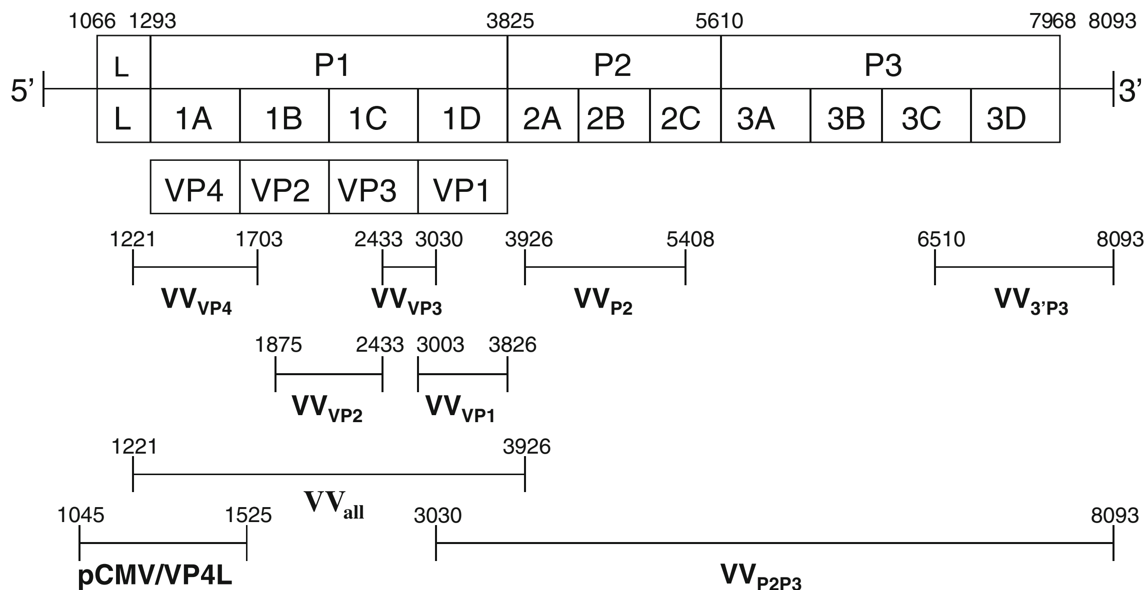


Fig. 1 Recombinant VV and the plasmid construct encoding TMEV proteins. The TMEV genome encodes a leader protein (L), the structural proteins [P1 (capsid region)], and the non-structural proteins [P2 (midsection) and P3 (right portion)]. The genomic coding regions *1A*, *1B*, *1C*, and *1D* encode the capsid proteins VP4, VP2, VP3, and VP1, respectively. The numbering of the nucleotide sequence is according to the GenBank sequence for the DA strain of TMEV (accession number M20301)

Therefore, we found that vaccination against surface capsid proteins (VV_{VP3} and VV_{all}), non-surface capsid protein (VV_{VP4}), and non-structural proteins (VV_{P2}) can elicit immune responses to virus or modulate subsequent viral-induced CNS disease.

Materials and methods

Viruses, plasmid, and animal experiments

A working stock of the DA strain of TMEV was prepared in baby hamster kidney (BHK)-21 cells, as described previously (Zurbriggen and Fujinami 1989). We used recombinant VV encoding all or portions of the capsid proteins (VV_{VP1}, VV_{VP2}, VV_{VP3}, VV_{VP4}, and VV_{all}), most of the P2 region (VV_{P2}), the 3′ portion of the P3 region ($VV_{3'P3}$) or most of the VP1, P2 and P3 regions (VV_{P2P3}) of the DA strain of TMEV (Fig. 1). Recombinant viruses were prepared using methods described previously (Chakrabarti et al. 1985). Briefly, the various regions of the DA strain of TMEV were excised from the plasmid pDAFL3, containing the infectious complementary DNA for DA (Roos et al. 1989), using appropriate restriction enzymes, and cloned into the *Sma*I site of the pNVV3 plasmid, described previously (Chakrabarti et al. 1985). The resulting plasmids contained the various regions of TMEV, with internal start sites, downstream from the early/late vaccinia promoter P7.5 and with a stop codon in the polylinker of the vector. The pNVV3 plasmids now containing the various regions of TMEV were transformed into cells infected with wild-type vaccinia (strain WR) and recombinant vaccinia viruses were selected via β-galactosidase production and growth in the presence of bromo-deoxyuridine. Recombinant viruses containing the various regions of TMEV were verified by DNA sequencing and Northern blots (data not shown). In addition, BHK-21 cells were infected with the VV_{all} and VV_{VP1} viral constructs, and immunohistochemistry using hyperimmune rabbit serum against TMEV demonstrated the production of viral antigens (data not shown). Furthermore, the production of viral antigens by the VV_{all} viral construct was confirmed through the successful use of VV_{all}-infected and irradiated spleen cells as antigen presenting cells, which stimulated spleen cells, from mice infected with DA, for use as effector cells in a ^{51}Cr release assay (Tsunoda et al. 2006). As a control, we used recombinant VV encoding β-galactosidase (VVsc11). Groups of 4–5 week-old female SJL/J mice (Jackson Laboratory, Bar Harbor, ME, USA) were injected i.v. with 5×10^6 plaque forming units (PFU) of each recombinant VV or phosphate-buffered saline (PBS). Three weeks after i.v. injection, half of each group of mice was infected i.c. with 2×10^5 PFU of the DA strain of TMEV. As controls, some groups of mice received PBS i.c. or complete Freund's adjuvant (CFA) subcutaneously (s.c.) with or without two i.v. injections of 5×10^9 *Bordetella pertussis* cells (Michigan Department of Public health, Lansing, MI, USA). The mice were weighed and observed for clinical signs and killed 100 days after i.c. infection.

The entire VP4 and leader region (VP4L) of the DA strain of TMEV (Roos et al. 1989) was cloned into the *Not*I site of the pCMV plasmid, as described previously (Tolley et al. 1999), resulting in an expression plasmid named pCMV/VP4L, which encoded both the leader protein and the VP4 capsid protein (Fig. 1). The pCMV vector, derived by excision of the *Escherichia coli* β-galactosidase gene from pCMVβ (Clontech, Palo Alto, CA, USA), contains the strong immediate-early gene promoter/enhancer from cytomegalovirus and the polyadenylation signal from simian virus 40. Mice were injected twice with the pCMV/VP4L plasmid or with the pCMV vector as a control. Each injection contained 100 μg of endotoxin-free plasmid DNA in 100 μl of saline introduced equally into each tibialis anterior muscle (Tolley et al. 1999; Tsunoda et al. 1998). Two weeks following the final plasmid injection, each mouse was challenged i.c. with 2×10^5 PFU of the DA strain of TMEV. The mice were killed 2 months after TMEV infection.

Clinical signs of disease in mice were evaluated by an impaired righting reflex test (Rauch et al. 1987; Tsunoda et al. 2001). When the proximal end of the mouse's tail is grasped and twisted to the right and then to the left, a healthy mouse resists being turned over (score of 0). If the mouse is flipped onto its back but immediately rights itself on one side or both sides, it is given a score of 1 or 1.5, respectively. If it rights itself in 1 to 5 s, the score is 2. If righting takes more than 5 s, the score is 3.

Histology

Killed mice were perfused with PBS followed by 4% paraformaldehyde. Harvested brains were divided coronally into five portions, and spinal cords were divided transversely into 12 portions. Tissues were embedded in paraffin. Four-micrometer-thick tissue sections were cut and stained with Luxol fast blue for myelin visualization. Histological scoring was performed as previously described (Tsunoda et al. 2001). Brain sections were scored for meningitis (0, no meningitis; 1, mild cellular infiltrates; 2, moderate cellular infiltrates; and 3, severe cellular infiltrates), perivascular cuffing (0, no cuffing; 1, 1–10 lesions; 2, 11–20 lesions; 3, 21–30 lesions; 4, 31–40 lesions; 5, over 40 lesions), and demyelination (0, no demyelination; 1, mild demyelination; 2, moderate demyelination; and 3, severe demyelination). Each score for the brain was combined for a maximum score of 11 per mouse. For scoring of spinal cord sections, each spinal cord segment was divided into four quadrants: the anterior funiculus, the posterior funiculus, and each lateral

funiculus. Any quadrant containing meningitis, perivascular cuffing, or demyelination was given a score of 1 in that pathologic class. The total number of positive quadrants for each pathologic class was determined, 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. An overall pathologic score was also determined by giving a positive score if any pathology was present in the quadrant. This was also presented as percent involvement.

Antibodies

Before immunization (preimmune), 3 weeks after each recombinant VV injection and 100 days after TMEV infection, sera were collected from the mice and stored at −20°C. Titers of TMEV-specific antibodies in the sera were determined by an enzyme-linked immunosorbent assay (ELISA), as described previously (Tsunoda et al. 2001). TMEV antigen was prepared by infecting BHK-21 cells with the DA strain of TMEV at a multiplicity of infection of 0.1 PFU/cell. For the ELISA, 96-well plates were coated overnight with TMEV antigen at 4°C. After blocking with diluent (PBS containing 10% fetal bovine serum and 0.2% Tween 20), twofold dilutions of the mouse sera beginning at $1{:}2^7$ were added to the plates and incubated at room temperature for 90 min. After washing with PBS containing 0.2% Tween 20, the plates were incubated with a peroxidase-labeled goat antimouse antibody in diluent for 90 min. The plates were colorized with *o*-phenylenediamine dihydrochloride (Sigma, St. Louis, MO, USA) and were read at 492 nm on a Titertek Multiskan Plus MK II spectrophotometer (Flow Laboratories, McLean, VA, USA). The endpoint of the assay was determined as the reciprocal of the highest dilution that gave an optical density reading that was two standard deviations above the control baseline from preimmune sera.

Results

VV_{P2} immunization induces mild CNS disease

During the first 3 weeks following immunization with each recombinant VV, mice were observed to determine whether recombinant VV alone could induce clinical signs. Six of 17 mice immunized with VV_{P2} showed a mild impaired righting reflex and the mean righting reflex score of the VV_{P2} immunized mouse group was statistically higher compared to the other groups of mice [Fig. 2a, $p<0.01$, analysis of variance (ANOVA)]. Although a few mice immunized with other recombinant VV also showed mild impaired righting reflex, no statistical differences were found compared with controls. Histologically, VV_{P2} immunized mice had perivascular cuffing in the brain parenchyma (Fig. 2d, arrow) and mild meningitis in the spinal cord (Fig. 2b, arrowheads) and brain (Fig. 2d, arrowheads), while mice in the other groups had no detectable lesions at the time of killing (Fig. 2c, e).

The recombinant VV-immunized mice were also observed for 5 months to investigate whether mice developed chronic disease. For each recombinant VV, five mice were immunized, three of which were injected with PBS (i.c.) 3 weeks after VV immunization. Among 45 mice immunized with recombinant VV, no mice showed any clinical signs except for one VV_{VP1} immunized mouse with PBS (i.c.) injection, which showed ataxic clinical signs 4 months after VV_{VP1} immunization. Twenty mice were then immunized with (1) VV_{VP1} alone, (2) VV_{VP1} with PBS (i.c.), (3) VV_{VP1} with CFA (s.c.), or (4) VV_{VP1} with CFA (s.c.) and *B. pertussis* (i.v.) and observed for clinical signs for 5 months. None of the 20 mice immunized with VV_{VP1} developed clinical disease. Histologically, no lesions were found in any of the 65 mice immunized with recombinant VV, with the exception of a small collection of mononuclear cells in a few mice (data not shown).

In summary, mice immunized with recombinant VV were observed for 3 weeks and 5 months for the development of acute and chronic disease, respectively. Only VV_{P2} induced mild acute CNS disease in immunized mice, and no mice developed chronic disease. Therefore, the immunizations themselves were not greatly detrimental to the health of the mice.

VV_{all} immunization induces anti-TMEV immune responses

In TMEV infection, antibody epitopes are specific for capsid proteins (Inoue et al. 1994). Among recombinant VV constructs, only VV_{all}, which encodes all TMEV capsid proteins, induced a significant amount of anti-TMEV antibody in the sera compared with controls (Fig. 3, $p<0.01$, ANOVA). Anti-TMEV antibody titers in the sera of mice immunized with the other recombinant VV, including the ones encoding each individual capsid protein, were comparable to controls. Therefore, a humoral immune response to TMEV was significantly induced in immunized mice only when all of the structural proteins of TMEV were present, as are found in VV_{all}.

VV_{VP4} immunization ameliorates TMEV-induced chronic disease

We tested whether immunization with recombinant VV could alter the clinical course of the subsequent TMEV infection. Groups of mice were immunized with the recombinant VV encoding different TMEV proteins. Control mice were injected with either PBS or VVsc11. Three weeks after immunization, mice were infected with TMEV and observed

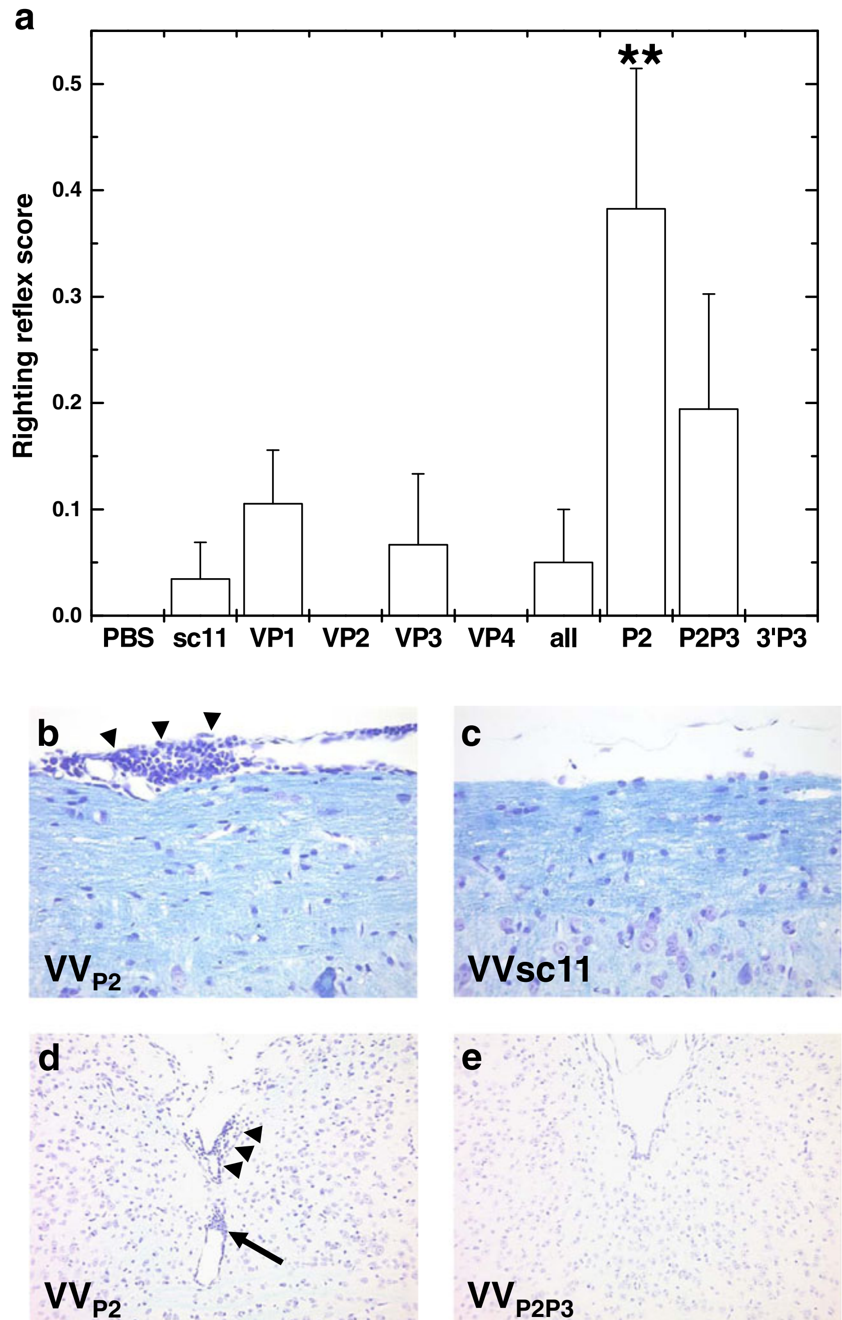


Fig. 2 Righting reflex and neuropathology following recombinant VV immunization. Mice were injected intravenously with PBS, VVsc11 (sc11), VV_{VP1} (VP1), VV_{VP2} (VP2), VV_{VP3} (VP3), VV_{VP4} (VP4), VV_{all} (all), VV_{P2} (P2), VV_{P2P3} (P2P3), or $VV_{3'P3}$ (3′P3) and observed for clinical signs for 3 weeks. **a** Mice immunized with VV_{P2} showed a significantly higher mean righting reflex score than the other groups (p<0.01, ANOVA). Data represent means+standard errors of the means (SEM) for groups consisting of 15–38 mice. **b**–**e** VV_{P2} immunization induced meningitis (*arrowheads*) in the spinal cord (**b**) and meningitis (*arrowheads*) and perivascular cuffing (*arrow*) in the midbrain (**d**), while other VV constructs did not induce obvious neuropathology in the spinal cord (**c**, VVsc11) or in the midbrain (**e**, VV_{P2P3}). **b**–**e** Luxol fast blue stain. Magnification: **b** and **c**, **x** 100; **d**, **e**, ×70

for clinical signs for 100 days. There were at least 10 mice per group, and experiments were repeated at least twice. Only VV_{VP4} immunized mice developed chronic disease that was significantly less severe than chronic disease in control groups of mice (Fig. 4, p<0.05, t test). The amelioration, however, was mild overall, and there were no statistical difference in weight changes between VV_{VP4} immunized mice versus control mice (data not shown). In addition, the VV_{VP4} immunized mice had higher clinical scores during acute infection than control groups, but the differences were not significant (Fig. 4).

As for the other recombinant VV immunizations, although no statistical differences were noted compared with control groups, VV_{VP3} and VV_{P2P3} immunizations also tended to ameliorate TMEV-induced disease (data not shown). VV_{VP2} immunization had no effect on disease course (data not shown). VV_{VP1}, VV_{all}, $VV_{3'P3}$, and VV_{P2} immunizations tended to exacerbate the chronic disease (data not shown). There was no difference in clinical signs between control groups injected with PBS versus VVsc11 (data not shown).

Here, we found that, although immunization with recombinant VV could alter the clinical course of the subsequent

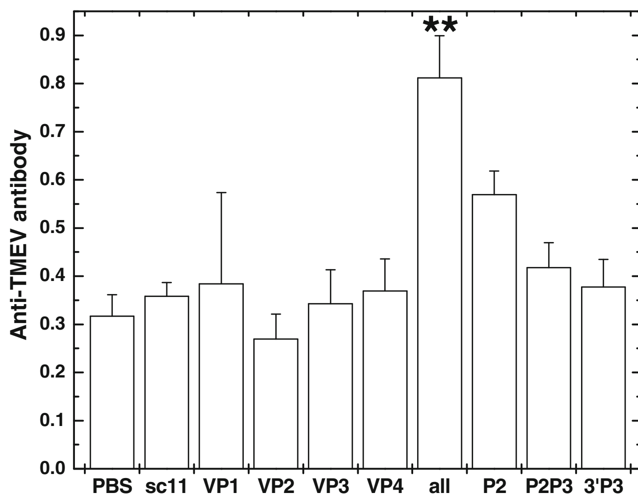


Fig. 3 Serum anti-TMEV antibody responses following recombinant VV immunization. Mice were injected intravenously with recombinant VV that encode each or all capsid proteins of TMEV [VV_{VP1} (VP1), VV_{VP2} (VP2), VV_{VP3} (VP3), VV_{VP4} (VP4), and VV_{all} (all)] or the non-structural P2 and P3 regions of TMEV [VV_{P2} (P2), VV_{P2P3} (P2P3), and $VV_{3'P3}$ (3′P3)] and anti-TMEV antibody titers were determined by ELISA at 3 weeks after injection. Control mice were injected with PBS or VV encoding β-galactosidase [VVsc11 (sc11)]. Significant anti-TMEV antibody responses were detected in mice immunized with VV_{all} ($p<0.01$, ANOVA). Values are mean optical density $(OD)_{492\ nm}$+SEM of groups of 5–20 mice

TMEV infection, only immunization with VV_{VP4} ameliorated the chronic disease to any significant degree. Immunization

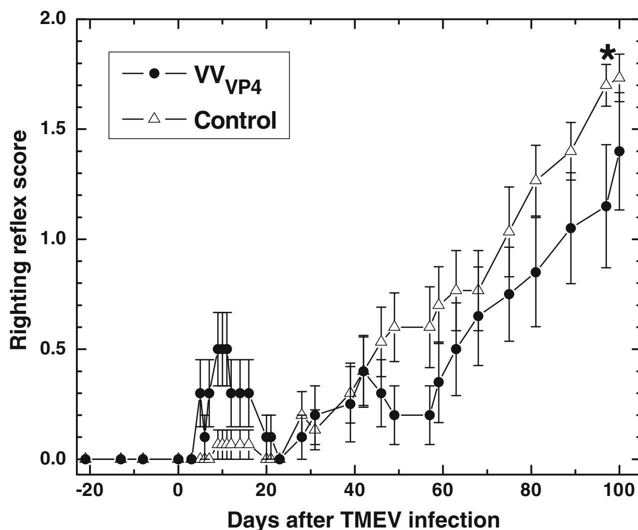


Fig. 4 Clinical course of TMEV-infected mice with or without VV_{VP4} immunization. Mice were injected intravenously with VV encoding the VP4 (VV_{VP4}) capsid protein of TMEV. Control mice were injected with VVsc11 or PBS. Three weeks after immunization, mice were infected with TMEV and impairment of righting reflex was scored. VV_{VP4} immunized mice showed less severe chronic disease ($p<0.05$, *t* test). Data represent the mean righting reflex scores ± SEM of the results from two independent experiments. Each group contained 10–15 mice

with the other recombinant VV either had no effect, mildly ameliorated, or mildly exacerbated the disease.

VV_{VP3} immunization ameliorates TMEV-induced demyelination

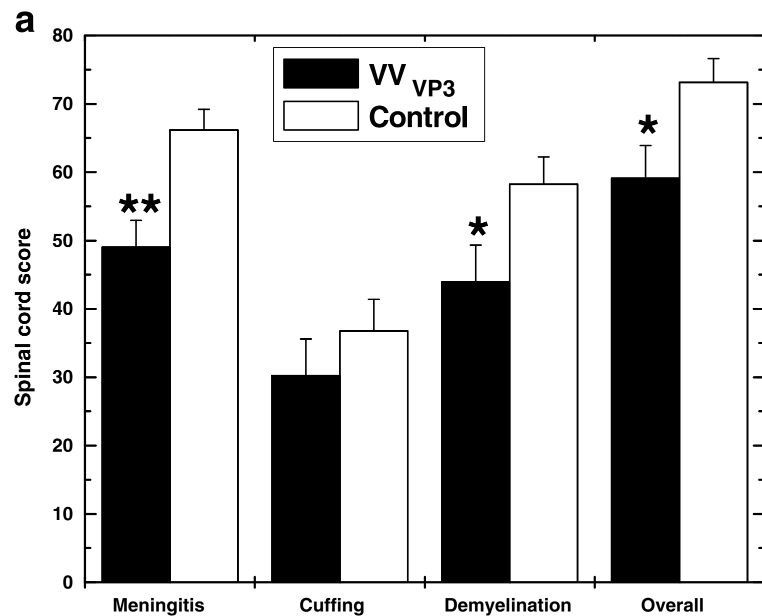
We compared the neuropathology found in brains and spinal cords harvested 100 days after TMEV infection for groups of mice immunized with the various VV recombinants. In the brain, we detected mild meningitis and perivascular cuffing mainly in the brainstem. Demyelination was rare in the brain. Although the cerebellum is known to be spared in TMEV infection, we sometimes found lesions in the cerebellum at this late stage. No differences were found in the extent or distribution of the lesions between groups of mice (5–18 mice per group) immunized with recombinant VV (data not shown).

In the spinal cord, we found severe inflammatory demyelinating lesions in all groups of mice. No statistical differences were seen in pathology scores between the groups (data not shown) except for VV_{VP3}-immunized mice compared to control groups (Fig. 5). VV_{VP3}-immunized mice (Fig. 5b) developed less severe inflammatory demyelination (arrows) and meningitis (arrowheads) than control mice (Fig. 5c) and had statistically lower pathology scores in meningitis ($p<0.01$, *t* test), demyelination and overall pathology ($p<0.05$, *t* test), compared with control mice (Fig. 5a).

Upon examination of demyelination within the CNS, only immunization with VV_{VP3} ameliorated demyelination, found only in the spinal cord, to any significant degree. Immunization with the other recombinant VV had no effect on demyelination.

Recombinant VV immunization followed by TMEV infection induces anti-TMEV antibody responses

When mice were killed (day 100), blood was collected, and anti-TMEV antibody responses were compared between groups of mice immunized with the various VV recombinants and infected with TMEV. In all groups of mice (4–23 mice per group), high anti-TMEV antibody titers were detected (data not shown). However, there were no significant differences in anti-TMEV antibody titers between the groups ($p>0.05$, ANOVA). Thus, although only VV_{all} immunization induced a humoral immune response to TMEV (Fig. 3), subsequent TMEV infection induced a humoral immune response to TMEV in mice immunized with all of the various VV recombinants, to include the VVsc11 control, and in those mice immunized with PBS. Therefore, no immunizations tested here were detrimental to the development of a humoral immune response upon subsequent TMEV infection.



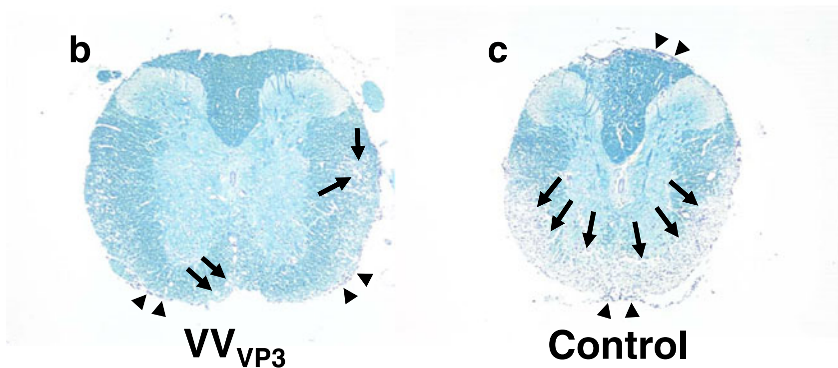


Fig. 5 Neuropathology of TMEV infected mice with VV_{VP3} immunization. **a** VV_{VP3} immunized mice had statistically lower spinal cord pathology scores for meningitis, demyelination and overall pathology, compared to control groups (*$p<0.05$; **$p<0.01$, *t* test). Data represent the mean pathology scores + SEM of results from two independent experiments. Each group contained 8–13 mice. **b** VV_{VP3} immunized mice developed mild vacuolar demyelination (*arrows*) and meningitis (*arrowheads*). **c** Control mice developed large demyelinating lesions (*arrows*) in the subpial area and severe meningitis (*arrowheads*). **b**, **c** Luxol fast blue stain

DNA immunization against VP4 ameliorates TMEV-induced disease

To further clarify the role of VP4, a eukaryotic expression vector, pCMV/VP4L, encoding the TMEV capsid protein VP4 was constructed. After i.m. immunization with pCMV/VP4L or vector, mice were infected i.c. with TMEV. Mice injected twice with pCMV/VP4L had significantly greater weight gain than mice injected twice with vector alone (Fig. 6a, $p<0.05$, $p<0.01$, *t* test), although there were no differences in mean righting reflex scores between the groups (data not shown).

Histologically, mice treated with pCMV/VP4L had less meningitis, perivascular cuffing, and overall pathology, although there were no statistical differences compared with control mice (Fig. 6b). Using ELISA, we found no anti-TMEV antibody in the sera of mice immunized with pCMV/VP4L prior to TMEV infection (data not shown). After TMEV infection, mice injected with pCMV/VP4L or vector developed high anti-TMEV antibody responses, and there were no statistical differences in the titers between the groups (data not shown).

Therefore, we have demonstrated amelioration of the TMEV-induced chronic disease through VP4 vaccination via two different methods. Mice immunized with VV_{VP4} demonstrated an improved righting reflex score (Fig. 4), while mice immunized with pCMV/VP4L demonstrated an improved weight gain (Fig. 6a), both of which are clinical signs of the chronic disease.

Discussion

Transgenic mouse studies, in which the mice (SJL/J background) express the entire P1 region (leader and VP1-4) of the TMEV genome, have shown that the level of T cell immunity against viral capsid proteins is a factor for the pathogenesis of demyelinating disease in SJL/J mice (Myoung et al. 2008). The P1-transgenic mice showed transient reductions in both $CD4^+$ and $CD8^+$ viral-specific T cell

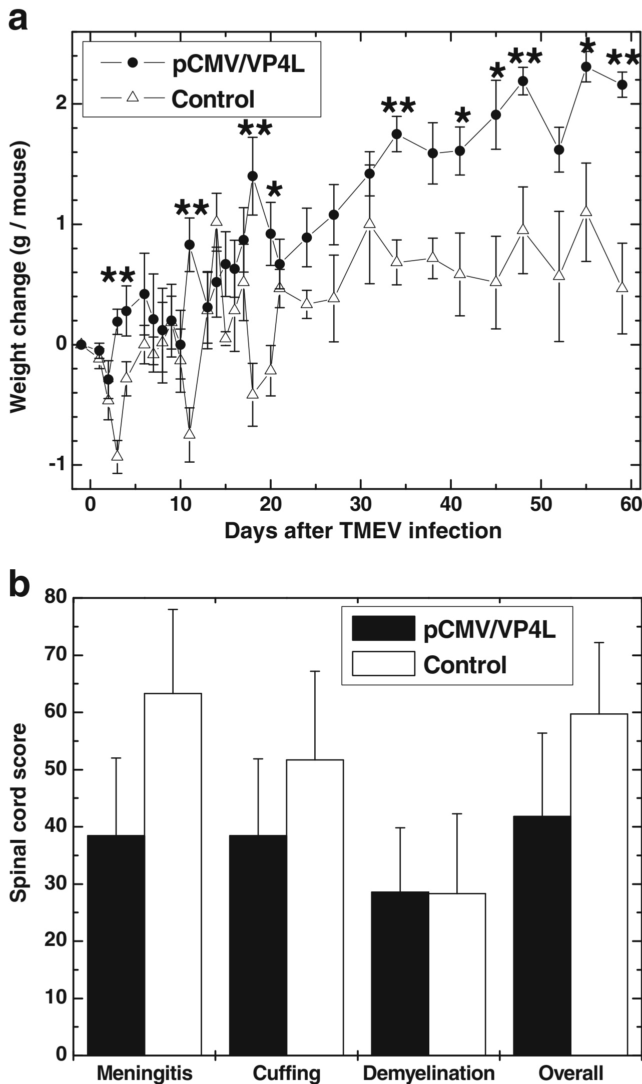


Fig. 6 Immunization with plasmid DNA encoding VP4, pCMV/VP4L, in TMEV infection. After injection with pCMV/VP4L or vector (control), mice were infected with TMEV. **a** Mice immunized twice with pCMV/VP4L (*circle*) gained significantly more weight than control mice (*triangle*) (*$p<0.05$; **$p<0.01$, *t* test). Data represent the means ± SEM for groups consisting of three to five mice. **b** Although mice injected with pCMV/VP4L tended to show lower spinal cord pathology scores, no statistical differences were found between controls and pCMV/VP4L immunized mice ($p>0.05$, *t* test)

responses and greatly increased levels of viral persistence in the CNS following infection with TMEV. Despite the high viral loads, these mice had significantly reduced levels of neurological disease (demyelination, disease incidence, and severity), suggested to be due to the reduced antiviral immune response (Myoung et al. 2008). Therefore, since the immune response to viral capsid can induce pathology, vaccination against subsequent TMEV infection through the use of TMEV capsid proteins is problematic.

Previously, our group explored i.m. immunization with DNA encoding the VP1-3 capsid proteins of TMEV as a vaccination strategy against subsequent TMEV infection (Tolley et al. 1999). It was found that the modulation of the outcome of the subsequent TMEV infection varied both with the number of DNA immunizations and with the capsid protein used. A single injection, but not two or three, of DNA encoding VP1 exacerbated disease while injection of DNA encoding VP2 or VP3 protected against disease in a dose-dependent manner (Tolley et al. 1999). Therefore, we found that we were unable to predict whether DNA immunization would result in protection from or exacerbation of disease.

As an alternative to DNA immunization, recombinant VV were constructed that encoded each or all capsid proteins of TMEV (VV_{VP1}, VV_{VP2}, VV_{VP3}, VV_{VP4}, and VV_{all}) or the non-structural P2 and P3 regions of TMEV (VV_{P2}, VV_{P2P3}, and $VV_{3'P3}$). Immunization (i.v.) with these recombinant VV constructs was explored as a vaccination strategy against subsequent TMEV infection. Without subsequent TMEV infection, no mice immunized with any of the recombinant VV constructs developed demyelinating disease. Immunization with VV_{all} resulted in the production of TMEV-specific antibodies, which is considered beneficial for a vaccine (NIH 2008); however, immunization with VV_{P2} induced mild meningitis, which is considered detrimental for a vaccine. Finally, immunization with recombinant VV encoding VP3 or VP4 protected against disease induced by subsequent TMEV infection.

It is interesting that, although immunization with VV_{all} induced a high anti-TMEV antibody response in mice (Fig. 3), this immunization did not protect the subsequently infected mice from developing demyelinating disease. Previous work by our group demonstrated that although passive administration of neutralizing monoclonal antibody into athymic (*nu/nu*) mice greatly increased the survival of these mice beyond 4 weeks post-TMEV infection, 50% of these mice still had significant demyelination (Fujinami et al. 1989). We also showed that antibody titers as measured by ELISA do not correlate with disease modulation induced by DNA vaccination (Tolley et al. 1999). Others have shown that not all antibodies with specificity for the six major linear antibody epitopes on the capsid proteins of TMEV are able to neutralize viral infection in vitro (Inoue et al. 1994) and that antibodies to only certain linear epitopes are able to protect mice from TMEV-induced demyelination (Yahikozawa et al. 1997). Therefore, the high anti-TMEV titer may reflect a high percentage of non-neutralizing antibodies and the antibodies represented, be them neutralizing or non-neutralizing, may not prevent demyelination. Furthermore, it was found that $CD4^+$ T cells were required, in addition to antibody-secreting B cells, for the development of protective immunity (Kurtz et al. 1995a,b). Therefore, it seems that antibodies alone are not sufficient for protection. In addition, TMEV-specific antibody produced within

infected mice may actively take part in the pathogenesis of demyelination (Yamada et al. 1990). In fact, an antibody to a particular linear epitope was found to have high neutralizing activity in vitro, but the levels of this antibody within infected mice correlated with disease progression, not protection (Yahikozawa et al. 1997).

Comparison of the results from the previous study with DNA immunization (Tolley et al. 1999) and the current study with recombinant VV immunization demonstrates that, in both cases, vaccination with constructs encoding VP3 protected against disease induced by subsequent TMEV infection. Therefore, further studies into the use of VP3 as a vaccination strategy against subsequent TMEV infection would be worth pursuing.

An immunogenic role for the VP4 capsid protein of TMEV has not previously been examined in SJL/J mice. Although the VP4 protein is buried in the natural virus, once it has been processed intracellularly, peptides from VP4 can be presented on major histocompatibility complex class I or II molecules. Indeed, in TMEV-resistant C57BL/6 mice infected with the BeAn strain of TMEV, interferon (IFN)-γ enzyme-linked immunospot assays and flow cytometry for intracellular IFN-γ production using truncated peptides indicated that one of the epitope regions recognized by CNS-infiltrating $CD4^+$ T cells was $VP4_{25–38}$ (Kang et al. 2005). Therefore, alteration of disease course may be due to cellular responses to VP4 epitopes. Additionally, although not demonstrated in this study, antibodies specific for VP4 of another picornavirus, poliovirus, are found to neutralize the virus at 37°C (Li et al. 1994). Thus, it would not be unreasonable for antibodies against VP4 to also be produced in TMEV infection, leading to virus neutralization. In fact, sera from SJL/J mice infected with TMEV were shown to react with the VP4 protein (Cameron et al. 2001).

The protective effect of VP4 was confirmed through DNA immunization. Clinical amelioration of TMEV infection was seen in mice with two injections of pCMV/VP4L, but not in mice with a single or three injections of pCMV/VP4L (data not shown). The effect is presumed to be conferred by the VP4 region and not the leader region included in the DNA construct, as it is very unlikely that the leader protein is functionally expressed since there is no viral protease associated with the construct. In addition, no epitope regions recognized by $CD8^+$ cytotoxic T lymphocytes (CTL) were identified in the leader protein in TMEV-resistant C57BL/6 mice or TMEV-susceptible SJL/J mice infected with the BeAn strain of TMEV (Kang et al. 2002b; Lyman et al. 2002) nor have any epitope regions recognized by $CD4^+$ T cells been identified in the leader protein in C57BL/6 mice infected with the BeAn strain of TMEV (Kang et al. 2005). These three studies were the only studies to include the leader protein in a peptide library used for screening for $CD8^+$ and $CD4^+$ T cell epitopes within TMEV. In addition, a novel molecular screening approach did not detect any epitopes within the leader protein of DA that were recognizable to $CD8^+$ CTL in C57BL/6 mice (McDole et al. 2008).

Although in some DNA vaccination studies, immune responses have been shown to increase after boosting with additional plasmid injections, the effect of DNA immunization does not depend on the number of immunizations. Decreased $CD8^+$ CTL responses following additional plasmid immunizations have been reported in DNA vaccinations against influenza virus (Pertmer et al. 1996) and HIV (Fuller and Haynes 1994). In DNA vaccination against *Plasmodium yoelii*, initial interleukin-4 production was replaced by IFN-γ production upon boosting (Mor et al. 1995). In TMEV infection, we previously showed that the mice immunized with DNA encoding VP1 showed exacerbation of demyelinating disease only after a single plasmid injection, not after two or three injections, while DNA immunizations against VP2 and VP3 showed dose-dependent protection against TMEV-induced demyelinating disease (Tolley et al. 1999).

In this study, we found that vaccination against surface capsid proteins (VV_{VP3} and VV_{all}), non-surface capsid protein (VV_{VP4}) and non-structural proteins (VV_{P2}) can elicit immune responses to virus or modulate subsequent TMEV-induced CNS disease. This is some of the first work, to our knowledge, examining the function of capsid protein VP4 in TMEV infection of susceptible SJL/J mice. Further studies into the use of VP3 and/or VP4 as a vaccination strategy against subsequent TMEV infection would be worth pursuing.

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