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West Nile Virus-Specific CD4 T Cells Exhibit Direct Antiviral Cytokine Secretion and Cytotoxicity and Are Sufficient for Antiviral Protection[1]

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CD4 T cells have been shown to be necessary for the prevention of encephalitis during West Nile virus (WNV) infection. However, the mechanisms used by Ag-specific CD4 T cells to protect mice from WNV encephalitis remain incompletely understood. Contrary to the belief that CD4 T cells are protective because they merely maintain the CD8 T cell response and improve Ab production, in this study we provide evidence for the direct antiviral activity of CD4 T cells that functions to protect the host from WNV encephalitis. In adoptive transfers, naive CD4 T cells protected a significant number of lethally infected RAG$^{-/-}$ mice, demonstrating the protective effect of CD4 T cells independent of B cells and CD8 T cells. To shed light on the mechanism of this protection, we defined the peptide specificities of the CD4 T cells responding to WNV infection in C57BL/6 (H-2^b) mice, and used these peptides to characterize the in vivo function of antiviral CD4 T cells. WNV-specific CD4 T cells produced IFN-γ and IL-2, but also showed potential for in vivo and ex vivo cytotoxicity. Furthermore, peptide vaccination using CD4 epitopes conferred protection against lethal WNV infection in immunocompetent mice. These results demonstrate the role of direct effector function of Ag-specific CD4 T cells in preventing severe WNV disease. ***The Journal of Immunology,*** **2008, 181: 8568–8575.**

West Nile virus (WNV)[4] is a small, enveloped arbovirus of the *Flaviviradae* family that persists in an enzootic cycle between mosquitoes and birds, with humans and many other animals as incidental hosts. Because WNV appeared on the Eastern seaboard of the U.S. in 1999 (1, 2), it has spread through all 48 continental states, infecting more than 27,551 people, and has been directly linked to the death of 1,077 people (3–6). WNV leads to systemic disease in ~20% infected individuals, and the most severe disease is caused by viral neuroinvasion resulting in meningitis and encephalitis (7, 8), occurring in >5% of the patients. T cells play an essential role in preventing meningitis and encephalitis upon primary infection, and limiting disease severity upon potential reinfection (9–14). It has been shown that both CD4 and CD8 T cells are required for the control and clearance of WNV (11, 13–16). Still, the relative importance of each cell population at different stages of infection and the critical antiviral mechanisms used in controlling systemic and CNS infection remain to be fully elucidated.

Sitati and Diamond (14) have demonstrated that CD4 T cells are required for survival following WNV infection; however, the mechanism of protection provide by CD4 T cells during WNV infection was not explored. This group demonstrated that CD4 T cell-deficient mice, generated by continued Ab depletion, exhibited high viral titers for over 50 days within the CNS, eventually leading to death (14). In these same mice, viral titers in the spleen were not altered, suggesting that CNS, but not systemic, virus control requires CD4 cells (14). Moreover, these same experiments suggested that CD4 T cells are responsible for aiding in the survival and proliferation of CD8 T cells and the priming of B cells (14), but that hypothesis was not formally tested. Prior work has identified a requirement for CD4 T cells in controlling other flavivirus infections, including those with the Japanese encephalitis virus (JEV) (17) and yellow fever virus (18), but again a direct role for CD4 T cell effector function has not been previously investigated.

Although it is well established that CD4 T cells play an accessory role providing help to both CD8 T cells and B cells, there is evidence that CD4 T cells can also exhibit a direct effector response during a viral infection (reviewed in Ref. 19). Thus, during influenza infection, CD4 T cells use perforin-mediated cytotoxicity to clear virus from the periphery (20), whereas measles-specific CD4 T cells use IFN-γ to control virus within the CNS (21, 22).

In this study, we show that by themselves, CD4 T cells are sufficient for the control of WNV infection in RAG-1$^{-/-}$ mice. WNV-specific CD4 T cells secreted cytokines and lysed infected cells following WNV infection. Because the vaccination of mice with CD4 epitopes increased protection, the direct CD4 T cell effector function may be a relevant target for future vaccine studies. We propose the potential beneficial role of CD4 T cells may be more important for the protective vaccination of the elderly, a population adversely affected by WNV infection. These results demonstrate that CD4 T cells contribute to protection during primary

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[4] Abbreviations used in this paper: WNV, West Nile virus; FCM, flow cytofluorometry; GzB, granzyme B; ICCS, intracellular cytokine staining; JEV, Japanese encephalitis virus; NS3, nonstructural protein 3.

WNV infection via direct effector function, albeit they do not exclude other modes of CD4 T cell action.

Materials and Methods

Mice

Adult (2- to 6-mo-old) male C57BL/6 (B6) mice were purchased from the National Cancer Institute Breeding Program. B6.Rag-1$^{-/-}$, B6.perforin$^{-/-}$ mice were purchased from The Jackson Laboratory, and bred at the Vaccine and Gene Therapy Institute vivarium (Oregon Health and Science University); they were used at 2–4 mo of age. B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ, commonly referred to as B6.Ly-5.1 congenic mice, were purchased from the National Cancer Institute and were used at 2–4 mo of age. B6.Fas-deficient (*Lpr/Lpr*), B6.Fas × perforin$^{-/-}$-double-deficient, and B6.CD4$^{-/-}$ mice were bred at the Washington University School of Medicine. All animals were housed and bred under specific pathogen-free conditions at either the Oregon Health and Science University or the Washington University School of Medicine. All WNV experiments were completed within U.S. Department of Agriculture-approved Biosafety Level 3 facilities, and were approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee in accordance with the applicable federal, state, and local regulations.

Virus, peptides, and cell lines

WNV strains 31A and 385-99 were used, and both virus strains yielded similar results. WNV strain 385-99 was a gift from R. Tesh (University of Texas Medical Branch, Galveston, TX); strain 31A was provided by the U.S. Department of Agriculture reagent program. An overlapping peptide library covering the entire length of the viral polyprotein (15-mers overlapping by 10 aa) was obtained from Sigma-Aldrich. Additional synthetic peptides were purchased at >95% purity from Sigma-Aldrich and 21st Century Biochemicals, diluted in 10% H_2O/90% DMSO, stored at −80°C, and subsequently used at indicated concentrations. Virus was grown in mycoplasma-negative Vero cells, cultured under aseptic conditions, as described previously (15); mycoplasma-negative IC-21 cells were used in stimulation assays. Cells were infected using variable multiplicity of infection, as indicated.

Infection and vaccination

All mice were infected s.c. between the shoulder blades with indicated doses (20–1200 PFU) of WNV in 100 μl of 1× PBS + 2% FBS. On specified days postinfection, splenocytes were isolated and subjected to flow cytofluorometry (FCM), intracellular cytokine staining (ICCS), or CTL assay analysis, as described below.

For peptide vaccinations, 20 μl of 1 mg/ml peptide/PBS solution containing peptides nonstructural protein 3 $(NS3)_{1616}$ and $NS3_{2066}$ was emulsified in equal volume of the adjuvant TiterMax Gold (Sigma-Aldrich), by vortex mixing for 30 min. Mice were immunized twice (s.c.) at 21-day intervals, at the base of the tail.

Adoptive transfer and virus challenge experiments

Naive CD4$^+$ T cells were isolated from spleens of 4-wk-old B6 mice. Briefly, splenic T cells were coated with anti-CD8, B220, NK1.1-coated beads (Miltenyi Biotec), and CD4$^+$ cells were isolated to 80–95% purity; such preparations contained <0.5% of B and CD8 T cells, and were overwhelmingly of the naive phenotype, containing <5% CD44^{high} (memory) cells; most of the contaminating cells were of the macrophage/monocyte lineage. CD4 T cells were transferred i.v. (at 5–10 × 10^6 cells/recipient); transfers were monitored and infected, as described above. Virus-specific CD4 T cell lines were generated by in vitro restimulation of WNV-primed spleen cells, were purified to deplete CD8$^+$ and B220$^+$ cells to <1%, and were injected (2–5 × 10^5 cells/recipient) i.v. into RAG-1$^{-/-}$ recipients. Engraftment success was evaluated by FCM 24 h later, at which time the animals were infected with WNV, as described above. Survival was scored on a daily basis. Death occurred between days 10 and 18, and all animals surviving this period remained disease free for 60–90 days, at which point the experiment was terminated.

Ab depletion of C57BL/6 mice

C57BL/6 mice were given two injections of 0.1 mg of anti-CD4 (GK1.5) Ab i.p. two times, on days −3 and 0. Ab depletion was confirmed by testing PBLs for the presence of CD4 T cells (clone RM4-5) using flow cytometry, as described below.

Determination of viral titer

Viral titer was determined by plaque assay in which a virus sample was serially diluted onto Vero cells. After coculture of the virus with the cells for 2 h, agarose overlay was added. Two days after the initial overlay, cells were overlayed with additional agarose-containing Neutral Red (0.2%). Plaques were then counted to determine viral load.

To evaluate a potential chronic virus infection, infected mice were perfused to avoid blood-borne contamination and brains were homogenized in RPMI 1640 using a beadbeater-96 (BioSpec Products). The cellular suspension was seeded onto a monolayer of Vero cells, in triplicate, and cultured for 7 days, while monitored for cytopathic effect. Cytopathic effect was monitored visually and defined as the rounding up of cells and loss of a monolayer. At the end of the culture period, cells and supernatant were transferred to a second monolayer of Vero cells and incubated for 48 h, after which they were trypsinized, fixed, permeabilized, and stained intracellularly, for the expression of WNV envelope protein using an E16-Alexa647 (23)-conjugated Ab, as described below, for intracellular cytokine staining.

Intracellular cytokine and surface FCM staining

Cytokine-producing T cells were detected using the Cytofix-Cytoperm kit (BD Pharmingen), as described below. Single-cell splenocyte suspension was depleted of RBC using ammonium chloride, incubated with 1 μM peptide, or infected with WNV in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich) for 6 h at 37°C, except when looking at production of IL-4, in which monensin (Sigma-Aldrich) was substituted for brefeldin A. After 6 h, the cells were washed and blocked with Fc block (anti-mouse FcγRI/III; BD Pharmingen) and incubated overnight in the presence of a saturating dose of surface Abs against CD8, CD3, CD4, CD11a, CD43 (clone 1B11), CD44, and CD62L (BD Pharmingen). After washing, the cells were fixed and permeabilized, and intracellular Abs (anti-IFN-γ, anti-TNF-α, anti-IL-4, or anti- IL-2; BD Pharmingen) were added for 30 min. For detection of granzyme B (GzB), splenocytes were isolated and kept on ice, surface stained, fixed, and permeabilized, as described above, without stimulation. Cells were stained with GzB Alexa647 (clone: gb11; BD Pharmingen). The samples were then washed and analyzed using either a FACSCalibur or LSR II cytometer (BD Immunocytometry Systems) instrument. FCM analysis was performed by collecting a minimum of 5 × 10^4 events and gates set on lymphocyte population based on forward and orthogonal light scatter, followed by marker positioning to denote fluorescence greater than that of control stained or unstained cells.

CTL assays

For ex vivo CTL assays, CD4$^+$ T cells were isolated from spleens of B6 mice 7 days postinfection with WNV, by negative selection using anti-CD8, B220, NK1.1-coated beads (Miltenyi Biotec). CD4$^+$ T cells were isolated at 80–95% purity with <0.5% CD8$^+$ cells present in the isolate. Direct ex vivo CTL activity was determined using radioactively labeled peptide-coated IC21 cells as targets. A total of 1 × 10^5 IC21 cells (1 × 10^4 cells/well) was pulsed with ^{51}CR and peptides (1 μM $NS3_{1616+2066}$ or 1 μM OTII peptide-$OVA_{323–339}$) overnight in a 96-well plate. Cells were washed three times with warm medium (5% FBS plus RPMI 1640), and purified CD4 T cells were serially diluted, then placed into the 96-well plate at indicated E:T ratio for 6 h. After 6 h, 30 μl of supernatant was removed and added to a lumaplate (Packard Instrument). Radioactivity was measured using TopCount Packard δ/γ radioactivity reader (Packard Instrument). Percent specific lysis was calculated as: ((E − S)/(M − S)) times 100, where E (experimental release) equals the cpm released from targets incubated with lymphocytes, S (spontaneous release) equals the cpm released from target cells incubated with no lymphocytes, and M (maximal release) equals the cpm released from cells after lysis with 1% Nonidet P-40 (USB; Affymetrix).

In vivo CTL assays were performed, as previously described (24). Briefly, splenocytes from B6.Ly-5.2 mice were isolated, then labeled with CFSE to produce fluorescein^{high} (1 μM)- and fluorescein^{low} (10 nM)-labeled population using standard CFSE-labeling protocol (Molecular Probes). After CFSE labeling, cells with different fluorescence intensity were peptide pulsed for 1 h at 37°C with 1 μM $NS3_{1616+2066}$ or 1 μM OTII peptide ($OVA_{323–339}$). Cells were counted, and equal numbers of fluorescein-high or -low labeled cells, coated with OTII or $NS3_{1616}$, respectively, were mixed and injected i.v. into infected and naive mice. After 12 h, mice were sacrificed and splenocytes were gated on Ly-5.2$^+$ cells (detected using mAb clone A20) and MHC class II$^+$ double-positive cells. In experiments in which donor mice were not on a Ly5.1 background, PKH26 (2

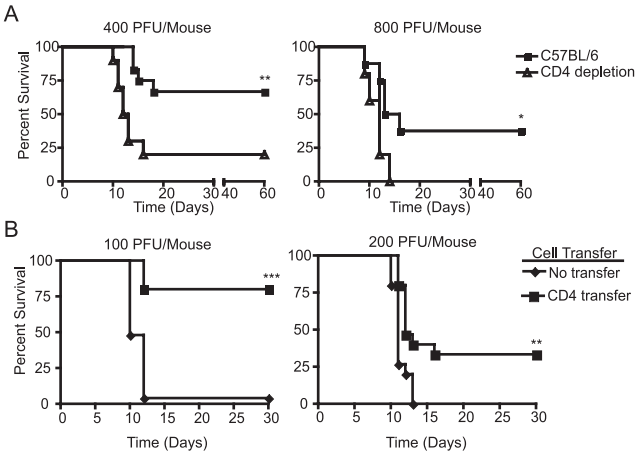

FIGURE 1. Protective effect of naive CD4 T cells. *A*, Ab depletion of CD4 T cells (triangles) renders C57BL/6 mice significantly (**, $p < 0.005$; *, $p < 0.04$) more susceptible to WNV-induced mortality compared with controls (squares). CD4 T cells were depleted using two doses of GK1.5 Ab on days −3 and 0, then infected with either 400 PFU/mouse (*left*) or 800 PFU/mouse (*right*). Results of one experiment with $n = 10$ mice per group are shown, representative of two independent experiments. *B*, Adoptive transfer of naive CD4 T cells provides protection to RAG-$1^{-/-}$ mice against lethal WNV infection. Splenic $CD4^+$ T cells ($5–10 \times 10^6$) from naive C57BL/6 mice were isolated by negative selection (80–95% purity) and transferred to C57BL/6 RAG-$1^{-/-}$ mice. Twenty-four hours after transfer, mice were challenged with 100 or 200 PFU WNV s.c. Survival in these groups was significantly different according to the log-rank test (***, $p < 0.0005$; *left*) (**, $p < 0.002$; *right*). Two individual experiments are shown; $n = 25$ mice per group are shown, representative of a total of four experiments completed.

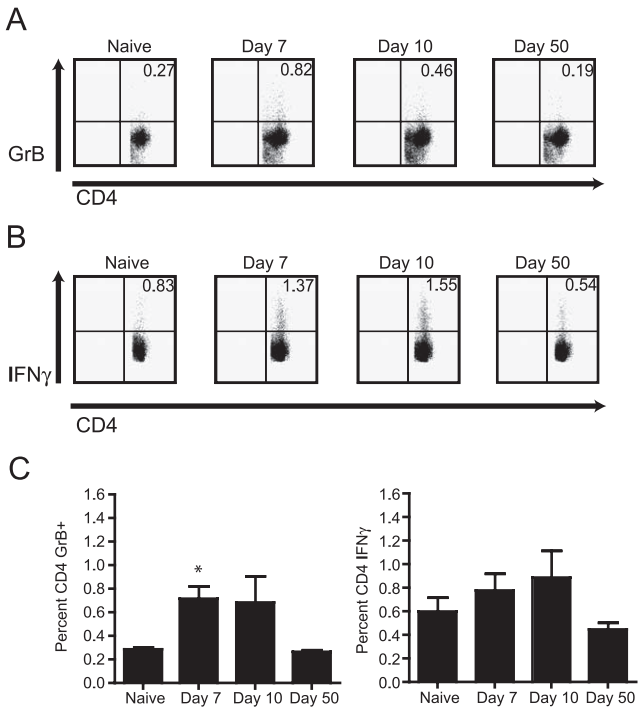

FIGURE 2. CD4 T cell response during WNV infection. *A*, Representative example of GzB expression by CD4 T cells after WNV infection as measured by direct ex vivo intracellular FCM, without in vitro stimulation. A naive animal is shown as a control. Results are from one representative mouse of four mice from each time point. One experiment of two is shown. *B*, Representative example of IFN-γ expression in CD4 T cells after WNV infection, measured by ICCS upon stimulation with 0.5 μg/ml anti-CD3ε (clone 2c11); a naive animal is shown as a control. Results are from one mouse of four. Data are representative one experiment of two. *C*, *Left panel*, Aggregate analysis of GzB expression in CD4 T cells, determined as in *A*. Percentage of CD4 GzB^+ T cells for the time points given above. There is a significant induction of GzB in CD4 T cells on day 7 ($p < 0.02$), but not on day 10 ($p > 0.05$). Panels show average of four mice per time point (× ±SEM), representative of two experiments. *Right panel*, Aggregate analysis of IFN-γ expression in CD4 T cells, determined as in *B*. Percentage of CD4 IFN-γ^+ T cells for the time points given above. Average of four mice per time point (× ±SEM), representative of two experiments.

μM) was used to label all cells using the standard labeling protocol included within the kit (Sigma-Aldrich), then labeled with CFSE. The percentage of killing was calculated as follows: (1 – (ratio immune/ratio naive)) × 100. Ratio = number of events $NS3_{1616+2066}$ peptide-coated target/number of events reference target (24).

Statistical analysis

All statistical significance for survival experiments was determined using log-rank test. All statistical significance for flow cytometry results was completed using the Mann-Whitney *U* test. All calculations were done using the Prism (GraphPad) software.

Results

Naive CD4 T cells protect mice from WNV severe disease

To confirm the CD4 T cell requirement for control of WNV infection, we depleted CD4 T cells in B6 mice before WNV infection. The CD4 T cells were depleted using the mAb GK1.5, and depletion was confirmed by FCM analysis of PBMC, in which there were fewer than 0.05% CD4 T cells remaining (data not shown). Ab depletion of CD4 T cells in C57BL/6 mice during WNV infection with 400 PFU/mouse resulted in a significant increase in the mortality rate ($p < 0.005$) (Fig. 1*A*). At that dose, 66% of the C57BL/6 mice survived compared with only 20% of the CD4 T cell-depleted mice. Overall mortality was higher in mice infected with 800 PFU, documenting the limits of protection against WNV. Still, no CD4 T cell-depleted mice survived when infected with 800 PFU of WNV, in contrast to 37% of the C57BL/6 control mice ($p < 0.04$). CD4-depleted mice exhibited slightly shorter, but not statistically significant difference in the mean survival time (14 days B6 vs 12 days CD4-depleted B6). Given the time of deaths, which coincided with the published action of adaptive immune system and the presence of the virus in the CNS (9–11, 13–15, 25–27), our data suggest that CD4 T cells do not alter the rate of disease, but rather may reduce the viral load below a threshold, which causes high incidence of mortality (see below). From this result, we concluded that within an immunocompetent animal, CD4 T cells are necessary for protection against severe WNV disease.

We next sought to determine whether naive CD4 T cells were sufficient for direct control of WNV disease in the absence of CD8 T cells or B cells. Others and we have previously shown that RAG-$1^{-/-}$ mice, which contain no T and B cells, are extremely sensitive to WNV infection (9, 15). Therefore, RAG-$1^{-/-}$ mice can serve as an excellent host to determine the relative contribution of different lymphocyte subsets in protecting from disease upon adoptive transfer. Naive CD4 T cells were purified by negative selection using magnetic beads. The resulting population was 85–95% $CD4^+$, but never contained more than 0.5% contaminating $CD8^+$ or $CD19^+$ cells. We transferred 5×10^6–1×10^7 CD4 T cells from naive C57BL/6 mice into RAG-$1^{-/-}$ deficient mice and challenged these mice 24 h later with WNV. Adoptively transferred naive CD4 T cells significantly protected the RAG-$1^{-/-}$ mice from mortality following s.c. WNV challenge with 100 PFU ($p < 0.0005$) or 200 PFU ($p < 0.002$) (Fig. 1*B*). Indeed, 80% of the

Table I. *Identification of WNV epitopes eliciting CD4$^+$ T cell response in B6 mice*

Day Postinfection	Peptide Identified	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
Part 1: 15-mer identification[a]						
No. of mice		$n = 4$	$n = 3$	$n = 4$	$n = 5$	$n = 2$
Anti-CD3		1.16	1.136	0.52	ND	0.911
E431–445	IFVHGPTTVESHGNY	0.105	0.179	0.03	0.14	0.152
E641–655	PVGRLVTVNPFVSVA	0.505	0.326	0.1	0.41	0.273
NS3 1616–1630	TKPGVFKTPEGEIGA	0.317	0.499	ND	0.30	ND
NS3 1866–1880	WFVPSVKMGNEIALC	0.082	0.089	0.01	0.13	ND
NS3 2066–2080	RRWCFDGPRTNTILE	0.527	0.406	0.487	0.49	0.176
NS3 2081–2095	DNNEVEVITKLGERK	0.096	0.029	0.01	0.14	ND
Part 2: Optimal peptide identification[b]						
No. of mice		$n = 4$	$n = 3$	$n = 4$	$n = 5$	$n = 2$
Anti-CD3		1.16	1.14	0.52	1.33	0.91
E646–660		ND	0.32	ND	0.29	0.16
E641–655		0.51	0.33	0.10	0.41	0.27
NS3 1616–1630		0.32	0.50	0.15	0.30	0.14
1617–1627		ND	0.11	ND	ND	0.08
1618–1628		ND	0.10	ND	0.17	0.08
1619–1629		ND	0.15	ND	0.28	0.37
1620–1630		ND	0.16	ND	ND	ND
NS3 2066–2080		0.53	0.41	0.41	0.49	0.43
2068–2078		ND	0.25	ND	0.24	ND
2070–2080		ND	0.20	0.01	0.34	0.08

[a] List of identified CD4 T cell 15-mer epitopes. Amino acid numbers and sequence of the CD4 T cell epitopes are listed for peptides that elicited IFN-γ, as measured by intracellular cytokine staining of splenocytes on day 7 postinfection.

[b] List of truncated CD4 T cell peptides. This table lists the truncated CD4 T cell peptides used to determine the optimal epitope. The optimal epitope is shown in bold. For both parts, responses were determined by ICCS for IFN-γ production by splenocytes on day 7 postinfection as described in *Materials and Methods*. Levels of cytokine responses (in percentages of total CD4 T cells) are indicated in the table as mean values from indicated numbers of mice.

RAG-1$^{-/-}$ mice that received naive CD4 T cells survived, as compared with only 4% of the RAG-1$^{-/-}$ controls when challenged with 100 PFU/mouse of WNV. Transferred CD4 T cells also significantly protected ($p < 0.0001$) the RAG-1$^{-/-}$ mice following i.p. WNV challenge (data not shown). Moreover, 60 days postinfection, we could not detect infectious WNV in the brains of RAG-1$^{-/-}$ mice that had received CD4 T cells by either plaque assay or coculture (level of detection 100 and 10 PFU, respectively, in contrast to the levels of 10^{5-7} PFU/organ detected in brains of moribund B6 or RAG-1$^{-/-}$ mice).

To ascertain that the above effects can be directly ascribed to the sole activity of CD4 T cells, we had to exclude the possibility of contamination by CD8 T cells or B cells. As mentioned above, maximum potential contamination in the CD4 inoculum was <0.5%, or <2.5–5 × 10^4 of either cell subset. Given that the prevalent frequencies of Ag-specific T and B cell precursors are in the 10^{-5} range, it was not likely that this contamination will play a major role. To address this experimentally, we bled all mice at the peak of the immune response, 7 days postinfection (8 days posttransfer) and enumerated CD8 and B cell contaminants by FCM (data not shown). If a contaminating population was playing a role in protection, the cell population would have expanded by 7 days postinfection. Such an expansion was detected in only 1 mouse of 30, and that animal was eliminated from the study. Moreover, upon necropsy of a random sample of animals (4/group), we found no evidence of expansion of CD8 or B cells in the spleen and peripheral lymph node (<0.2% cells positive for CD8β, B220, or CD19), either at 24 h or at 7 days postinfection (data not shown). Altogether, our data indicate that naive CD4 T cells are both necessary and sufficient to protect mice from a lethal WNV challenge. Finally, we also examined surviving Rag-knockout mice that received CD4 T cells and could not find detectable infectious virus in the organs of these animals (detection limit 10 PFU; data not shown). This result suggests that CD4 T cells are able to provide sterilizing immunity. Overall, we conclude that protection from WNV challenge in the model used above was the result of direct antiviral action of CD4 T cells.

Ex vivo analysis of effector CD4 T cell function

To evaluate the potential of antiviral CD4 T cells for direct anti-WNV action in the course of WNV infection of B6 mice, we chose to monitor GzB expression levels in this T cell subset. We focused our analysis upon CD4 T cells by selective gating; CD4 T cells represented between 25 and 37% of total splenic population, and exhibited consistent response to WNV across experiments. GzB is expressed on effector T cells with lytic potential, and its expression does not require in vitro stimulation, limiting any potential misinterpretation of data due to in vitro bias (28). FCM analysis revealed that a significant percentage of CD4 T cells up-regulated GzB content on day 7 ($p < 0.02$) post-WNV infection compared with naive controls (Fig. 2, *A* and *C*). By day 10 postinfection we began to see a decrease in GzB content, and by day 50 postinfection the percentage of CD4 T cells that contained GzB returned to baseline levels (Fig. 2, *A* and *C*). The kinetics of IFN-γ production by CD4 T cells was similar to what we observed for GzB during the course of WNV infection, except that the detection required brief in vitro stimulation of CD4 T cells with anti-CD3ε in vitro (Fig. 2*B*). From the results shown in Fig. 2, we concluded that in response to WNV infection, CD4 T cells express molecules involved in direct effector T cell function, including lytic granules and effector cytokine.

Functional characteristics of WNV Ag-specific CD4 T cells

To gain a firmer understanding of the mechanism by which CD4 T cells protect WNV-infected mice, we developed tools to track and study WNV-specific T cells. To that effect, we identified multiple CD4 T cell epitopes encoded by WNV using pools of overlapping peptides that cover the entire WNV polyprotein (Table I, part 1). For the strongest three epitopes, several peptide truncations were synthesized to determine optimal epitopes, which conformed to the expected MHC class II I-A^b-binding motifs (Table I, part 2). All

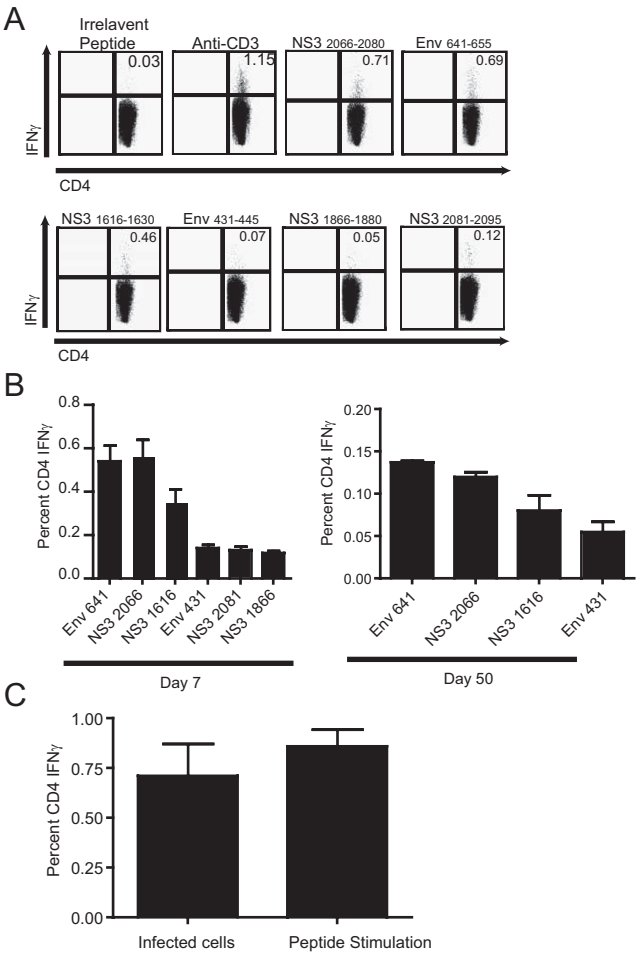

FIGURE 3. Ag-specific CD4 T cell IFN-γ response to class II epitopes. *A*, Representative example of CD4 T cell IFN-γ ICCS response to the three immunodominant and three subdominant CD4 T cell epitopes as measured by 6-h ICCS. CD4 T cells were from mice 7 days postinfection and were stimulated with the peptide (10^{-6} M) indicated above each plot. One mouse of five is shown for one experiment, representative of three experiments. *B*, Aggregate analysis of IFN-γ expression in primary and memory responses. *Left panel*, Quantification of the CD4 T cell IFN-γ ICCS response 7 days postinfection, shown in *A*. Average of five mice per time point (× ±SEM), representative of three experiments. *Right panel*, Quantification of day 50 CD4 T cell IFN-γ response. Average of five mice per time point (× ±SEM), representative of three experiments. *C*, Quantification of day 7 CD4 T cell IFN-γ response (measured by ICCS) to either WNV-infected IC-21s (multiplicity of infection: 40) or peptide-pulsed IC-21s 10^{-6} M (Env_{641} and $NS3_{1616+2066}$) peptide. CD4 T cells were stimulated for 6 h in the presence of brefeldin A. Results depict average values of four mice per time point (× ±SEM), representative of two experiments.

peptides described are cited with their inclusive amino acid numbers the first time in the text, as well as in Table I. Subsequently, abbreviated nomenclature is used based upon designation of the protein component from which the peptide is derived, e.g., NS3, followed by the initial amino acid at which the peptide begins counting from the beginning of the polyprotein. Therefore, the NS3 peptide 2066–2080 is designated $NS3_{2066}$. Of note, none of the described peptides elicited IFN-γ responses (<0.03%) by cells of any other phenotype (CD8 T cells or double-negative cells; data not shown). A summary of the results for all identified CD4 T cell epitopes is listed in Table I. Through this process, we identified in B6 mice three dominant epitopes, $Env_{641-655}$, $NS3_{2066-2080}$, and $NS3_{1616-1630}$ (Fig. 3, *A* and *B*) and three subdominant epitopes (Fig. 3, *A* and *B*). Epitopes Env_{641} and $NS3_{2066}$ are each responsible for 30% of the total response; $NS3_{1616}$ is responsible for 20% of the total response; and $Env_{431-445}$, $NS3_{2081-2095}$, and $NS3_{1866-1880}$ are each responsible for less than 8% of the total response. The response to Env_{641}, $NS3_{2066}$, and $NS3_{1616}$ peptide pool was always at least equal to, and often significantly larger than, the response to WNV-infected cells (Fig. 3*C*), probably due to peptide competition, unequal temporal expression of all epitopes, or a combination of these and other, unknown, factors. Importantly, Fig. 3*C* demonstrates direct recognition of infected targets by CD4 T cells, showing that CD4 T cells react to relevant, endogenously processed viral fragments, and not just to peptides themselves. These data suggested that we have indeed identified the vast majority of WNV epitopes restricted by the $I\text{-}A^b$.

Ag-specific CD4 T cell cytokine secretion

Using these newly identified epitopes, we sought to determine the functional capabilities of the WNV-specific CD4 T cells. As shown in Fig. 3, the peptide stimulation of splenocytes isolated from mice 7 days after infection resulted in a robust IFN-γ response. We also observed spontaneous IL-4 production by CD4 T cells from day 7 infected animals (infected mice CD4 $IL\text{-}4^+$ = 0.91%, naive mice CD4 $IL\text{-}4^+$ = 0.20%, $n = 4$ mice per group), which may be due to a bystander effect, because no increase in IL-4 production was induced following stimulation with TCR (anti-CD3ε), purified peptides (Fig. 4*A*), or infected cell lysate (data not shown). The results of the cytokine profile produced by the WNV-specific CD4 T cells directly ex vivo suggest that the WNV CD4 T cell response shows a Th1 bias. Our data do not indicate that the IL-4 production is generated by Ag-specific CD4 T cells; however, at this point it is unclear whether this IL-4 response is targeted directly against WNV in these animals, which is possible in light of the role of CD4 T cells as boosters of B cell immunity and Ab production (14).

We next used these epitopes to track the percentage of the Ag-specific CD4 T cells within the B6 spleen during the course of an infection (Fig. 4*B*). Using ICCS, we were able to define the time course of the systemic Ag-specific CD4 T cell responses (Fig. 4*B*). We could start to detect the Ag-specific response by day 4, and the peak of the response in the spleen occurred at day 10 (Fig. 4*B*). As expected, upon peptide stimulation, memory CD4 T cells were capable of immediately producing both IFN-γ and, to a lower extent, IL-2 (Fig. 4*B*).

In vivo CD4 T cell cytotoxicity

Because we saw a strong induction of GzB in the CD4 T cell population during infection, we next wanted to determine whether the CD4 T cells elicited in B6 mice were capable of in vivo cytotoxicity (Fig. 4*C*). We chose to use a CFSE- based CD4 in vivo cytotoxicity assay, as initially described by Jellison et al. (24). In these experiments, we adoptively transferred peptide-pulsed splenocytes from B6 Ly-5.1 congenic mice (target cells), into day 6 WNV-infected B6 mice, in which the congenic marker was used to identify transferred cells and two concentrations of CFSE labeling were used to differentiate specific targets from the control ones. The in vivo CTL assays showed the cytotoxic capacity of the Ag-specific CD4 T cells, in which 39% of the MHC class II^+ targets were killed within a 12-h time period ($p < 0.008$) (Fig. 4*C*, *middle panel*).

Additional in vivo CTL assays were used to determine the mechanism of cytolytic clearance. PKH26 was used to label transferred cells, and two different concentrations of CFSE were used to differentiate targets. In these experiments, we showed that targets sensitized with class II-restricted peptides were not as efficiently

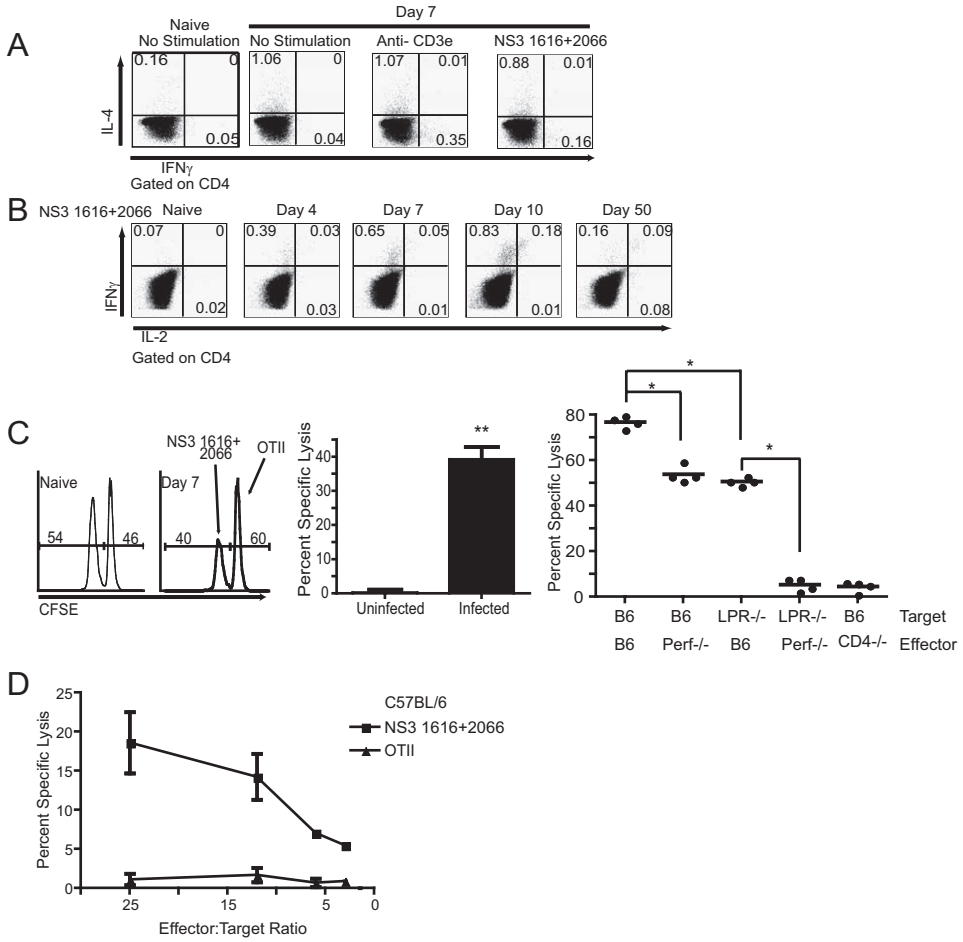

FIGURE 4. Functional potential of Ag-specific CD4 T cells. *A*, Representative example of a CD4 T cell cytokine response 7 days postinfection. Following gating on CD4, IFN-γ and IL-4 were measured after 6-h stimulation in the presence of monensin. Cells were stimulated with medium, 2c11 (0.5 μg/ml), or $NS3_{1616+2066}$ (10^{-6} M), and a naive mouse was used as a control. One mouse of four is shown, from one representative experiment of two. *B*, Representative example of CD4 T cell ICCS during the course of infection, as indicated above each plot. Following gating on CD4, IFN-γ and IL-2 were measured after 6-h stimulation with $NS3_{1616+2066}$ (10^{-6} M) in brefeldin A. Controls and repetitions were as in *A*. *C*, In vivo CD4 T cell CFSE cytotoxicity assay. *Left panel*, Representative histogram of transferred (donor, Ly-5.1^{+}) splenocytes 12 h after adoptive transfer of target cells into naive (*left histogram*) and infected (*right histogram*) mice. *Middle panel*, Aggregate quantification of in vivo CD4 T cell CFSE-based cytotoxicity assay. CD4 T cells were cytolytic in vivo on day 7 post-WNV infection (**, $p < 0.008$). Results represent the average of five mice per group, and are representative of three independent experiments. *Right panel*, Quantification of in vivo CD4 T cell CFSE-based cytotoxicity assay completed within WNV-infected perforin$^{-/-}$, CD4$^{-/-}$, and B6 control mice. B6 or LPR$^{-/-}$ splenocytes were coated with WNV or control peptides, labeled with CFSE, and used as targets with different hosts: the *x*-axis legend denotes which molecule(s) was missing during the interaction of CTL with their targets. B6 (targets) transferred into perforin$^{-/-}$ mice as well as the transfer of LPR$^{-/-}$-deficient targets lead to a reduction in cytotoxicity (*, <0.03). Transfer of LPR$^{-/-}$ targets into perforin$^{-/-}$ mice leads to a complete ablation of cytotoxicity ($p < 0.03$). Results represent the compilation of two independent experiments. *D*, In vitro CTL activity of purified CD4 T cells against targets coated with indicated WNV peptides or the control class II-restricted OVA peptide (OT-II, $OVA_{323–339}$). IC21 cells were coated with the indicated peptides and labeled with ^{51}Cr. The cells were incubated with CD4 T cells purified from spleens of B6 mice infected with WNV 7 days earlier, as described, with minimal purity of 89% and contaminating CD8 or B cells at <0.5%. Chromium release assay was performed, as described in *Materials and Methods*. Results are representative of three experiments.

eliminated in vivo in mice lacking perforin ($p < 0.03$ vs B6) or targets lacking Fas (*lpr/lpr*) ($p < 0.03$ vs B6) vs B6 mice. Most importantly, when Fas$^{-/-}$ targets were transferred into perforin-deficient mice, thereby eliminating both of the main cytolytic mechanisms simultaneously, CD4 T cells were no longer able to clear peptide-coated targets ($p < 0.03$ vs LPR$^{-/-}$→B6) (Fig. 4*C*, *right panel*). As an additional control for specificity, when sensitized targets were transferred into CD4-deficient mice, no cytolytic activity was seen (Fig. 4*C*, *right panel*). We therefore conclude that both Fas/FasL- and perforin-mediated mechanisms are involved in CD4 cytotoxicity, and that they have a level of redundancy for CD4 T cell-mediated cytolytic clearance of WNV peptide-coated targets in vivo.

Furthermore, direct ex vivo CD4 ^{51}Cr release assays were also completed using peptide-pulsed IC-21 cells, a macrophage cell line, in which we again readily observed specific lysis of WNV-coated, but not OVA-coated (OT-II) targets (Fig. 4*D*). These data strongly suggest that during the course of WNV infection, CD4 T cells differentiate into in vivo cytotoxic effectors capable of killing infected cells.

In vivo relevance of Ag-specific CD4 T cell response

Based upon adoptive transfer of naive CD4 T cells and the direct ex vivo functions of Ag-specific T cells, we proposed that Ag-specific CD4 T cells use both their cytolytic and cytokine capacity

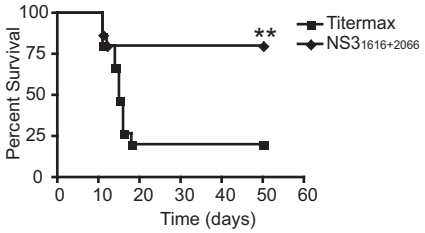

FIGURE 5. Ag-specific CD4 T cell responses are essential for protection against WNV. CD4 T cell peptide vaccination leads to a significant ($p < 0.03$) increase in protection of WNV-infected mice. Mice were vaccinated using 20 μg of $NS3_{1616+2066}$ or 20 μg of OTII control peptide in Titermax Gold emulsion. Ten mice per group were challenged with 1200 PFU of WNV. One representative experiment of three is shown.

to control WNV infection, preventing the development of encephalitis/meningitis. To look at the direct protective capacity of Ag-specific CD4 T cells, we used a peptide vaccination approach. B6 mice were vaccinated twice with an emulsion of $NS3_{1616}$ and $NS3_{2066}$ peptide in adjuvant TiterMax Gold (29). Twenty days after the last vaccination, mice were challenged with a high dose of WNV (1200 PFU s.c.) and were observed for 60 days. The mice that received the vaccination with WNV epitopes $NS3_{1616}$ + $NS3_{2066}$ were protected significantly better then the mice vaccinated with $OVA_{323-339}$ ($p < 0.03$) (Fig. 5). These data demonstrate that the dominant peptide epitopes recognized by CD4 T cells can be used as a vaccine to protect mice given a lethal dose of WNV.

Discussion

In this study, we show that naive CD4 T cells differentiate into primary effector cells and protect RAG-$1^{-/-}$ mice from lethal WNV infection. This indicates that not only do CD4 T cells have the ability to directly control WNV infection, but that they are sufficient to measurably protect RAG-$1^{-/-}$ mice from WNV. In this study, we also defined, in the H-2^b haplotype, most, if not all, CD4 T cell epitopes that develop during the course of a WNV infection. More importantly, we used these CD4 T cell epitopes as a vaccine to show that the generation of memory CD4 T cell response in intact B6 mice can be protective.

Previous reports studying antiviral immune responses to flaviviruses, including WNV, have indicated that T cells are required to prevent the development of encephalitis/meningitis (17, 18, 30). More recent work indicates a requirement for CD8 T cells in protection from lineage I strains of WNV (13, 15, 16, 18). Although CD4 T cells are not required during primary Dengue virus infection of mice (12), there is an absolute requirement for CD4 T cells during primary WNV infection (14) (data above). Our studies indicate that whereas the CD4 Ag-specific response may be important for the protection by other mechanisms, such as CD8 T cells and Ab/B cells, as originally described by Sitati and Diamond (14), they also can protect in vivo at least in part by direct effector function, and that this is sufficient to confer a significant degree of anti-WNV protection to adoptive hosts.

There has been a long-standing interest in the CD4 T cell functional response to many of the flaviviruses that infect humans. It has been shown, predominantly with T cell lines, that CD4 T cells responding to Dengue virus, JEV, YF, and WNV can proliferate, produce IFN-γ and IL-2, and are cytotoxic in response to viral Ags (11, 30–33). It has been recently shown that during resolution of JEV infection, the presence of a strong Th1 T cell response, including IFN-γ production, results in reduction of neurological sequelae (34). However, the CD4 T cell response to lineage I WNV infection in real time has not been analyzed in these studies. We provide what we believe to be the first description of a CD4 T cell Ag-specific response to WNV and a list of WNV determinants recognized by CD4 T cells. Our data indicate that the Ag-specific CD4 T cells respond in a Th1 fashion, including pronounced IFN-γ and low amounts of IL-2 production.

The one report that examined the direct effector role of CD4 T cells during a lineage I WNV infection did observe a protective effect upon the transfer of CD4 T cells, but did not observe a difference in the protective capacity of CD4 T cells that lacked IFN-γ, perforin, or Fas-FasL (14). The authors interpreted this as evidence for the lack of direct effector function. Our results suggest that CD4 T cells can lyse peptide-coated targets in vivo using a combination of perforin- and Fas-FasL-mediated pathway. The use of the perforin-mediated pathway would be consistent with our results on the expression of GzB. It is possible that WNV-specific CD4 effector T cells in vivo use all three effector mechanisms, IFN-γ, perforin, and Fas-FasL, with a level of redundancy built into the Ag-specific response to control WNV replication and spread. However, CD4 T cells deficient in one effector function may not be sufficient to reduce their antiviral activity and to observe a difference in survival in an adoptive transfer model. If so, that would explain why Sitati and Diamond (14) saw a similar increase in survival in mice receiving CD4 T cells, regardless of whether they were wild type, IFN-$\gamma^{-/-}$, perforin$^{-/-}$, or FasL$^{-/-}$.

Several studies have identified proper T cell trafficking as critical for protection from WNV neurological disease. CXCL10- and CD40-deficient mice have impaired trafficking of CD4 T cells into the CNS (10, 16). In B6 mice, WNV infection of the CNS begins ~3–4 days postinfection, which is the earliest time point when we could detect Ag-specific CD4 T cells that are prepared to secrete antiviral cytokines. However, control of viral spread within the CNS requires the use of noncytolytic mechanisms of viral clearance for host survival (35). The secretion of IFN-γ by lymphocytes is required for the clearance of neurotropic viral infections, such as Sindbis virus (36) and yellow fever (18), to keep neurons intact. Although neurons are not known to express MHC class II in situ, they do constitutively express IFN-γR (37), and upon exposure to IFN-γ secreted by WNV-specific CD4 T cells can up-regulate essential antiviral molecules such as RNaseL (38). Therefore, one could expect that the secretion of IFN-γ by WNV-specific CD4 T cells would play a role in containing viral pathology/loads in the CNS. Experiments are in progress to address this issue.

In summary, our experiments show that CD4 T cells are sufficient for controlling and clearing WNV from RAG-$1^{-/-}$ mice. Ag-specific CD4 T cells rapidly respond to infection within the periphery by secreting a multitude of cytokines. In addition to cytokine production, our data show that CD4 T cells have in vivo cytotoxic capabilities. These Ag-specific CD4 T cells most likely use both cytokine production and cytotoxicity as mechanisms to prevent WNV encephalitis/meningitis during viral challenge. Together with the published results on other immune mechanisms deployed against WNV (9–11, 13–15, 25–27, 32, 38), including other components of the adaptive immune system (notably CD8 T cells and B cells), the above results illustrate a very broad collaboration of diverse innate and adaptive effector mechanisms that protect against this virus.

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Disclosures

The authors have no financial conflict of interest.

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