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Single-Chain HLA-A2 MHC Trimers That Incorporate an Immunodominant Peptide Elicit Protective T Cell Immunity against Lethal West Nile Virus Infection

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The generation of a robust CD8⁺ T cell response is an ongoing challenge for the development of DNA vaccines. One problem encountered with classical DNA plasmid immunization is that peptides produced are noncovalently and transiently associated with MHC class I molecules and thus may not durably stimulate CD8⁺ T cell responses. To address this and enhance the expression and presentation of the antigenic peptide/MHC complexes, we generated single-chain trimers (SCTs) composed of a single polypeptide chain with a linear composition of antigenic peptide, β_2 -microglobulin, and H chain connected by flexible linkers. In this study, we test whether the preassembled nature of the SCT makes them effective for eliciting protective CD8⁺ T cell responses against pathogens. A DNA plasmid was constructed encoding an SCT incorporating the human MHC class I molecule HLA-A2 and the immunodominant peptide SVG9 derived from the envelope protein of West Nile virus (WNV). HLA-A2 transgenic mice vaccinated with the DNA encoding the SVG9/HLA-A2 SCT generated a robust epitope-specific CD8⁺ T cell response and showed enhanced survival rate and lower viral burden in the brain after lethal WNV challenge. Inclusion of a CD4⁺ Th cell epitope within the SCT did not increase the frequency of SVG9-specific CD8⁺ T cells, but did enhance protection against WNV challenge. Overall, these findings demonstrate that the SCT platform can induce protective CD8⁺ T cell responses against lethal virus infection and may be paired with immunogens that elicit robust neutralizing Ab responses to generate vaccines that optimally activate all facets of adaptive immunity. *The Journal of Immunology*, 2010, 184: 4423–4430.

Vaccination strategies to elicit protection against virus infection have largely focused on humoral immunity and Ab neutralization of virus. However, T cells also have a significant function in viral immunity. For example, cytotoxic memory CD8⁺ T cells have been found in several model systems to have prominent roles in the clearance of virus by producing antiviral cytokines or lysing virus-infected cells. Over the last several years, several immunodominant viral epitopes presented by MHC class I (MHC I) molecules to CD8⁺ T cells have been defined in mice and humans. These viral immunodominant epitopes are capable of specific ex vivo reactivation of CD8⁺ T cells from infected patients or animals. Unfortunately, immunodo-

minant peptides have not been particularly effective in stimulating primary CD8⁺ T cell immune responses in vivo, thus limiting their vaccine applications. There are no currently available peptide-based or plasmid DNA vaccines encoding only immunodominant CD8⁺ T cell epitopes.

Failure to elicit a robust CD8⁺ T cell response postimmunization with class I peptide epitopes in part stems from the lack of concomitant help from CD4⁺ T cells. In several experimental systems, CD4⁺ T cell help is required for generating and sustaining long-term CD8⁺ T cell memory (1). An additional problem with immunizing with class I binding peptides by themselves is the difficulty in maintaining a sufficient level of Ag presentation required for CD8⁺ T cell activation (2). Peptides are inherently unstable in vivo, and exogenous peptides introduced by vaccination compete with an extensive pool of endogenous peptides for loading onto MHC I molecules and presentation to CD8⁺ T cells. To circumvent these problems, we and others have engineered fully assembled MHC molecules with a linker-attached peptide that can be expressed as membrane proteins on the cell surface (3, 4). These fully assembled MHC molecules, termed single-chain trimers (SCTs), are composed of an immunodominant peptide, an amino acid linker, β_2 -microglobulin (β_2m), a second amino acid linker, and the H chain of a given MHC I molecule.

As a result of high peptide occupancy, SCTs are stably expressed at the cell surface and are potent stimulators of CD8⁺ T cells (3, 5). The SCT format has been widely applicable to several different mouse and human MHC I/peptide complexes (6). Furthermore, when expressed by DNA vaccination, SCTs elicit robust CD8⁺ T cell responses in animal model systems (7–11). Importantly, SCT-based DNA vaccines appeared more effective at generating CD8⁺ T cell immunity than subunit- and epitope-only DNA vaccines even when targeted to the

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Abbreviations used in this paper: β_2m , β_2 -microglobulin; DC, dendritic cell; Env, envelope; MFI, mean fluorescence intensity; MHC I, MHC class I; NS, nonstructural; PADRE, pan HLA-DR-reactive epitope; PFA, paraformaldehyde; SCT, single-chain trimer; SS, signal sequence; TCRm, TCR-mimic; WNV, West Nile virus.

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endoplasmic reticulum lumen, due to incorporation of a preprocessed and preloaded peptide (7, 8). Indeed, when tested preclinically in a mouse tumor model system, SCT-based DNA vaccines conferred protection against tumors (7). Despite this apparent success, up to now, there have been no reports of pathogen protection after DNA vaccine expression of SCTs.

To test the efficacy of an SCT DNA vaccine, we developed a clinically relevant human HLA-transgenic mouse model of West Nile virus (WNV) infection. WNV is mosquito-borne RNA flavivirus, and infection in humans and other vertebrate animals can progress to paralysis, meningitis, encephalitis, and death, especially in the elderly and immunocompromised (12). Although the threat of continued WNV epidemics has stimulated vaccine development, there are currently no commercial human vaccines available (13). Recent studies have suggested that induction of a strong CD8⁺ T cell immune response can enhance the protective activity of a WNV vaccine against challenge (14). Thus, vaccines targeting CD8 immunity to WNV are clearly relevant.

In this report, we study vaccine efficacy of WNV-derived peptides previously shown to bind human HLA-A2 molecules and trigger CD8⁺ T cell responses in patients after WNV infection (15). We show that the same WNV immunodominant peptide in patients is also immunodominant in HLA-A2 transgenic mice, thus validating this model system for study of WNV vaccine efficacy. The immunodominant WNV peptide (designated SVG9) is expressed at high levels on the surface of infected cells as determined using a novel TCR-mimic (TCRm) mAb (J.A. Weidanz, unpublished data). DNA vaccination with plasmids encoding an SCT with the SVG9 peptide bound to HLA-A2 elicited robust CD8⁺ T cell responses that conferred protective immunity against WNV in the absence of pre-existing Ab. Our data suggest that an SCT-based DNA vaccine can elicit a potent antiviral CD8⁺ T cell response and thus should be considered for inclusion in a composite vaccine targeting WNV or other pathogens.

Materials and Methods

Viruses and mouse infection

The WNV strain 3000.0259 was isolated in New York in 2000 and passaged once in C6/36 cells. The WNV-Kunjin (WNV-KUN) strain (16-532) propagated in C6/36 cells has been described previously (16). HHDII transgenic [B6; Cg-B2M^{tm1Unc} H2-D1^{tm1Bpe}Tg (HLA-A/H2-D/B2M)1Bpe] mice were obtained from Dr. Beatriz Carreno (Washington University, St. Louis, MO) with permission of Dr. François A. Lemonnier (Institut Pasteur, Paris, France) (17). These mice express the transgene Tg (HLA-A/H2-D/B2M) 1Bpe in a mixed background involving B2M^{tm1Unc/tm1Unc} and H2-D1^{tm1Bpe/tm1Bpe}. For simplicity, these mice are designated HHDII mice for the rest of the paper. For inoculation of mice, virus was diluted in HBSS containing 1% heat-inactivated FBS. Mice were infected with 10² PFUs of WNV s.c. by footpad injection. All mice were bred and housed at Washington University Animal Facility (St. Louis, MO), and all animal procedures were approved by the Animal Studies Committee at Washington University.

Flow cytometry

IFN- γ production and tetramer binding of splenocytes and surface expression of viral epitope/HLA-A2 complexes on virus-infected cells were monitored by flow cytometry. For intracellular IFN- γ staining, splenocytes were stimulated *in vitro* with each peptide at 0.1 μ g/ml in the presence of GolgiPlug (BD Biosciences, San Jose, CA) for 4 h at 37°C. Cells were washed, stained with FITC-conjugated anti-CD8 α mAb or rat IgG2a (BD Biosciences) and fixed with 1% paraformaldehyde (PFA). After washing twice with buffer containing 0.1% saponin, cells were stained with APC-conjugated anti-IFN- γ mAb or rat IgG1 (BD Biosciences).

PE-conjugated SVG9 peptide/HLA-A*0201 tetramer was obtained from the National Institute of Allergy and Infectious Diseases tetramer facility (Emory University, Atlanta, GA). Cells were stained with tetramers for 40 min at 37°C, and subsequently FITC-conjugated anti-CD8 α mAb was added for additional 20 min at 4°C. Propidium iodide was added shortly

before flow cytometry so that dead cells could be gated. Cells were acquired on FACSCalibur (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Detection of viral epitope/HLA-A2 complex expression

Primary human monocyte-derived dendritic cells (DCs) at day 7 in the culture with GM-CSF and IL-4 were plated at 1 \times 10⁶ cells/ml/well in a non-tissue culture-treated six-well plate. The following day, cells were infected with WNV at a multiplicity of infection of 3 for 48 h. For controls, 10 μ M peptide SVG9 or SLF9 was added to uninfected cells for the last hour. Cells were washed three times and incubated with TCRm, RL14C, or RL15A (mIgG2a) for 40 min on ice. After washing, cells were stained with PE-conjugated goat anti-mouse Ig Ab (BD Biosciences). To confirm the infection, aliquots of cells were stained with anti-WNV envelope mAb E24. Prior to flow cytometry, cells were fixed with PFA.

CTL assays

For *in vitro* CTL assay, CTLs were generated from WNV-KUN-immunized HHDII spleen cells after *in vitro* SVG9 peptide stimulation for 2–4 wk. To perform a [⁵¹Cr]-release assay, target cells labeled with 0.2 mCi of [⁵¹Cr] (PerkinElmer Life Sciences, Wellesley, MA) were incubated with CTLs with or without peptides for 4.5 h at 37°C. To determine maximum lysis, Triton-X 100 was added to control wells. To determine spontaneous lysis, target cells were incubated without CTLs. Supernatants were collected and read by an Isomedic γ -counter (ICN Biomedicals, Huntsville, AL). The percentage of [⁵¹Cr] release was calculated by the formula: [(experimental [⁵¹Cr] release – spontaneous [⁵¹Cr] release)/(maximum [⁵¹Cr] release – spontaneous [⁵¹Cr] release)] \times 100.

For *in vivo* CTL assay, recipient HHDII mice were left uninfected or challenged with 10² PFU of WNV-NY 1 wk before adoptive transfer of target cells. For target cells, naive HHDII splenocytes were stained with 2 μ M PKH26 (Sigma-Aldrich, St. Louis, MO) and incubated with 1 μ M SVG9 or control peptide for 1 h at 37°C. After washing, SVG9- or control peptide-pulsed cells were labeled with 1 μ M or 50 nM CFSE, respectively. Equal numbers of SVG9- and control peptide-pulsed cells were mixed in HBSS, and 2 \times 10⁷ total labeled target cells were transferred *i.v.* into the recipient mice. Six hours later, spleen cells were harvested, fixed with PFA, and evaluated by flow cytometry. Target cells were distinguished from recipient cells based on PKH26 staining and from one another based on CFSE labeling. Gating on PKH26⁺ cells, the percentage of killing was calculated as follows: [1 – ($r_{naive}/r_{infected}$)] \times 100, where r is the percentage of CFSE^{low} cells/percentage of CFSE^{hi} cells.

SCT DNA constructs

The SVG9/HLA-A2*0201 SCT consists of the leader sequence of β_2m followed by the SVGGVFTSV sequence, then a linker of 15 residues (G₄S)₃, and the mature human β_2m sequence. The β_2m was followed by the second linker of 20 residues (G₄S)₄, and the chimeric HLA-A*0201 sequence of which the $\alpha 3$ domain was replaced by that of H-2K^b. For a control SCT, the SVG9 sequence was replaced with the mammoglobin A epitope, LIYDSSLCDL, sequence. The construct was expressed from the pIRESneo vector. The pan HLA-DR-reactive epitope (PADRE) sequence (AKFVAAWTLKAAA) flanked by ATG and TAA was inserted before the leader sequence.

DNA immunization

DNA-coated gold particle-mediated DNA vaccination was performed using a helium-driven gene gun (Bio-Rad, Hercules, CA). DNA-coated gold particles and cartridges were prepared according to the manufacturer's instructions so that each cartridge contained 1 μ g DNA. The DNA-coated gold particles were delivered to the shaved abdomen of mice with a discharge pressure of 400 ψ . Mice were immunized with 4 μ g DNA three times at 3 d intervals.

Detection of IFN- γ response after DNA immunization

Spleens were harvested from mice 5 d after the last DNA immunization. Single-cell suspensions were incubated with 1 μ g/ml SVG9 or control peptides in a plate precoated with 15 μ g/ml of anti-IFN- γ capture Ab (Mabtech, Cincinnati, OH). After overnight stimulation, IFN- γ production was measured by ELISPOT.

Detection of WNV-specific Abs in serum

Serum was collected and added to microtiter plates preabsorbed with purified rWNV E protein (5 μ g/ml). After blocking, incubation of primary and secondary Abs, WNV-specific Ab titers were measured by ELISA. Endpoint titers were determined using GraphPad Prism 5 (GraphPad, San Diego, CA).

Quantification of viral burden

For analysis of virus, brains were recovered after cardiac perfusion with PBS, homogenized, and titrated for virus by a plaque assay using BHK21 cells (18). Virus concentrations were determined as PFUs per gram.

Results

Identification of an immunodominant peptide in HLA-A2 transgenic mice

To identify WNV-derived peptides presented by HLA-A2 MHC I molecules postinfection, members of our group previously eluted peptides from affinity-purified HLA-A2 molecules isolated from uninfected or infected cells (15). Comparative analyses using mass spectrometry identified six virus-derived peptides (Table I). When tested in reactivation assays using CD8⁺ T cells from infected individuals, the WNV E-derived peptide (SVG9) was immunodominant. To determine whether these findings could be replicated in a mouse model of WNV infection, we compared CD8⁺ T cell responses to these six WNV peptides using the HHDII transgenic mice. These mice express a chimeric monochain of HLA-A*0201 ($\alpha 1/\alpha 2$ domains), mouse D^b ($\alpha 3$ domain), and linker-attached human $\beta 2m$ (17) (see *Materials and Methods*). To restrict the development of CD8⁺ T cells to only HLA-A*0201, HHDII mice are also genetically deficient in D^b and mouse $\beta 2m$ and have been useful for preclinical vaccine studies against tumors and viruses (19, 20).

To assess the T cell responses against the six WNV peptides, HHDII mice were infected with 10² PFU of a North American isolate of WNV (strain New York 1999 [WNV-NY]). At 7 d postinfection, splenocytes were harvested and stimulated *ex vivo* with each peptide and then stained for levels of intracellular IFN- γ . Only the SVG9 peptide induced a strong IFN- γ response in CD8⁺ T cells from infected mice (Fig. 1A). On average, ~3% of CD8⁺ T cells produced IFN- γ after restimulation with the SVG9 peptide, whereas a much smaller percentage of CD8⁺ T cells produced IFN- γ in response to other peptides. Consistent with this finding, staining with SVG9 peptide/HLA-A*0201 tetramers confirmed the presence of SVG9-specific CD8⁺ T cells in infected spleens (Fig. 1B). Thus, of the six viral peptides bound to HLA-A*0201, the SVG9 peptide is immunodominant in HLA-A*0201 transgenic mice, results that parallel what was observed in infected humans (15). This concordance supports the relevance of using the HHDII transgenic model to test vaccine efficacy for protection against WNV.

Epitope expression on WNV-infected cells

The immunodominance of the SVG9 peptide suggested that it might be expressed at high levels on virus-infected cells. The six viral peptides listed in Table I, including the immunodominant SVG9 peptide, had been isolated from soluble HLA-A*0201 secreted by infected human HeLa tumor cells. To monitor SVG9 presentation in a more physiologically relevant cell type, human DCs from HLA-A*0201 positive individuals were used. Human DCs are professional APCs and likely a primary cell type infected

Table I. Six identified WNV-derived peptides bound to HLA-A*0201

	Peptide	Sequence	Protein
1	RLD10	RLDDDGNFQL	NS2b
2	ATW9	ATWAENIQV	NS5
3	SVG9	SVGGVFTSV	Env
4	YTM9	YTMDGEYRL	NS3
5	SLT9	SLTSINVQA	NS4b
6	SLF9	SLFGQRIEV	NS4b

Data were adapted from the previous publication by McMurtrey et al. (15). Env, envelope; NS, nonstructural.

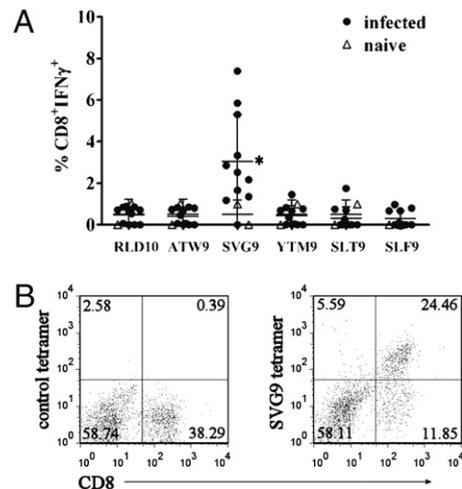


FIGURE 1. Identification of an immunodominant peptide in HLA-A*0201 transgenic mice. HHDII mice were infected with 10² PFU of WNV, and 7 d later spleen cells were harvested. *A*, Cells were stimulated *in vitro* with peptides for 4 h in the presence of Golgi-blocking agent. Then cells were stained for CD8 and intracellular IFN- γ . The percentages of IFN- γ ⁺ of CD8⁺ T cells after each peptide stimulation (*x*-axis) from infected (●) or naive (Δ) mice are shown. *B*, Cells were stimulated *in vitro* with SVG9 peptide for 7 d and stained with anti-CD8 Ab and SVG9/HLA-A*0201 or control tetramers. Cells were gated on propidium iodide-negative events. Numbers indicate the percentage of cells in each quadrant of total cells. **p* < 0.05 (unpaired *t* test).

by WNV *in vivo* (21). To detect the Ag presentation of the viral epitope, TCRm mAbs that are specific for peptide/HLA-A*0201 complexes were generated by immunization of mice with tetramers of peptide/MHC. Two mAbs with different specificities, RL15A and RL14C, were isolated. RL15A recognizes the SVG9/HLA-A*0201 and RL14C the SLF9/HLA-A*0201 complex (J.A. Weidanz, unpublished data).

To monitor WNV epitope display after viral infection, human DCs were infected *in vitro* with WNV and stained with mAbs, RL15A, or RL14C. As a positive control, uninfected DCs were pulsed with each peptide prior to staining; as expected, the TCRm mAbs bound to peptide-pulsed cells specifically (Fig. 2A). The degree of infection was confirmed using an anti-WNV E protein Ab, E24 (Fig. 2B). Both SVG9 and SLF9/HLA-A*0201 complexes were detected on the cell surface of infected cells at high levels, but not on uninfected cells (Fig. 2C). This experiment confirmed that the immunodominant epitope SVG9 is processed and presented in the context of HLA-A*0201 on the surface of APCs after virus infection. To establish the specificity of TCRm mAb RL15A in functional assays, we tested whether it could block SVG9-specific lysis of target cells by CD8⁺ T cells. SVG9-specific CTLs were incubated with target cells pulsed with SVG9 peptide in the presence of RL15A or RL14C. Notably, RL15A completely blocked SVG9-specific killing, whereas RL14C, which has the same IgG1 isotype, did not alter killing (Fig. 2D). Thus, RL15A not only binds to SVG9/HLA-A*0201 complexes presented on infected APCs, but also inhibits TCR-mediated lysis of targets by CD8⁺ T cells.

Cytotoxic activity of SVG9-specific T cells and CTL-mediated protection against infection

To determine whether functional cytolytic CD8⁺ T cells specific for the SVG9 epitope are induced during viral infection, both *in vitro* and *in vivo* assays were employed. For *in vitro* killing assays, bulk CD8⁺ T cells were generated from WNV-KUN immune spleen cells from HHDII mice after *in vitro* restimulation with SVG9 peptide.

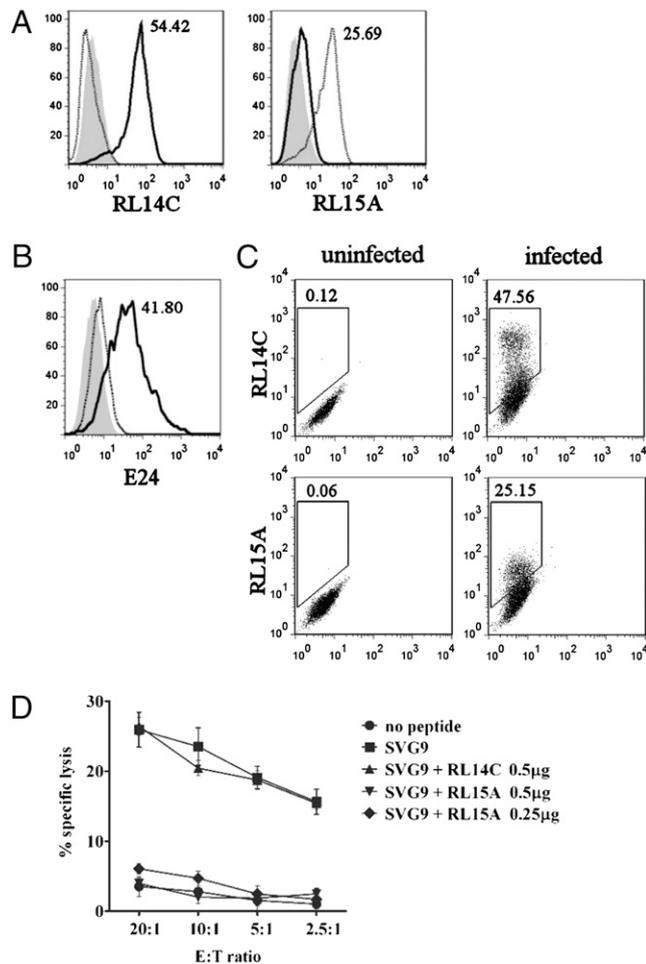


FIGURE 2. Epitope expression on WNV-infected cells. *A*, Primary DCs derived from HLA-A*0201-positive PBMCs were incubated with SVG9 (dotted line) or SLF9 (solid line) peptide for 4 h and stained with TCRm Ab, RL14C, or RL15A. Numbers indicate mean fluorescence intensity (MFI) of each histogram. Shaded histogram indicates unpulsed cells. *B*, Primary DCs were infected with WNV at a multiplicity of infection of 1. Forty-eight hours later, cells were stained with anti-WNV E24 (solid line) or isotype control mAb (dotted line). Shaded histogram indicates uninfected cells. *C*, Uninfected (left panels) or infected (right panels) DCs were stained with RL14C (upper panels) or RL15A (lower panels). Numbers indicate the percentages of TCRm-positive cells among total cells. *D*, A [51 Cr]-release assay was performed in the presence of TCRm Ab. HLA-A*0201-expressing HeLa (HeLaA2) cells were used as targets. SVG9-specific bulk CTLs were incubated with 1 μ M SVG9-pulsed HeLaA2 cells for 4.5 h in the presence of RL14C or RL15A. All data are representative of at least two independent experiments with similar results.

We used WNV-KUN because the mortality rate with WNV-NY approached 100% in the HHDII mice. WNV-KUN is a related lineage I WNV strain with reduced virulence in mice and humans and has 97.6% sequence identity with WNV-NY 1999, including 100% identity of SVG9 peptide. In [51 Cr]-release assays, bulk CD8⁺ T cells from WNV-KUN immune mice lysed SVG9 peptide-loaded target cells as well as WNV-KUN infected cells (Fig. 3A, 3B). To determine whether SVG9-specific CD8⁺ T cells were cytolytic in vivo, a 1:1 mixture of differentially CFSE-labeled naive syngeneic splenocytes preloaded with SVG9 or an irrelevant peptide were adoptively transferred into naive mice or mice infected with WNV. Six hours later, the splenocytes were harvested and analyzed by flow cytometry for levels of CFSE expression. In naive recipient mice, the 1:1 ratio of SVG9-loaded targets and irrelevant

peptide-loaded targets remained the same (Fig. 3C). However, in infected mice, 58% of the SVG9-loaded targets were eliminated, but irrelevant peptide-loaded targets were not, indicating SVG9-specific killing of targets by T cells in vivo. Thus, during WNV infection, SVG9-specific CD8⁺ T cells are generated and lyse virus-infected cells.

Previous studies have established that CD8⁺ T cells contribute to viral clearance of WNV-infected mice. Mice lacking CD8⁺ T cells or MHC I developed higher CNS viral burdens and increased mortality rates postinfection (22). Based on these findings, we assessed whether SVG9-specific CD8⁺ T cells could control WNV infection in vivo. Initially, an adoptive transfer experiment of SVG9-specific CD8⁺ T cells into WNV-infected mice was performed. Mice that received naive CD8⁺ T cells had a 100% mortality rate within 10 d of infection (Fig. 3D). In contrast, mice that received SVG9-restimulated CD8⁺ T cells exhibited a 40% survival rate ($p < 0.0005$; $n = 10-11$). Thus, enhanced CD8⁺ T cell responses specific

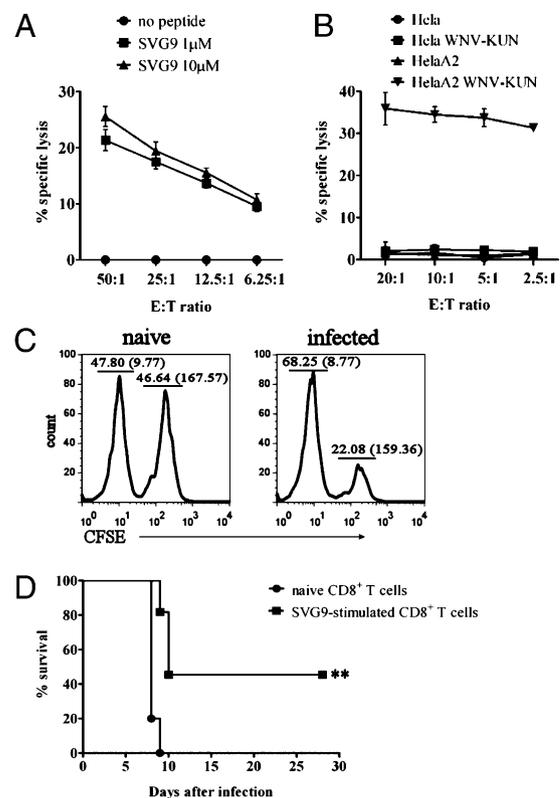


FIGURE 3. Cytotoxic activity of the identified epitope-specific T cells in vitro and in vivo. *A* and *B*, A [51 Cr]-release assay was performed with SVG9-specific CTLs as effectors. *A*, HeLaA2 (HLA-A2-transduced HeLa) targets were incubated with CTLs for 4.5 h in the presence of 1 or 10 μ M of SVG9 peptide. *B*, HeLa or HeLaA2 cells infected with WNV-KUN were used as targets. *C*, In vivo cytotoxic assay. For targets, PKH26-labeled naive HHDII splenocytes were pulsed with SVG9 or irrelevant peptide and labeled with 1 μ M or 50 nM of CFSE, respectively. Differentially labeled targets were mixed at 1:1 ratio and transferred i.v. into a HHDII mouse at day 7 postinfection. Six hours later, spleen cells from the recipients were analyzed. Histograms were gated on PKH26⁺ events. The percentage of CFSE^{hi} or CFSE^{lo} population of PKH26⁺ cells is indicated with MFI in parentheses. All data are representative of at least two independent experiments with similar results. *D*, Donor cells were obtained from HHDII mice that had been infected with 10² PFU of WNV. Cells were restimulated in vitro for 7 d with 0.1 μ M of SVG9 peptide. CD8⁺ T cells were then isolated by negative selection and transferred into HHDII mice at day 1 postinfection with 10² PFU of WNV (2 \times 10⁶ CD8⁺ T cells per mouse). Survival was monitored over 28 d. The results were combined from two independent experiments ($n = 10-11$ per group). ** $p < 0.0005$ (log-rank test).

for an immunodominant epitope SVG9 can provide protection against lethal WNV infection *in vivo*. Importantly, the SVG9 (SVG9VFTSV) sequence is completely conserved in most contemporary and historical WNV isolates available in the National Center for Biotechnology Information database, including WNV-KUN, Egypt 101, the original Uganda 1937 lineage 2 isolate, and all Western Hemisphere strains that have been sequenced to date. Thus, this epitope is an excellent candidate to develop a CD8⁺ T cell-based WNV vaccine for HLA-A2 individuals.

Generation of CD8 T cell responses by DNA vaccination with an SCT incorporating HLA-A*0201 and the SVG9 peptide

Although peptide/MHC SCTs efficiently prime CD8⁺ T cell responses *in vivo* against various Ags (3, 7, 8), it has not been determined whether the SCT-based vaccination can provide protection against pathogens. Because we identified the immunodominant epitope of WNV and showed that increased CD8⁺ T cell responses against the epitope can protect mice against infection, we next tested the efficacy of SCTs containing this epitope as a DNA vaccine for viral infection. An SVG9/HLA-A*0201 SCT (SVG9 SCT) DNA construct was generated. The SCT construct consisted of a short leader sequence followed by the SVG9 peptide, a 15-aa linker, human β_2m , a 20-aa linker, and chimeric HLA-A*0201 H chain containing the $\alpha 3$ domain of K^b (Fig. 4A). As a control DNA, a mamoglobin A epitope, which is a human breast tumor Ag known to bind HLA-A*0201 (10), was inserted in place of the SVG9 sequence in the same plasmid. We also incorporated a CD4⁺ Th epitope, PADRE, sequence into the construct to enhance CD8⁺ T cell responses (23).

To determine whether the SVG9 SCT was properly folded and expressed on the cell surface, HeLa cells were transfected with SVG9 SCT DNA and stained with anti-HLA-A*0201 Ab (BB7.2) or RL15A. SVG9/HLA-A*0201 complexes were detected on the cell surface by both Abs (Fig. 4B), suggesting stable expression of the complexes with native folding. Also, in a cytotoxicity assay, SVG9-specific CD8⁺ T cells lysed SVG9 SCT-expressing target cells *in vitro* comparably to that of peptide-pulsed target cells (Fig. 4C), confirming that SCTs are recognized by Ag-specific CD8⁺ T cells. We then tested whether immunization with SVG9 SCT DNA can elicit specific CD8⁺ T cell responses. SVG9 or control SCT DNA plasmid was injected intradermally three times into the abdomen of naive HHDII mice using a gene-gun approach (24). At day 5 after the last immunization, splenocytes were harvested and stained with SVG9/HLA-A*0201 tetramers or restimulated *in vitro* with SVG9 peptide to induce IFN- γ production. Immunization with SVG9 SCT DNA induced an average of 27% of splenic CD8⁺ T cells to become Ag-specific, as judged by tetramer-positive reactivity (Fig. 4D) and production of IFN- γ from ~20% of splenic CD8⁺ T cells after SVG9 peptide stimulation (Fig. 4E). These findings demonstrate that SVG9 SCT DNA vaccination induces a robust SVG9-specific CD8⁺ T cell response *in vivo*.

Protection against lethal WNV infection by SCT DNA vaccination

To test whether vaccination with SCT DNA can protect mice against lethal infection, naive HHDII mice were vaccinated with SVG9 or control SCT DNA plasmids and subsequently infected with WNV-NY at day 5 after the last immunization. Mice vaccinated with control SCT DNA developed the expected CNS disease symptoms beginning at day 8 until death, which occurred 2–5 d later (100% mortality rate). In contrast, of mice vaccinated with SVG9 SCT DNA, ~30% developed disease symptoms and 25% became moribund by days 10–13 postinfection. The remainder of immunized mice never developed disease symptoms and were healthy for 60 d postinfection (75% survival rate; Fig. 5A). This result clearly es-

tablishes that T cell immunity induced by SVG9 SCT DNA vaccination in the absence of a humoral response protects mice from lethal WNV infection. We next assessed whether SCT vaccine efficacy required coexpression of the CD4⁺ Th cell epitope, PADRE. Interestingly, immunization of the SVG9 SCT DNA with or without PADRE resulted in a comparable number of SVG9-specific T cells by IFN- γ production (Fig. 5B, 5C), but coexpression of SCT with PADRE was significantly more effective at conferring protective immunity (Fig. 5D; $p < 0.05$; $n = 8-9$).

To define the mechanism of disease protection, the viral burden in the brains of SVG9 and control SCT-vaccinated mice was compared

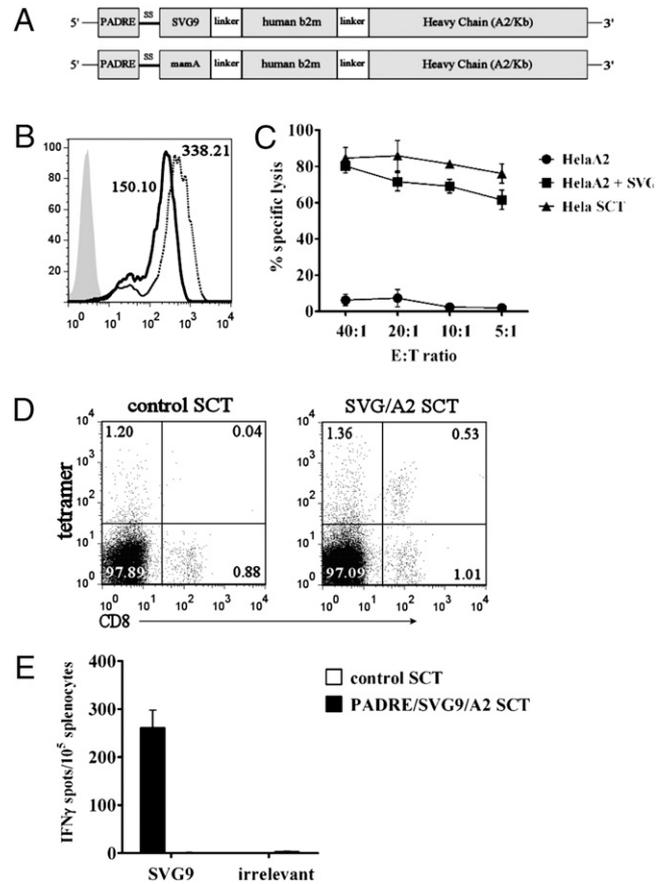


FIGURE 4. Generation of SVG9/HLA-A*0201 SCTs and specific CD8⁺ T cell responses. *A*, Diagram of the SCT DNA encoding SVG9 or mamoglobin A (mamA) peptide/chimeric HLA-A*0201 complex. *B*, Cell surface expression of SVG9 SCT. HeLa cells were transfected with SVG9 SCT DNA and stained with anti-HLA-A2 Ab (BB7.2, dotted line), RL15A (solid line), or isotype control Ab (shaded histogram). Numbers indicate MFI of each histogram. *C*, Lysis of target cells expressing SVG9 SCT. SVG9 SCT-expressing HeLa cells were used as targets in a [⁵¹Cr]-release assay with SVG9-specific CTLs. For controls, HeLaA2 cells or HeLaA2 cells pulsed with SVG9 peptide were also used as targets. *D* and *E*, Generation of specific CD8⁺ T cell responses *in vivo*. HHDII mice were immunized with SVG9 or control SCT DNA three times at 3 d intervals. At 5 d after the last immunization, splenocytes were harvested. *D*, Splenocytes from control (left panel) or SVG9 SCT-immunized mice (right panel) were stained with anti-CD8 Ab and SVG9/HLA-A*0201 tetramers. Numbers indicate the percentage of cells in each quadrant among total cells. Representative figures of the flow cytometry data. *E*, Splenocytes from each group were stimulated *in vitro* with SVG9 or irrelevant peptide overnight, and IFN- γ secretion was measured by ELISPOT. Error bars indicate SE of the experiment. The data presented are from one representative experiment of two performed independently with each group containing four to five mice. SS, signal sequence.

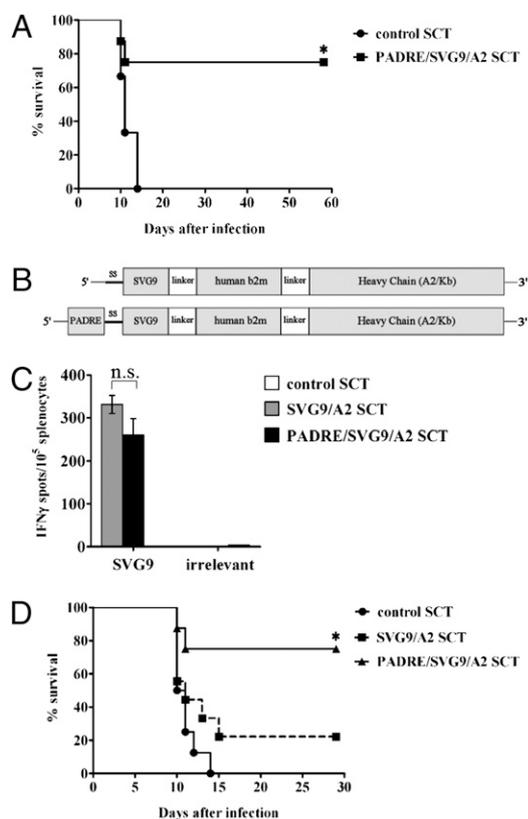


FIGURE 5. Protection against viral infection by SVG9 SCT DNA vaccination. **A**, HHDII mice were immunized with SVG9 or control SCT DNA with PADRE. At 5 d after the last immunization, mice were infected with 10^2 PFU of WNV. Mice were monitored over 60 d ($n = 11$ –12 per group). $*p < 0.05$ (log-rank test). **B**, Diagram of a SVG9 SCT DNA construct with or without an insertion of PADRE sequence. **C** and **D**, HHDII mice were immunized with SVG9 SCT with or without PADRE or control SCT DNA. **C**, At 5 d after the last immunization, splenocytes were stimulated in vitro with 1 μ M SVG9 or irrelevant peptide overnight. IFN- γ secretion was measured by ELISPOT. Error bars indicate SD with $n = 5$ mice per group. n.s., unpaired t test. **D**, At 5 d after the last immunization, mice were infected with 10^2 PFU of WNV and monitored over 30 d ($n = 8$ –9 per group). $*p < 0.05$ (unpaired t test) compared with SVG9 SCT without PADRE. SS, signal sequence.

on day 8, the time point at which infected HHDII mice became noticeably ill. Consistent with the clinical phenotype and decreased lethality, SVG9 SCT-vaccinated mice had 100-fold lower levels of WNV in the brain than control SCT-vaccinated mice (Fig. 6A). These results suggest that SVG9 SCT vaccination increased survival rate by limiting virus spread to the brain and/or by accelerating virus clearance. This effect was independent of humoral responses because the levels of anti-WNV E IgG and IgM in the serum were comparable between the two groups (Fig. 6B). This finding was expected because the chemical stability of the SCT makes it unlikely that the SVG9 epitope would be released to induce epitope-specific Abs. Moreover, the SVG9 epitope, which is located beyond domain III at residues 430–438 of E protein, is not part of any known B cell epitopes for neutralizing Ab in mice or humans (25, 26). We also examined whether the SCT-vaccinated mice that survived viral infection developed memory CD8⁺ T cells and protective Abs against the virus. Mice were vaccinated with SVG9 SCT DNA, then infected with WNV, and, at day 65, splenocytes and serum were analyzed for the presence of SVG9/HLA-A*0201 tetramer-positive cells and the ability to produce IFN- γ upon restimulation with SVG9 peptide and for levels of specific Abs against WNV E protein. About 12% of

CD8⁺ T cells in the spleens of vaccinated mice stained positive with SVG9-specific tetramers and were CD44^{hi}, consistent with a memory phenotype (Fig. 6C). Accordingly, ~11% of CD8⁺ T cells produced IFN- γ after restimulation with the SVG9 peptide (Fig. 6D). WNV-specific IgG responses were detected in all SVG9 SCT-vaccinated mice, indicating nonsterilizing immunity and the induction of durable humoral responses postchallenge (Fig. 6E).

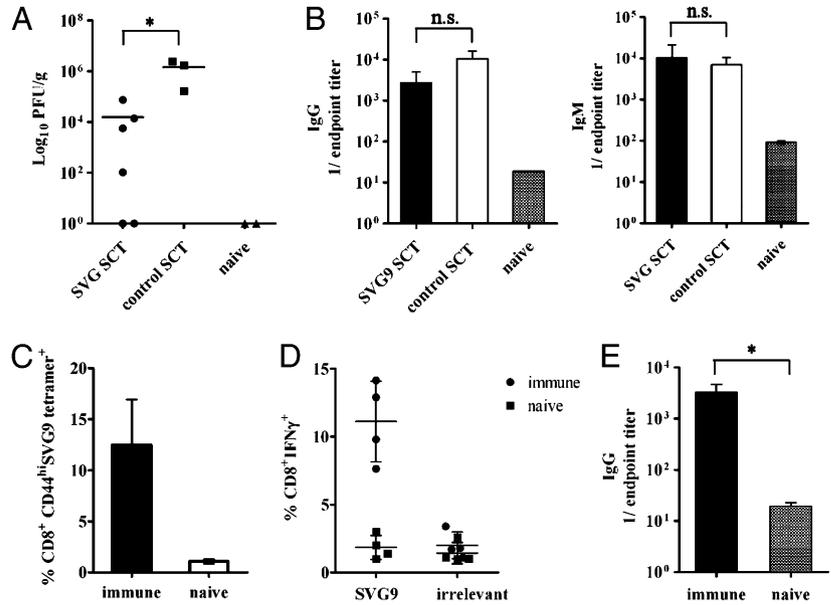
Finally, whether DNA vaccination provides long-lasting protective immunity was addressed. Mice were vaccinated with SVG9 or control SCT DNA with PADRE as described above, rested for 45 d, and then challenged with 10^2 PFU of WNV. SVG9 SCT DNA vaccination conferred 46% survival against lethal WNV infection (Fig. 7; $p < 0.05$; $n = 11$ –13), demonstrating functional memory T cell responses in vaccinated mice. In conclusion, SVG9 SCT DNA vaccination protects mice from lethal WNV infection by inducing strong and long-lasting Ag-specific CD8⁺ T cell immunity and that optimal protection requires coexpression of a Th cell determinant.

Discussion

DNA vaccines are an attractive approach to generate humoral and cellular immunity because plasmids are relatively safe, easy to produce, and readily amenable to technological improvements (27). Of particular relevance for pathogen immunity, DNA vaccines induce CD8⁺ T cell immunity that may not be elicited by inactivated or protein subunit-based vaccine platforms. DNA vaccines do not have the risks associated with production of live attenuated viral vaccines, especially for administration to the elderly or immunocompromised, the likely targets of diseases of many severe acute infections including WNV and seasonal influenza. Within the last few years, several technical innovations in DNA vaccination have occurred, including more effective delivery strategies, immune enhancements using adjuvants, and targeting to specific intracellular pathways. Even with these advances, a major hurdle for improving DNA vaccines is increasing immunogenicity. This issue is particularly relevant for larger animals and humans in whom the DNA platform has proven less effective than in mice. In this study, we used a unique approach to improve CD8⁺ T cell responses by targeting an immunodominant WNV epitope to the MHC I Ag presentation pathway with SCT-based technology.

Several properties of SCTs likely contribute to their effectiveness in eliciting a CD8⁺ T cell response. The expressed SCT polypeptide includes all three MHC I components, peptide, β_2m , and H chain attached sequentially with flexible linkers (3). The SCT platform appears readily applicable to different mouse and human MHC I/peptide complexes (8, 28). The preassembled nature of SCTs allows them to rapidly fold, exclude the binding of competitive peptides, and expediently transit to the cell surface. Furthermore, the linker-controlled peptide occupancy renders the SCT stable at the cell surface. Once on the cell surface, SCTs are recognized by CD8⁺ T cells in a manner comparable to native class I/peptide complexes (3). Thus, immunization of SCT induces the stable display of specific class I/peptide complexes for a duration sufficient to elicit a robust CD8⁺ T cell response. Indeed, there are now several published examples of plasmids encoding SCT that elicit robust CD8⁺ T cell responses in model systems, and SCT-based vaccines confer protection against tumor challenge in mice (7, 8). Although SCTs incorporating viral epitopes have been shown to elicit CD8⁺ T cells, prior to our study, no previous reports have documented the utility of SCT-based vaccines in conferring protection against lethal challenge by a pathogen. Application of SCTs to pathogen immunity is attractive given that CD8⁺ T cells detect unique pathogen-derived epitopes, whereas tumor-associated epitopes are typically overexpressed self-proteins. It is also notable that immune evasion mechanisms can mitigate CD8⁺

FIGURE 6. Viral clearance and induction of non-sterilizing immunity by SCT DNA immunization. *A* and *B*, Brain tissues and serum were collected at day 8 after viral challenge of HHDII mice that had been immunized with SVG9 or control SCT DNA. *A*, Viral burdens in brain tissues were determined by a plaque assay. Naive mice were used as a negative control. *B*, The levels of anti-WNV IgG (*left panel*) and IgM (*right panel*) in the serum were measured by ELISA. n.s., unpaired *t* test. *C–E*, Spleen and serum samples were taken from SVG9 SCT DNA-vaccinated mice at day 65 post-WNV infection. Naive mice were used as a negative control. *C*, Splenocytes were stained with anti-CD8, anti-CD44 mAb, and SVG9/HLA-A*0201 tetramers. Mean percentage of tetramer-positive CD44^{hi}CD8⁺ T cells of four mice in each group was shown. Error bars indicate SD. *D*, Splenocytes were stained for CD8 and intracellular IFN- γ after 4 h stimulation *in vitro* with SVG9 or irrelevant peptide in the presence of Golgi-blocking agent. *E*, WNV-specific IgG titers in the serum were measured by ELISA. Data were expressed as reciprocal log endpoint titers after regression analysis. **p* < 0.05 (unpaired *t* test).



T cell responses to pathogens by mechanisms that include blocking Ag presentation. Indeed, commonly used viral immune evasion mechanisms prevent peptide generation by inhibiting proteasome function or peptide loading by inhibiting TAP function (29). The preprocessed and preassembled nature of the SCT makes their expression impervious to these mechanisms of immune evasion (9).

The ongoing discovery of pathogen-derived peptide epitopes presented by different HLA molecules will allow construction of tailored SCT-based vaccines. The effectiveness of SCT vaccines may be related to the immunodominance of particular CD8⁺ T cell epitopes. Mechanistically, immunodominance is believed to be affected by: 1) peptide abundance as determined by the turnover rate of the donor protein and the peptide-processing efficiency; 2) peptide-binding affinity to MHC I molecules; and 3) the CD8⁺ T cell repertoire, which is determined during thymic selection. Although six different WNV peptides were eluted from HLA-A2 molecules, the E-derived peptide SVG9 was immunodominant in HLA-A2 transgenic mice and humans. This concordance suggests that the SVG9 peptide is efficiently processed and presented by both mouse and human APCs. In support of this, high levels of SVG9/HLA-A2 complexes were displayed on the surface of mouse or human cells infected *in vitro* with WNV. Interestingly, comparably high levels of HLA-A2 bound by the SLF9 peptide were also displayed on the cell surface of WNV-infected cells (Fig. 2C), even though few if any SLF9-specific CD8⁺ T cells were detected in

restimulation assays. Thus, the level of epitope presentation on the APC alone does not explain immunodominance. We speculate the T cell repertoire must also contribute to the immunodominance of the SVG9 peptide. Plasmid DNA encoding an SCT with the SVG9 peptide elicited a robust CD8⁺ T cell response as determined by intracellular IFN- γ production and protective immunity against WNV infection. Coexpression of a helper epitope with the SCT did not affect the level of SVG9-specific T cells, but was required for optimal protective immunity. This observation is consistent with the previous findings showing that CD4 deficiency did not affect the primary CD8⁺ T responses but did compromise the responses at later time points after WNV infection (30). Thus, the inclusion of the helper epitope in the SCT likely facilitates development of central and effector memory or the presence of memory T cells in tissues. Our studies also confirm the importance of CD8⁺ T cells in controlling WNV during primary infection (22). Indeed, the induction of WNV-specific CD8⁺ T cells was sufficient to control infection in the complete absence of humoral immunity. These findings also extend earlier studies in which adoptive transfer of bulk CD8⁺ T cells from infected mice protected naive mice from WNV challenge and immunization with a D^b-restricted immunodominant NS4b peptide limited WNV-induced disease and mortality (16, 31).

Although SCT vaccines elicit Ag-specific CD8⁺ T cells, it is likely that their effectiveness can be enhanced once the immune response to DNA vaccination is better understood. For example, the identity of the critical cells expressing Ags after DNA vaccination remains controversial as well as the relative importance of direct versus cross-presentation for CD8⁺ T cell activation (27, 32, 33). To realize the full potential of SCT vaccine approaches, it will be necessary to define the precise cellular and molecular mechanism of Ag presentation after DNA plasmid vaccination. Then, expression of the SCT can be targeted to appropriate cell types to provide maximal CD8⁺ T responses. In summary, SCT-based DNA vaccines have the potential to induce pathogen-specific CD8⁺ T cell responses. Incorporation of an SCT vaccine with other classical approaches that elicit robust humoral immunity may provide a focused approach to stimulating both arms of the adaptive response and generating protective immunity against a range of infectious diseases.

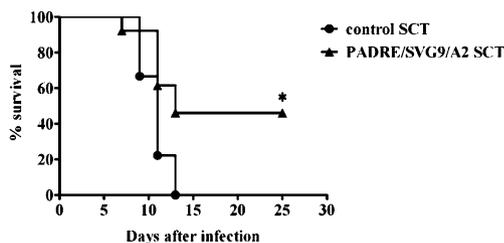


FIGURE 7. Long-term immunity provided by SVG9 SCT DNA vaccination. HHDII mice were vaccinated with SVG9 or control SCT DNA with PADRE as above and, at 45 d after the last immunization, challenged with 10² PFU of WNV. Mice were monitored over 25 d (*n* = 11–13 per group). **p* < 0.05 (log-rank test).

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Disclosures

J.A.W. is associated with Receptor Logic, Inc., a company that develops TCRm mAbs for possible commercial development.

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