Differential Outcome of Tolerance Induction in Naive versus Activated Theiler's Virus Epitope-Specific CD8⁺ Cytotoxic T Cells[▽]

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Tolerance induced by the intravenous injection of peptide-pulsed, ethylene carbodiimide (ECDI)-fixed splenic antigen-presenting cells (Ag-SP) is a safe and effective method of inducing specific unresponsiveness in CD4⁺ T cells for the prevention and treatment of a variety of autoimmune diseases. We determined whether Ag-SP tolerance could also be used to tolerize CD8⁺ T cells. We show in the Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease model of multiple sclerosis that CD8⁺ T cells specific for both dominant and subdominant epitopes can be rendered tolerant. Interestingly, although virus clearance was delayed, lack of the virus-specific cytotoxic T-lymphocyte response did not result in the conversion of normally TMEV-resistant C57BL/6 mice to a susceptible phenotype. Importantly, we found that Ag-SP tolerance may not be a practical treatment for human diseases in which CD8⁺ T cells play a major role in pathogenesis, as tolerance induction in mice previously infected with TMEV led to a severe, often fatal reaction.

Multiple sclerosis (MS) is a demyelinating disease in which autoreactive CD4⁺ and CD8⁺ T cells are believed to be responsible for much of the damage to myelin sheaths (5, 10). Theiler's murine encephalomyelitis virus (TMEV) causes a CD4⁺ T-cell-mediated demyelinating disease in susceptible mice and serves as a model of MS. Upon TMEV infection, some strains of mice (e.g., SJL/J) remain persistently infected and succumb to a paralytic demyelinating disease known as TMEV-induced demyelinating disease (TMEV-IDD), while others (e.g., C57BL/6) rapidly clear the virus from the central nervous system (CNS) and are completely protected from disease (2, 6). Susceptibility has been linked to the *H-2* locus, suggesting that CD8⁺ T cells are, to some degree, involved in protection from TMEV-IDD (9, 32, 34). Specifically, the *H-2D* genes have been associated with resistance (1, 21).

Unlike TMEV-susceptible SJL mice, B6 mice are reported to develop a strong CD8⁺ T-cell response to TMEV that migrates efficiently to the CNS and peaks 7 to 8 days postinfection (p.i.). This response is directed against an immunodominant epitope, VP2₁₂₁₋₁₃₀, and two subdominant epitopes, VP2₁₆₅₋₁₇₃ and VP3₁₁₀₋₁₂₀ (23). The immunodominant VP2₁₂₁₋₁₃₀ response is a potent producer of gamma interferon (IFN- γ) and induces a highly lytic response, whereas the VP2₁₆₅₋₁₇₃ response is a potent IFN- γ producer but does not induce a lytic response and the VP3₁₁₀₋₁₂₀ response is somewhat lytic but produces little IFN- γ . Several studies have addressed the role of the TMEV-specific CD8⁺ T-cell response in viral clearance and protection from disease, but most of these studies have addressed the role of the CD8⁺ T-cell response by using CD8 depletion or knockout mice that lack various aspects of the CD8⁺ T-cell response (12, 27, 31, 33). We sought to study the

virus-specific CD8⁺ T-cell response in a more precise way by using antigen-specific tolerance.

Antigen-specific tolerance would be an ideal therapy for human autoimmune diseases such as MS and type 1 diabetes, for which current therapies are nonspecific and generally ineffective (10, 39). Antigen-specific tolerance can be induced by several methods, including injection of soluble peptide to delete or anergize specific cells or injection of altered peptide ligands designed to elicit antiinflammatory responses from self-reactive T cells. These therapies have been shown to lead to undesirable side effects such as toxicity and anaphylactic shock when used in a therapeutic mode (16, 36). The ideal tolerance therapy would work through anergy or deletion of specific cells and not through mechanisms such as immune deviation, which could have suppressive effects on bystander T cells. Furthermore, this ideal therapy would be associated with an extremely low risk of adverse effects. Tolerance induced by intravenous (i.v.) injection of peptide-pulsed, ethylene carbodiimide (ECDI)-fixed splenic antigen-presenting cells (Ag-SP) has proven to be a safe and highly effective method of inducing antigen-specific unresponsiveness in CD4⁺ T cells for the prevention and treatment of a variety of autoimmune diseases (17, 18, 25, 36).

In autoimmune diseases such as MS and type 1 diabetes, CD4⁺ T cells have traditionally been thought to be the main mediators of tissue damage. More recently, however, CD8⁺ T cells have been implicated in the pathogenesis of these diseases (4, 19, 20, 37). In MS, for example, significant numbers of CD8⁺ T cells are present in active MS lesions, and myelin-reactive CD8⁺ T cells have been isolated from MS patients (3, 26, 41). Patients with other CD8⁺ T-cell-mediated diseases, autoimmune or otherwise, would also benefit from antigen-specific therapies (19, 26). We therefore set out to determine if Ag-SP tolerance could efficiently tolerize CD8⁺ T-cell responses. We show that CTL responses to TMEV epitopes can, in fact, be completely tolerized by Ag-SP injection prior to virus infection. Surprisingly, the lack of virus-specific CD8⁺ T

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cells was shown to only delay TMEV clearance in the CNS by a few days in resistant C57BL/6 mice. An important goal of these studies was to determine if induction of Ag-SP tolerance could be applicable to therapy for human diseases in which CD8⁺ T cells are pathogenic. We show that Ag-SP tolerance may not be a safe option for targeting primed or memory CD8⁺ T cells in ongoing disease, as TMEV-infected mice that are subsequently tolerized with VP2₁₂₁₋₁₃₀-SP underwent a severe, often fatal systemic cytokine-mediated reaction.

MATERIALS AND METHODS

Mice. C57BL/6 (B6) mice and IFN- γ ^{-/-} mice on the C57BL/6 background were purchased from Jackson Laboratories, Bar Harbor, ME. All mice were housed in the Center for Comparative Medicine, Northwestern University, Chicago, IL, under the guidelines of the Animal Care and Use Committee.

Peptides. All synthetic peptides were obtained from Genemed Synthesis, San Francisco, CA. These included TMEV peptides VP2₁₂₁₋₁₃₀ (FHAGSLVFM), VP2₁₆₅₋₁₇₃ (TGYRYDSRT), and VP3₁₁₀₋₁₂₀ (NFLFVFTGAAM), as well as simian virus 40 (SV40) epitope I (SAINNYAQKL) and lymphocytic choriomeningitis virus (LCMV) NP₂₀₅₋₂₁₂ (YTVKYPNL). All peptides were purified by high-performance liquid chromatography and certified to be $\geq 95\%$ pure.

TMEV inoculation and evaluation of TMEV-induced disease. Mice were infected by intracerebral injection with 4×10^6 to 10×10^6 PFU of TMEV strain BeAn 8386 in a volume of 30 μ l of serum-free Dulbecco's modified Eagle's medium. For long-term experiments, mice were monitored and assessed for disease severity every 3 to 4 days. The following grading scale was used as a guideline: 0, asymptomatic; 1, lowered hind end and/or mild-to-moderate waddling gait; 2, lowered hind end with splayed legs and much difficulty walking; 3, paralysis of one limb; 4, paralysis of more than one limb, severe difficulty in movement; 5, moribund. However, mice in the present experiments did not progress beyond a score of 1.

Priming of mice with peptide or adjuvant. Mice were primed with an emulsion containing 1 mg/ml peptide (VP2₁₂₁ or LCMV NP₂₀₅) or phosphate-buffered saline (PBS) and complete Freund's adjuvant (CFA) containing 2 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). A 100- μ l volume of emulsion was injected subcutaneously divided among three sites on the flank of each mouse.

Plaque assays. Mice were anesthetized with 5 mg/ml pentobarbital sodium (Nembutal) and perfused. Spinal cords and brains were collected, weighed, and homogenized with a Polytron System PT1200C tissue homogenizer (Kinematic AG, Littau-Lucerne, Switzerland). Homogenates were serially diluted and added to tissue culture-treated plates (Nunc, Roskilde, Denmark) of confluent BHK-21 cells for incubation at room temperature for 1 h with periodic gentle rocking. Cells were then covered with a medium-agar solution containing 1% Noble agar (BD, Sparks, MD). Following a 5-day incubation at 34°C, the agar was removed and the BHK-21 cell monolayer was fixed with formalin or methanol (Fisher Scientific, Fair Lawn, NJ). Plaques were visualized by staining with crystal violet. To determine the number of PFU per milliliter of homogenate, the number of plaques on each plate was multiplied by the dilution factor of the homogenate and divided by the amount of homogenate added per plate. The number of PFU per milliliter was divided by the weight of the tissue to calculate the number of PFU per milligram of tissue.

Induction of peptide-specific tolerance. Splenocytes suspensions from naive B6 mice were treated with Tris-NH₄Cl to remove red blood cells and then suspended in PBS at a concentration of 3.2×10^6 cells/ml with the appropriate peptide at 1 mg/ml and ECDI (Calbiochem, La Jolla, CA) at 30.75 mg/ml. The mixture was incubated on ice for 1 h with constant shaking. Ag-SP were washed and filtered, and mice were injected i.v. with 5×10^7 cells in 300 μ l of PBS.

Soluble peptide administration. Mice were injected intravenously with 100 μ g of the indicated peptide in 200 μ l of PBS at the indicated time point relative to infection.

Isolation of CNS-infiltrating cells. Mice were anesthetized with pentobarbital sodium and perfused with PBS. Brains and spinal cords were harvested, minced with scissors, and treated with Liberase R1 (Roche, Indianapolis, IN) and DNase I (Invitrogen, Carlsbad, CA) for 45 min at 37°C. Samples were pushed through 40- μ m-pore-size Nytex to make a single-cell suspension. Mononuclear cells were isolated with a 30/70 Percoll gradient centrifuged at $2,500 \times g$ for 20 min.

Flow cytometric analysis. Cells to be analyzed by flow cytometry were blocked with anti-CD16/32 (BD Biosciences, San Jose, CA) and/or mouse or rat serum. For CD3 and CD8 labeling, cells were stained with 0.5 μ g of antibody per 10^6

cells with eBioscience (San Diego, CA) antibodies. For tetramer staining, H-2D^b:VP2₁₂₁₋₁₃₀ tetramers were procured from the NIH Tetramer Core facility (Atlanta, GA) and cells were stained with a 1:150 dilution of the tetramer. Samples were run on an LSRII flow cytometer with FACS Diva software (Becton Dickinson, Mountain View, CA) and analyzed with CellQuest Pro Software (Becton Dickinson).

Enzyme-linked immunospot (ELISPOT) assays. ELISPOT assays were carried out as previously described (38), with the following modifications. Unless otherwise noted, 5×10^5 bulk splenocytes were plated per well with 0 or 50 μ g of the indicated antigen. For some experiments, CD4⁺ and/or CD8⁺ cells were positively or negatively selected prior to plating by Auto-Macs bead separation (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. All ELISPOT data are presented as the mean number of spots per well $\pm$ the standard error of the mean.

In vivo cytotoxicity assays. Splenocytes were collected from naive animals, treated with NH₄Cl to lyse red blood cells, and divided into two populations. Each population was pulsed with either cognate or irrelevant peptide, and then the two populations were labeled with differential concentrations of 5-carboxy-fluorescein diacetate succinimidyl ester (CFSE). These two populations were injected in equal numbers into immune (infected) or naive animals at 6×10^6 to 10×10^6 total cells per mouse. After 15 to 18 h, splenocytes were collected from recipients and analyzed by flow cytometry for the presence and relative numbers of cells in each CFSE⁺ peak. Cells loaded with cognate antigen were lysed by antigen-specific CD8⁺ T cells in immune animals, and thus the corresponding peak was drastically reduced. To determine the percent lysis, an adjustment factor (*A*) was obtained from naive controls by dividing the percentage of cells within the cognate peptide peak by the percentage of cells within the irrelevant peptide peak. The average *A* from two or three naive mice was then used in the following equation: % lysis = [(% irrelevant peptide $\times A$) - (% cognate peptide)]/(% irrelevant peptide $\times A$).

Analysis of serum cytokines. Mice were anesthetized, and blood was collected by cardiac puncture. Serum was harvested and analyzed for cytokine content with a 10-plex multicytokine kit (QIAGEN, Valencia, CA) and a Luminex LiquiChip analyzer (QIAGEN, Valencia, CA).

RESULTS

VP2₁₂₁-SP tolerance effectively inhibits development of VP2₁₂₁₋₁₃₀-specific CD8⁺ T cells. We first determined if Ag-SP tolerance would be an effective method to tolerize CD8⁺ T cells. We injected 5×10^7 B6 splenocytes coupled with either the immunodominant TMEV epitope, VP2₁₂₁₋₁₃₀, or an irrelevant peptide into naive B6 recipient mice 7 days prior to TMEV infection and assessed their recall responses at various time points thereafter with multiple assays. In the periphery of VP2₁₂₁-SP-tolerized recipients, the frequency of IFN- γ -producing cells from bulk splenocytes (Fig. 1A) in response to VP2₁₂₁₋₁₃₀ was completely inhibited compared to that in sham-SP-tolerized recipients. Similarly, in the CNS, VP2₁₂₁₋₁₃₀-elicited IFN- γ production was significantly reduced in VP2₁₂₁-SP-tolerized mice (Fig. 1B). Tolerance of the IFN- γ response was long lasting, as it was maintained at >150 days p.i. (long after the virus has been cleared in control infected B6 mice), and unlike sham-SP recipients, VP2₁₂₁-SP recipients were unable to mount a memory response to VP2₁₂₁₋₁₃₀ (Fig. 1C). The frequency of IFN- γ -producing cells among splenocytes depleted of CD4⁺ T cells was inhibited similarly to that from bulk splenocytes (Fig. 1D), thus, the presence or absence of CD4⁺ T cells did not affect the ability of Ag-SP to tolerize CD8⁺ T cells. Tolerization with VP2₁₂₁-SP also inhibited the in vivo lytic response to VP2₁₂₁₋₁₃₀-loaded target cells compared to the response in sham-SP treated, TMEV-infected recipients (Fig. 2). Lastly, at day 6 p.i., while approximately 3% of the CD8⁺ T cells in the spleen and 35% of the CD8⁺ T cells in the CNS were specific for VP2₁₂₁₋₁₃₀ in sham-SP recipients, the percentage of VP2₁₂₁₋₁₃₀-specific CD8⁺ T cells in VP2₁₂₁-SP-

FIG. 1. Frequency of peptide-specific CD8⁺ IFN- γ -producing cells in the periphery and the CNS in sham-SP- and VP2₁₂₁-SP-tolerized mice following TMEV infection. B6 mice were tolerized or sham tolerized (five mice per group) with 5×10^7 VP2₁₂₁₋₁₃₀-coupled (VP2₁₂₁-SP) or sham-coupled (sham-SP) cells 7 days prior to infection. Cells producing IFN- γ in response to VP2₁₂₁₋₁₃₀ were enumerated by ELISPOT assay at day 15 p.i. from unseparated splenocytes (A) and brain-infiltrating mononuclear cells (B) in response to a challenge with both TMEV VP2₁₂₁₋₁₃₀ and an irrelevant H-2D^b-binding peptide (SV40 epitope I). The data in panels A and B are representative of three separate experiments at this time point, and similar results were obtained at other time points during the acute CD8⁺ T-cell response to TMEV. (C) Splenic IFN- γ ELISPOTs were enumerated at day 158 p.i. Data are representative of two separate experiments. (D) Splenocytes were collected on day 8 p.i. and depleted of CD4⁺ T cells with an Auto-Macs depletion system prior to plating. CD8⁺ cells made up $\geq 99.75\%$ of the T cells following separation. Data in this panel are representative of three separate experiments. *, $P < 0.01$; **, $P < 0.001$.

tolerized recipients was reduced to $<1\%$ in the spleen and $<2\%$ in the CNS (Fig. 3). Thus, not only were TMEV-specific effector functions vastly reduced in VP2₁₂₁-SP recipients, but the VP2₁₂₁₋₁₃₀-specific response was, in fact, unable to expand. Collectively, these results indicate that Ag-SP tolerance leads to a profound diminution of CD8⁺ T-cell responses to TMEV epitopes in both the periphery and the CNS.

Interestingly, we noted that mice tolerized with the immunodominant VP2₁₂₁₋₁₃₀ epitope also lost the ability to respond

to the two TMEV subdominant epitopes VP3₁₁₀₋₁₂₀ and VP2₁₆₅₋₁₇₃ following TMEV infection. This effect was apparent both in splenic IFN- γ ELISPOT assays (Fig. 4A) and in *in vivo* lysis of VP3₁₁₀₋₁₂₀-loaded target cells (Fig. 4B). Lysis of VP2₁₆₅₋₁₇₃-loaded cells is not detectable by this assay, so lysis of cells loaded with this peptide in VP2₁₂₁-SP recipient mice was not assessed. The lack of subdominant CD8⁺ T-cell responses in VP2₁₂₁-SP recipients can, in part, be attributed to the fact that the VP2₁₂₁₋₁₃₀ and VP2₁₆₅₋₁₇₃ peptides are par-

FIG. 2. Abrogated lysis of VP2₁₂₁-loaded target cells in VP2₁₂₁-SP-tolerized B6 mice infected with TMEV. Mice were tolerized to either VP2₁₂₁₋₁₃₀ or a sham peptide 6 days prior to TMEV infection. At 9 days p.i., an *in vivo* lysis assay was performed in which recipients of the target cells included naive mice, sham-tolerized mice, and VP2₁₂₁₋₁₃₀-tolerized mice. In each panel, the peak corresponding to the higher concentration of CFSE represents cells loaded with the irrelevant SV40 epitope I peptide and the peak corresponding to the lower CFSE concentration represents cells loaded with VP2₁₂₁₋₁₃₀. These data are representative of two or three mice per group analyzed in four separate experiments. Similar data were obtained when mice were assessed for lysis of VP2₁₂₁₋₁₃₀-loaded targets on day 6 or 7 p.i.

FIG. 3. Inhibition of induction of VP2₁₂₁₋₁₃₀-H-2D^b tetramer-staining cells in VP2₁₂₁-SP-tolerized B6 mice infected with TMEV. Three or four B6 mice were tolerized with either sham-SP (B and E) or VP2₁₂₁₋₁₃₀-SP (C and F) 7 days before TMEV infection. On day 6 p.i., naïve and tolerized-infected mice were perfused and their spleens (A to C) or brains (D to F) were prepared and stained for CD3, CD8, and VP2₁₂₁₋₁₃₀-H-2D^b tetramers. Each plot is gated on live CD3⁺ CD8⁺ cells. Percentages indicate the number of CD3⁺ CD8⁺ T cells that are tetramer positive. Data are representative of three or four mice per group analyzed in three separate experiments.

tially cross-reactive, as CD8 T cells from B6 mice primed with VP2₁₂₁₋₁₃₀ in CFA produced IFN- γ in response to VP2₁₆₅₋₁₇₃, albeit a smaller amount than in TMEV-infected B6 mice (Fig. 4C). However, there was no detectable cross-reaction between VP2₁₂₁₋₁₃₀ and VP3₁₁₀₋₁₂₀ (Fig. 4C). This effect was specific to TMEV determinants, as VP2₁₂₁-SP-tolerized mice mounted a normal recall response to the LCMV CD8⁺ T-cell nucleoprotein (NP) epitope following priming with NP₂₀₅₋₂₁₂ in CFA (Fig. 4D).

Induction of tolerance in TMEV-specific CD8⁺ T cells prior to infection delays viral clearance but does not lead to viral persistence or demyelinating disease in B6 mice. We next determined the effect of a lack of TMEV-specific CD8⁺ T cells on the course of TMEV infection in B6 mice. We hypothesized that without the immunodominant CD8⁺ T-cell response to TMEV, B6 mice may develop TMEV-IDD or another CNS disease related to the inability to clear the infection. We observed the development of some mild, transient signs of motor dysfunction in a small percentage (9.5%) of wild-type (WT) B6 mice tolerized with VP2₁₂₁-SP; however, no signs of clinical disease were observed in most of the cases (Table 1). We also found no evidence of either CNS inflammation or demyelination at >150 days p.i. in either VP2₁₂₁-SP or sham-SP recipient mice (data not shown).

In TMEV-IDD-susceptible SJL mice, the virus persists in the CNS for the lifetime of the animal, while in TMEV-IDD-resistant B6 mice, the virus is cleared from the CNS by 2 to 3 weeks p.i. We thus assessed the effects of VP₁₂₁₋₁₃₀-specific tolerance on the CNS viral load at several time points and found that in the 2 weeks following infection, the viral load was significantly higher in the brains of tolerized mice than in those of sham-tolerized controls (Fig. 5A). In the spinal cord tissue of VP2₁₂₁-SP-tolerized mice, the virus load was particularly

high on day 10 p.i. and then the virus lingered at low titers for approximately a week after the virus was cleared in sham-SP-treated mice (Fig. 5B). Thus, the presence of a TMEV-specific CD8⁺ T-cell response clearly allows rapid clearance of TMEV from the CNS in B6 mice. However, the fact that by day 24 p.i. the virus was not detectable in CNS tissue from the tolerized mice indicates that the VP2₁₂₁₋₁₃₀-specific CD8⁺ T-cell response is not required for eventual clearance of TMEV from the CNS in B6 mice.

VP2₁₂₁-SP tolerance induction in TMEV-infected animals leads to a severe systemic reaction in more than 90% of mice. It was important to determine the effect of Ag-SP tolerance on previously activated CD8⁺ T cells if the method is to be potentially useful to treat human diseases mediated by pathogenic CD8⁺ T cells. B6 mice infected with TMEV 6 days previously were thus tolerized with 5×10^7 VP2₁₂₁₋₁₃₀-SP. Within 12 h, the mice exhibited drastically reduced activity, piloerection, and seizures. After 18 to 30 h, the mice became extremely dehydrated and 37.5% of them were moribund by 48 h postinjection (Table 2, experiment 1). Of note, no ill effects were observed in TMEV-infected mice injected with cells coupled to the subdominant TMEV epitope VP2₁₆₅₋₁₇₃ or VP3₁₁₀₋₁₂₀ (which also had no effect on the generation of a VP2₁₂₁-specific response, whether administered before or after TMEV infection), nor did we see any effect when either Ag-SP or soluble peptide tolerance to the immunodominant or any subdominant CD8⁺ T-cell epitope was induced in TMEV-infected SJL mice (data not shown). This effect in B6 mice was somewhat dose dependent, as mice injected with 40×10^6 coupled cells exhibited signs of fever and seizures but only 8.3% died (Table 2, experiment 2), and was antigen specific, as no mice injected with splenocytes coupled with irrelevant peptides developed symptoms (Table 2). Also, when tolerance was

FIG. 4. Tolerance to the dominant VP2₁₂₁₋₁₃₀ epitope also inhibits responses to the subdominant TMEV-specific CD8⁺ T-cell epitopes. (A and B) Four or five B6 mice were tolerized with 50×10^6 sham-SP or VP2₁₂₁-SP and infected with TMEV 6 days later. At 6 to 7 days p.i., the mice were assessed for splenic IFN- γ responses to VP2₁₂₁₋₁₃₀, VP2₁₆₅₋₁₇₃, and VP3₁₁₀₋₁₂₀ by ELISPOT assay (A) or in vivo lysis of VP3₁₁₀₋₁₂₀-loaded target cells (B). Response significantly less than that of sham-SP-tolerized control, *, $P < 0.01$. (C) B6 mice were infected with viable TMEV or primed with 100 μ g of VP2₁₂₁₋₁₃₀-CFA or PBS-CFA. Seven days later, splenic IFN- γ responses to all TMEV-specific CD8⁺ T-cell epitopes were assessed by ELISPOT assay. Response significantly greater than of appropriate naive or PBS-CFA-primed control: *, $P < 0.05$. (D) Four or five B6 mice were tolerized with 50×10^6 sham-SP or VP2₁₂₁-SP. Seven days later, mice were primed subcutaneously with 100 μ g of LCMV NP₂₀₅₋₂₁₂-CFA. Seven days postpriming, splenic IFN- γ ELISPOTs were enumerated in response to both TMEV epitopes and LCMV NP₂₀₅₋₂₁₂. Panels A and B are each representative of three or four experiments. Panels C and D are each representative of two separate experiments.

induced prior to TMEV infection, as well as 6 days after TMEV infection, no mice developed symptoms (Table 2, experiment 3), indicating that a large frequency of VP2₁₂₁-specific cells is required for this effect to occur.

Inflammatory cytokines can lead to fever-induced seizures in mice. Since the reaction we observed in these mice resembled

that of mice undergoing inflammatory cytokine-induced seizures, we analyzed serum cytokines from affected mice at various time points. IFN- γ , tumor necrosis factor alpha (TNF- α), and interleukin-6 (IL-6) were all highly produced in VP2₁₂₁-SP, but not sham-SP, recipient mice (Fig. 6). In addition, CD8⁺ T cells from TMEV-infected mice produce significant levels of TNF- α following peptide restimulation in vitro (data not shown). These cytokines were present at high levels 5 h after injection of VP2₁₂₁-SP and then recovered to levels similar to those in sham-SP recipients by 24 h postinduction of tolerance. IFN- γ levels were exceedingly high in VP2₁₂₁-SP recipients. However, the presence of this cytokine alone was not required to cause seizures or death, as VP2₁₂₁-SP treatment of TMEV-infected IFN- γ ^{-/-} mice on the B6 background led to identical symptoms and a slightly higher mortality rate compared to WT B6 mice (Table 2, experiment 4). This reaction was not a classic anaphylactic response, as mice did not exhibit elevated levels of IL-4 (Fig. 6) or serum histamine (data not shown).

TABLE 1. Clinical disease parameters of B6 mice lacking TMEV-specific CD8⁺ T cells

Treatment ^a	No. of mice affected/total	Mean time until onset (days)	Peak score	% of sick mice that recovered	Mean time to recovery (days)
Sham-SP	0/33				
VP2 ₁₂₁ -SP	4/42 ^b	36.3	1	50	67.5

^a WT B6 mice were tolerized with either 5×10^7 sham-SP or VP2₁₂₁₋₁₃₀-SP prior to TMEV infection and monitored for signs of clinical disease for 150 days p.i.

^b The percentage of VP2₁₂₁-SP recipient mice affected is not significantly different from that of sham-SP-tolerized controls ($P = 0.1258$, Fisher's exact test).

FIG. 5. VP2₁₂₁₋₁₃₀-SP tolerance delays CNS viral clearance in TMEV-infected B6 mice. B6 mice were tolerized with sham-SP or VP2₁₂₁₋₁₃₀-SP 7 days prior to TMEV infection. On days 4, 8, 10, 12, 14, 22, and 24 p.i., mice were perfused and brain and spinal cord tissues were harvested and homogenized. Viral titers of homogenates were determined by plaque assay. Data are presented as the mean number of PFU per milligram of tissue $\pm$ the standard error of the mean of three mice per group per time point and are representative of three separate experiments performed at similar time points. Titers significantly greater than sham-SP-injected controls: *, $P < 0.05$; **, $P < 0.005$.

TABLE 2. Tolerization of B6 mice on day 6 p.i. infection with TMEV by using VP2₁₂₁₋₁₃₀-SP induces a severe, often fatal reaction within 48 h of the injection

Expt no. and treatment ^a	No. of mice affected	No. of deaths/total
1		
50 $\times$ 10 ⁶ WT Ag-SP, day 6 p.i.		
Sham-SP	0/5	0/5
VP2 ₁₂₁₋₁₃₀ -SP	15/16 ^b	6/16
VP2 ₁₆₅₋₁₇₃ -SP	0/5	0/5
VP3 ₁₁₀₋₁₂₀ -SP	0/5	0/5
2		
40 $\times$ 10 ⁶ WT Ag-SP, day 6 p.i.		
Sham-SP	0/8	0/8
VP2 ₁₂₁₋₁₃₀ -SP	9/12 ^b	1/12
3		
50 $\times$ 10 ⁶ WT Ag-SP, days -6 and +6 p.i.		
Sham-SP/Sham-SP	0/3	0/3
VP2 ₁₂₁₋₁₃₀ -SP/Sham-SP	0/3	0/3
Sham-SP/VP2 ₁₂₁₋₁₃₀ -SP	4/6 ^c	4/6
VP2 ₁₂₁₋₁₃₀ -SP/VP2 ₁₂₁₋₁₃₀ -SP	0/6	0/6
4		
50 $\times$ 10 ⁶ IFN- γ KO Ag-SP, day -6 p.i.		
Sham-SP	0/7	0/7
VP2 ₁₂₁₋₁₃₀ -SP	12/13 ^b	10/13
5		
Soluble peptide, day 6 p.i.		
Sham	0/5	0/5
VP2 ₁₂₁₋₁₃₀	10/10 ^b	10/10
6		
50 $\times$ 10 ⁶ WT Ag-SP, day 18 p.i.		
Sham-SP	0/5	0/5
VP2 ₁₂₁₋₁₃₀ -SP	3/5	0/5

^a WT or IFN- γ B6 mice were tolerized with sham-SP, soluble VP2₁₂₁₋₁₃₀, or splenocytes coupled with VP2₁₂₁₋₁₃₀, VP2₁₆₅₋₁₇₃, or VP3₁₁₀₋₁₂₀ on the p.i. days indicated. The number of mice in a given group that were affected or that died following the treatment is shown.

^b The percentage of mice affected is significantly greater than that of sham-SP-tolerized controls ($P < 0.01$).

^c The percentage of mice affected is significantly greater than that of sham-SP-tolerized controls ($P < 0.05$).

A previous study also reported animal deaths following injection of soluble VP2₁₂₁₋₁₃₀ into TMEV-infected B6 mice but did not mention any seizures or describe the cytokine environment (14). We thus compared the effects of injection of soluble VP2₁₂₁₋₁₃₀ versus VP2₁₂₁₋₁₃₀-SP into TMEV-infected B6 mice at 6 days p.i. Mice injected with soluble VP2₁₂₁₋₁₃₀ exhibited seizures and behavior identical to those injected with VP2₁₂₁₋₁₃₀-SP. Death occurred more rapidly and in a higher percentage of mice injected with soluble peptide; this was likely related to the increased peptide dose (Table 2, experiment 5). Additionally, serum cytokine profiles in mice treated with soluble peptide were similar to those in recipients of coupled cells (Fig. 6).

To determine if the adverse effect of VP2₁₂₁₋₁₃₀-SP administration was related to the number of VP2₁₂₁₋₁₃₀-specific CD8⁺ T cells in the mouse at the time of tolerization (40 to 60% of the CD8⁺ T cells within the CNS are specific for VP2₁₂₁₋₁₃₀ at the peak of the response, days 6 to 8 p.i.), we induced tolerance at day 18 p.i., when the virus had been cleared (Fig. 5) and the VP2₁₂₁₋₁₃₀-specific response had waned considerably. TMEV-infected mice tolerized at day 18 exhibited only very mild signs of distress, in the form of reduced activity and slight piloerection in 60% of the mice and mild seizures in 20% of the mice, and no deaths were observed (Table 2, experiment 6). This clearly suggests that a threshold number of VP2₁₂₁₋₁₃₀-specific CD8⁺ T cells is required for this response to occur; however, it was of interest to determine the fate of these cells following an in vivo encounter with VP2₁₂₁₋₁₃₀-SP. It was possible that the encounter of activated CD8⁺ T cells with VP2₁₂₁₋₁₃₀-SP induced the death of the T cells and release of the inflammatory cytokines by a large number of VP2₁₂₁₋₁₃₀-specific cells dying en masse triggered the syndrome. Alternatively, the syndrome could be triggered by the further activation of a large number of specific cells, inducing release of perforin and other inflammatory cytokines, e.g., IL-6 and TNF- α . To address this question, we analyzed the frequency of VP2₁₂₁₋₁₃₀-specific IFN- γ -producing cells in mice that had received 5×10^7 sham-SP or VP2₁₂₁₋₁₃₀-SP on day 6 p.i. and which survived until day 10 p.i. Interestingly, the frequency of VP2₁₂₁₋₁₃₀-specific IFN- γ -producing cells in the surviving mice was approximately fivefold higher than the response in sham-SP recipients (Fig. 7), indicating that VP2₁₂₁₋₁₃₀-SP injection did not trigger massive death of

FIG. 6. VP2₁₂₁₋₁₃₀-SP injection into B6 mice previously infected with TMEV elicits release of high levels of proinflammatory cytokines into the serum. Six days after TMEV infection, mice were injected i.v. with sham-SP, VP2₁₂₁₋₁₃₀-SP, or soluble VP2₁₂₁₋₁₃₀. At 5 and 24 h postinjection, blood was collected and serum was assessed for inflammatory cytokines by LiquiChip analysis. Data are presented as picograms of cytokine per milliliter of pooled serum from three mice per group per time point and are representative of two separate experiments.

the CTLs but appeared to induce further expansion of the memory population.

DISCUSSION

Induction of tolerance to the immunodominant TMEV VP2₁₂₁₋₁₃₀ epitope with peptide-pulsed, ECDI-fixed splenocytes efficiently and specifically prevented the activation and expansion of virus epitope-specific CD8⁺ T cells, as assessed by a variety of in vitro and in vivo functional responses. Thus, Ag-SP-induced tolerance is as effective for TMEV-specific

CD8⁺ T cells as it has been shown to be for various specificities of CD4⁺ T cells. Tolerizing TMEV-specific CTLs resulted in an increased viral load in the CNS at early time points and delayed viral clearance but did not, however, result in clinical or histological signs of TMEV-IDD in B6 mice, which are normally resistant to TMEV-IDD. Interestingly, injection of VP2₁₂₁-SP led to a total lack of response to the two subdominant CD8⁺ T-cell responses to TMEV as well. This result is in contrast to what has been described as immunodominance, wherein CD8⁺ T-cell responses to subdominant epitopes can actually be enhanced in

FIG. 7. VP2₁₂₁-SP tolerance in B6 mice previously infected with TMEV leads to expansion of VP2₁₂₁₋₁₃₀-specific IFN- γ -producing cells. B6 mice were injected with VP2₁₂₁-SP or sham-SP 6 days p.i. Five sham-SP recipients and four surviving VP2₁₂₁-SP recipients were sacrificed 4 days postinjection of Ag-SP (day 10 p.i.). Frequencies of splenic IFN- γ -producing cells in response to VP2₁₂₁₋₁₃₀ restimulation were assessed by ELISPOT assay. Data are representative of two separate experiments. Response significantly greater than that in sham-SP injected controls: **, $P < 0.001$.

the absence of the dominant response (8, 28, 29). Our observation can, in part, be explained by partial cross-reactivity between VP2₁₂₁₋₁₃₀ and the subdominant epitope VP2₁₆₅₋₁₇₃. However, no cross-reactivity between VP2₁₂₁₋₁₃₀ and the second subdominant epitope, VP3₁₁₀₋₁₂₀, was observed. We have not observed a similar lack of response to subdominant TMEV epitopes in the absence of the dominant response in SJL mice (data not shown), indicating that this effect is strain or epitope specific. In SJL mice, the frequency of CD8⁺ T cells specific for the immunodominant epitope versus the subdominant epitopes is not as drastically skewed toward the dominant epitope, so the total lack of a subdominant response in VP2₁₂₁₋₁₃₀-tolerized B6 mice may be related to the stronger dominance of the VP2₁₂₁₋₁₃₀ response. This observation may also indicate that lysis of virus-infected cells mediated by VP2₁₂₁₋₁₃₀-specific CD8⁺ T cells may in some way lead to more efficient presentation of the subdominant epitopes; thus, interfering with the dominant response would then have downstream effects.

Mice that received VP2₁₂₁-SP prior to TMEV infection possessed drastically reduced numbers of TMEV-specific cells in both the periphery and the CNS. The small number of VP2₁₂₁-specific cells present within the CNS in these mice may contribute to the eventual clearance of TMEV from the CNS. However, the fact that the virus was cleared from the CNS in VP2₁₂₁-SP recipient mice despite the largely eradicated TMEV-specific CD8⁺ T-cell response suggests that mechanisms other than those mediated by CD8⁺ T cells are capable of clearing TMEV in B6 mice. Our findings were in contrast to studies in which the entire CD8⁺ cell population (which would include several cell types and CD8⁺ T cells of many specificities) was knocked out or depleted, and mice became at least partially susceptible to TMEV-induced disease and/or persistence of TMEV in the CNS (12, 27, 31, 33). Recently, it was shown that FVB mice, which are normally susceptible to TMEV-IDD, could be rendered resistant through transgenic expression of the *D^b* gene, and this resistance is abrogated when VP2₁₂₁₋₁₃₀-specific cells are depleted with soluble peptide prior to TMEV infection (24). In light of those experiments, we expected that our method of inducing antigen-spe-

cific tolerance in the B6 mouse would lead to TMEV-IDD or at least persistent virus infection; however, this was not the case. This discrepancy could be due to the fact that both the strains of mice (FVB versus B6) and the strains of TMEV (DA versus BeAn) differed in these two systems. In our model, it is clear that the vastly expanded TMEV-specific CD8⁺ T-cell response is not required for viral clearance in B6 mice that have not been genetically manipulated. Similarly, TMEV-specific CD8⁺ T cells were not required for the resistance of B6 mice to the clinical or histological signs of TMEV-IDD. These findings are in agreement with a recent paper in which antibody depletion of CD8⁺ cells had little effect on the resistance of B6 mice to TMEV-IDD induced by the BeAn strain (15). It should be noted that, in this study, although a lack of CD8⁺ cells alone did not lead to TMEV-IDD, a lack of both CD8⁺ cells and B cells resulted in a severe, nondemyelinating, paralytic disease, a finding that we have corroborated with the TMEV-specific CD8⁺ T-cell tolerance model (data not shown). Thus, it is clear that those few TMEV-specific CD8⁺ T cells remaining within the CNS in VP2₁₂₁-SP recipient mice are, at least in the absence of a B-cell response, unable to clear the virus.

Ag-SP tolerance has been pursued as an attractive specific therapy for the treatment of human autoimmune diseases. This method of tolerance has advantages over the use of soluble peptides. In animal models of CD4⁺ T-cell-mediated autoimmunity, it has been shown that soluble peptide injection can lead to anaphylactic shock (36) or other adverse reactions (14, 22), and in a clinical trial, parenteral injection of peptide led to hypersensitivity reactions (16). Thus, ECDI-coupled cell tolerance is of possible interest for the treatment of human diseases. Therefore, we were interested in determining whether previously activated CD8⁺ T cells could be tolerized by this method, a paradigm more relevant to the treatment of preexisting disease in humans. Unexpectedly, we found that tolerance induced by the i.v. injection of VP2₁₂₁-SP into B6 mice infected with TMEV 6 days previously led to seizures and, in some cases, death.

Analysis of the sera indicated that multiple cytokines were elevated in VP2₁₂₁-SP-injected mice as little as 5 h after injection. It is well recognized that TCR cross-linking, e.g., with anti-CD3 monoclonal antibody, can induce a cytokine release syndrome, leading to significant morbidity and mortality (11, 13) due to the release of proinflammatory cytokines, including IL-1, IL-6, and TNF- α , which can induce fever and seizure responses in mice (7, 30, 35). We hypothesized that encounter of a high frequency of activated CD8⁺ T cells with syngeneic cells coupled with a specific antigen (VP2₁₂₁₋₁₃₀) leads to release of high levels of inflammatory cytokines, resulting in cytokine-induced fever and seizure responses. Although IFN- γ was by far the most elevated cytokine of those detected, this cytokine alone was not responsible for seizures and death in tolerized mice, as IFN- γ ^{-/-} mice were as susceptible to the seizure reaction as WT mice and even more likely to die following treatment. Since this reaction is clearly CD8⁺ T cell mediated, the slightly protective property of IFN- γ in this case could be a result of its well-documented immunoregulatory functions (40).

Interestingly, this adverse response to coupled-cell tolerance occurred only when mice were tolerized to the immunodomi-

nant VP2₁₂₁₋₁₃₀ epitope and not to either of the subdominant CD8 epitopes. This is likely due to the significantly larger fraction of anti-TMEV CD8⁺ T cells specific for the VP2₁₂₁₋₁₃₀ epitope. In support of this hypothesis, only mild clinical symptoms were evident when B6 mice were injected with VP2₁₂₁₋₁₃₀-SP on day 18 p.i., a time when the virus infection had resolved and the VP2₁₂₁₋₁₃₀-specific CD8⁺ T-cell response was considerably diminished. Also, milder symptoms and a decreased percent mortality were evident when we decreased the number of Ag-SP administered per mouse, thereby decreasing the number of VP2₁₂₁-specific T cells encountering VP2₁₂₁-SP. The immunodominant VP2₁₂₁₋₁₃₀ peptide is, in our studies, the only one of the three B6 TMEV CTL epitopes that elicits both high levels of IFN- γ and rapid, efficient lysis of target cells, so the intensity of this response may also contribute to the syndrome. Additionally, the fact that administration of VP2₁₂₁-SP also affects the VP2₁₆₅- and VP3₁₁₀-specific responses may or may not exacerbate this reaction. A recent study found that a reaction similar to the one we have described occurred upon injection of soluble peptides corresponding to virus-specific CD8⁺ T-cell epitopes into mice previously infected with LCMV or vaccinia virus and that caspase inhibitors and a lack of TNF signaling each partially prevented mice from dying of the peptide-induced syndrome (22). Those authors injected peptide into mice in which 10 to 50% of the CD8⁺ T cells were specific for the cognate peptide (22). In addition, another group found that naive mice transgenic for a CD8⁺ T-cell epitope died upon injection of that peptide in soluble form (14). These data support our conclusion that the number of antigen-specific cells present in the host upon the injection of soluble peptide or Ag-SP is an important factor. A recent study also reported that injection of soluble VP2₁₂₁₋₁₃₀ into TMEV-infected B6 mice at day 8 p.i. resulted in death within 48 h of injection. This reaction was characterized by highly disrupted vascular systems in the brains of affected mice, and it was concluded that death (but not vascular disruption) was perforin dependent (14). There was no description of seizures in the peptide-treated mice; however, when we directly compared soluble peptide to peptide-coupled cell injection into TMEV-infected mice, the reactions were identical. Although we did not assess the contribution of perforin to the observed clinical responses, it is likely that injection of soluble VP2₁₂₁₋₁₃₀ and injection of VP2₁₂₁-SP lead to the same fatal reaction.

In an attempt to distinguish between the possibility that VP2₁₂₁-SP provoked activation-induced cell death in VP2₁₂₁₋₁₃₀-specific CD8⁺ T cells, resulting in a cytokine release syndrome, and the possibility that it heightened activation of the TMEV-specific CTLs in TMEV-infected mice, we determined the frequency of VP2₁₂₁₋₁₃₀-specific IFN- γ producers in the mice surviving the adverse reaction. The fact that the frequency of the VP2₁₂₁₋₁₃₀-specific response was nearly fivefold greater than that in sham-SP recipients leads us to conclude that the peptide-specific CTLs are not induced to die immediately upon encountering VP2₁₂₁-SP but rather to release cytokines and expand in numbers as a result of the encounter and remain responsive to subsequent peptide restimulation.

Collectively, our results indicate that peptide-pulsed, ECDI-fixed splenocytes efficiently and specifically prevent the activation and expansion of virus epitope-specific CD8⁺ T cells when administered prior to virus infection, resulting in an increased

viral load in the CNS at early time points and delayed viral clearance. In contrast, tolerization of mice with either soluble peptide or peptide-coupled cells following virus infection led to an unexpected, often fatal, cytokine release syndrome characterized by severe seizures. These results indicate that use of antigen-specific tolerance as a treatment for diseases mediated by activated CD8⁺ T cells should be approached with a great deal of caution. This method of tolerance may, however, be applicable to therapy for prevention of transplantation-induced organ or tissue rejection or for prevention of the development of spontaneous autoimmune diseases with a significant CD8 component such as type 1 diabetes in individuals at high genetic risk, situations where CD8⁺ T cells will neither be present at a particularly high frequency nor have been activated prior to tolerance induction.

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