# Key role of T cell defects in age-related vulnerability to West Nile virus

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**West Nile virus (WNV) infection causes a life-threatening meningoencephalitis that becomes increasingly more prevalent over the age of 50 and is 40–50× more prevalent in people over the age of 70, compared with adults under the age of 40. In a mouse model of age-related vulnerability to WNV, we demonstrate that death correlates with increased viral titers in the brain and that this loss of virus control with age was the result of defects in the CD4 and CD8 T cell response against WNV. Specific age-related defects in T cell responses against dominant WNV epitopes were detected at the level of cytokine and lytic granule production, each of which are essential for resistance against WNV, and in the ability to generate multifunctional anti-WNV effector T cells, which are believed to be critical for robust antiviral immunity. In contrast, at the peak of the response, old and adult T cells exhibited superimposable peptide sensitivity. Most importantly, although the adult CD4 or CD8 T cells readily protected immunodeficient mice upon adoptive transfer, old T cells of either subset were unable to provide WNV-specific protection. Consistent with a profound qualitative and quantitative defect in T cell immunity, old brains contained at least 12× fewer total effector CD8 T cells compared with adult mice at the peak of brain infection. These findings identify potential targets for immunomodulation and treatment to combat lethal WNV infection in the elderly.**

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Abbreviations used: GzB, granzyme B; IFNAR, IFN-αβ receptor; MST, mean survival time; WNV, West Nile virus.

West Nile virus (WNV) is a small enveloped single-stranded positive sense RNA-containing virus with a genome of ∼11 Kb that belongs to the family *flaviviridae* (Lindenbach and Rice, 2001). WNV is a typical arbovirus, cycling between its two natural hosts, mosquitoes and birds (Hayes, 2001). Mosquitoes transmit WNV to a wide range of other species (including humans), which cannot further spread the infection (dead-end hosts) but which, nevertheless, can exhibit significant morbidity and mortality. Since 1999, this virus has been responsible for >23,000 clinically registered human infections, leading to nearly 1,000 deaths in the United States alone (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm). Moreover, annual outbreaks in the United States have been registered in every year since 2000 and were marked by increased mortality rate in infected birds (Anderson et al., 1999) and horses (Ng et al., 2003) and by an increase in the frequency and clinical severity of WNV infection within the human population (Petersen and Marfin, 2002). These observations are consistent with findings elsewhere in the world since the mid 1990s (Tsai et al., 1998; Chowers et al., 2001; Platonov et al., 2001) and suggest that the US strains of WNV may exhibit an increase in virulence compared with the original old-world strains of WNV.

The incidence of WNV infection is fairly uniform with age (Mostashari et al., 2001) and, in most immunocompetent humans, the disease is asymptomatic (Weiss et al., 2001; Petersen and Marfin, 2002). However, severe WNV disease, which includes the involvement of the nervous system (meningitis and encephalitis), is a disease of old age, with a frequency of ∼1 in 150, a



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lethality of 10%, and a mean age at death of 78 yr (Mostashari et al., 2001; Murray et al., 2006). Persons between 50 and 59 yr of age exhibit a 10× higher incidence of severe WNV disease, whereas persons aged 80 yr or older exhibit a 43× higher incidence, compared with the adults between 20 and 40 yr of age (Nash et al., 2001; Murray et al., 2006). Moreover, many patients that suffer from WNV encephalitis require more than one and, possibly, several years to fully recover physically, functionally, and cognitively (Weiss et al., 2001), and the overall mortality in the first year after infection is significantly increased compared with age-matched controls (Murray et al., 2006).

Aging leads to a widespread but poorly understood state of immunodeficiency, which is associated with an increased incidence and severity of infectious disease in the elderly (Gardner, 1980). Many facets of innate and adaptive immunity have been shown to be altered by aging (Cambier, 2005; for reviews see Miller, 1996; Pawelec et al., 1998; Linton and Dorshkind, 2004), but at the present it is unclear which of these defects are critical to impaired immune defense. It is further unclear whether the critical age-related defects are constant or whether they vary depending on the biology of the encountered pathogen. It is of note that T cells have been shown to exhibit some of the most pronounced age-related defects (for reviews see Miller, 1996; Nikolich-Žugich, 2005), and reversion of T cell defects has been associated with improved immune function in old rodents and monkeys (Garcia and Miller, 2003; Haynes et al., 2004, 2005; Messaoudi et al., 2006). However, direct mechanistic links between a decline in T cell function and reduced immune defense against infectious diseases remain scarce. Therefore, we examined the immunological basis of the age-related increase in susceptibility to WNV, and we report in this paper that qualitative and quantitative impairment with age in effector T cell immunity at the site of critical affected tissue (the brain) leads to increased susceptibility to this virus.

## RESULTS

### Mouse model of age-related susceptibility to WNV

We established a robust mouse model of the age-related susceptibility to WNV. In our hands, old mice exhibited increased susceptibility to WNV regardless of the infection route (i.p. or s.c.), the viral isolate of WNV Ia (NY-99, 31A, or 385–99; Table S1, Fig. 1; see Figs. 2 and 5; and not depicted), or the mouse strain (Fig. 1, C57BL/6; and see Figs. 2 and 5, C57BL/6; or Fig. S1, BALB/c) used, although, as expected, i.p infection produced lethal effects at a lower dose (1–20 PFU) compared with the more physiological s.c. infection (50–1,000 PFU). Overall, old mice were at least six times more susceptible to WNV as measured by survival rates over many viral concentrations (Fig. 1 A and not depicted). At low viral doses, that difference was drastically reduced and, in some experiments, disappeared (Fig. 1 A, Table S1, and not depicted), whereas at the high doses both old and adult animals succumbed to infection, suggesting that the viral dose is one of the principal determinants of selective mortality of old mice within a specific dose window.

In principle, this age-related susceptibility could be the result of a generalized inability of old mice to control the virus at the level of both the innate and adaptive immune systems, of the generalized inability to control the virus by one of these two components, or of their focused inability to control the virus in selected target organs. To investigate this issue, infectious viral titer was determined in different organs of adult and old mice and at different time points after infection. After infection with a viral dose that kills most old but not adult mice, we found no significant difference in viral titers between adult and old mice between days 2 and 5 (Fig. 1 B and Fig. S2). Likewise, early time points showed no difference in spleen viral titers (Fig. S2), and our survey of other organs (kidney, gut and liver) also failed to show viral titer differences between old and adult animals between days 3 and 10. We could not detect WNV in the blood past day 5 or in any other organs past day 10, and certain organs (lung and skin, except the site of injection) were negative throughout the course of infection, with the exception of the central nervous system. Indeed, we found significantly higher viral titers in the brains of old mice compared with the adult counterparts on days 8 and 10 after infection (actually between days 7 and 12 [not depicted]; Fig. 1 C, left and middle). Two additional lines of evidence showed that viral titers within the brain directly correlated to mortality. First, once the animals became moribund, they exhibited equivalent and high brain WNV titers regardless of age (Fig. 1 B, right). Moreover, when higher infection doses of WNV, lethal to both adult and old mice, were used, both adult and old mice again exhibited comparable and high brain viral titers (unpublished data). Therefore, in all subsequent experiments where we sought to dissect the immunological basis of vulnerability to WNV, we used the viral doses at which most old animals had high viral titers in brain and died but at which the majority of adult mice exhibited low brain WNV titers and survived.

### Relative kinetics and roles of innate and adaptive immune responses in mediating defense against WNV infection

We next asked whether the differences mentioned in the previous section could be explained by age-related defects in innate or adaptive immunity. As a model of pronounced innate immunity defect, we used IFN-αβ receptor (IFNAR)–deficient mice ($IFNAR^{-/-}$; Müller et al., 1994), which are unable to respond to type I IFNs (IFN-I) and are known to be highly susceptible to numerous infections (for review see Pestka et al., 2004), including WNV (Samuel and Diamond, 2005). $Rag\text{-}1^{-/-}$ mice (Mombaerts et al., 1992), which have no T and B cells as a result of the lack of the recombinase essential for generation of T and B cell receptors and which are also susceptible to many infections, including WNV (Engle and Diamond, 2003), were used as a model of profoundly deficient adaptive immunity. Our results confirmed that both of these strains are highly susceptible to WNV (Fig. 2 A) but highlighted a difference in the mean survival times (MSTs; Fig. 2 A). Thus, $IFNAR^{-/-}$ mice died rapidly after infection (MST, 5 d), which is consistent with the lack of innate defensive

mechanisms, whereas the RAG1$^{-/-}$ mice died within the same temporal window as old and adult mice (MST, 13 d). Because the MST of old mice was 13–14 d, we concluded that innate immunity in these mice, unlike that in IFNAR$^{-/-}$ animals, was capable of containing the virus and fending off early WNV-mediated mortality. Therefore, any putative defects in innate immunity in old mice, if present, do not lead to early loss of viral control. To confirm this, we examined functional levels of IFN-I in the serum of adult and old animals. Although the results of this test cannot be taken as fully conclusive because of the low sensitivity of this assay (Fig. S3), we detected no major age-related differences within these confines. More importantly, any defect in innate immunity would have been expected to result in loss of viral control early after infection. However, this was not observed in any of the experiments designed to test early viremia and viral spread (Fig. 1 and Fig. S2).

Because the results suggested a key role for defects in adaptive immunity, we directly explored this possibility by performing adoptive transfers of old and adult spleen cells (Fig. 2 B, left) or T cells (CD4 and CD8; Fig. 2 B, right) into adult RAG1$^{-/-}$ mice. In this series of experiments, we found that adult spleen cells, as well as the adult T cells, conferred significant protection to RAG1$^{-/-}$ mice. In contrast, mice that received old cells were afforded weaker protection, which was not improved in the case of splenocytes and was significant for transferred old T cells ($P < 0.04$) compared with RAG1$^{-/-}$ mice receiving no cell transfer. Direct comparison between the protective capacity of adult and old T cells revealed significantly better protection by adult T cells ($P < 0.003$; Fig. 2 B). Because antigen presentation and priming are not affected by either age or by the targeted deletion of Rag-1 in the adult RAG1$^{-/-}$ mice, these results collectively strongly suggested that the process of aging impairs resistance to WNV at the level of generation of primary T cell responses.

### Age-related differences in WNV-specific T cell responses

IgM and the virus-specific CD8 and CD4 T cells were all implicated in affording protection against WNV in adult mice (Diamond et al., 2003; Shrestha and Diamond, 2004; Sitati and Diamond, 2006). Given the extensive literature on the decline of T cell immunity with aging and our results with T cell transfers, we initiated experiments to test possible defects in the T cell pool. We first examined signs of T cell activation

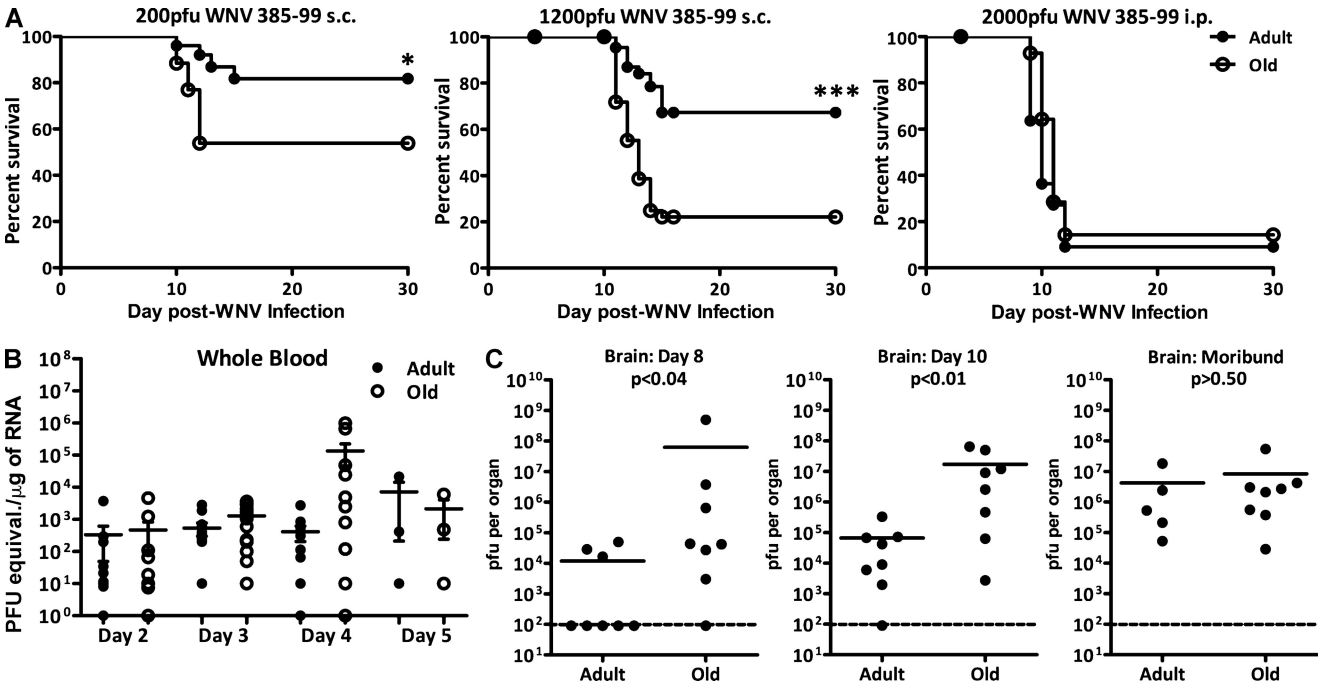


**Figure 1. Age-related susceptibility to WNV disease in old mice.** (A) Survival of adult (4–6 mo old) and old (18–22 mo old) mice after challenge with the indicated doses of WNV 385–99 s.c. or i.p. Old mice are five and six times more susceptible to WNV upon i.p. (MST, 13 d) and s.c. (MST, 14 d) infection, respectively, compared with adult controls. Statistical significance was evaluated by the Log-rank test (***, $P < 0.0005$; *, $P < 0.05$). Please note that difference between the old and adult mice disappears at the high viral challenge dose, where essentially all animals succumb to infection, and also at the low virus dose, where old animals appear to control the low viral loads well (left; see also Table S1 for additional experiments with other viral isolates and routes). Results depict mortality ($n = 12$ mice/group) and are representative of at least four experiments with similar results. (B and C) Viral titers within indicated tissues and organs of adult and old mice after s.c. infection (1,200 PFU/mouse) were determined by quantitative PCR (B, whole blood expressed in approximate PFU equivalents, based on quantitative PCR of a known WNV RNA standard) or by plaque assay (C, brain). The brains of old mice contained significantly more virus on both day 7 and day 10 after infection. In contrast, there was no difference between brain viral levels of old and adult moribund mice on day 12–16. For data on day 3 blood and spleen by plaque assay, please see Fig. S2. Horizontal bars indicate mean values of the unpaired Student's *t* test. Error bars represent SEM.

using multicolor flow cytofluorometric analysis. We followed quantitative and qualitative aspects of antigen-specific responses using peptide MHC class I tetramers, as well as the functional response measured by the ability to produce IFN-γ, TNF, and granzyme B (GzB) and express CD43, a molecule involved in T cell activation, costimulation, and effector function (Onami et al., 2002), and to perform lytic function in response to recently identified immunodominant WNV peptide epitopes that stimulate $CD8^+$(Brien et al., 2007) and $CD4^+$ T cells (Brien et al., 2008; Figs. 3 and 4). At the peak of the response (days 7–8 after infection), we detected a strongly significant reduction in both percentages and numbers of $CD8^+$ cells specific for the immunodominant class I–restricted epitope $NS4b_{2488}$ by both tetramer staining (Fig. 3 A) and IFN-γ production after brief in vitro stimulation (Fig. 3 B). In addition to these quantitative defects, the ratio between IFN-γ–producing and $NS4b_{2488}$ $Tet^+$ cells was also significantly reduced in old CD8 cells so that less than one-half of Ag-specific cells were also making the cytokine (Fig. 3 C). That suggested the existence of superimposed qualitative defects in immunity of old mice against WNV.

To analyze the quality of the anti-WNV response in depth, we examined several aspects of the response, including the ability to produce polyfunctional cells, the intensity of cytokine expression/cell, the level of activation of CD8 cells as measured by CD43 expression, and the ability to lyse peptide-coated cells (Fig. 4). By each and every measure, old CD8 T cells were significantly inferior to the adult counterparts. Thus, although nearly 40% of adult CD8 cells expressed IFN-γ, TNF, and GzB (Fig. 4 A, 3 Fxn), only 21% of adult cells did so. Second, expression of each of these molecules per cell was significantly lower in old CD8 T cells (Fig. 4 B). Third, only about half of the WNV-specific ($Tet^+$) CD8 T cells from old mice expressed high levels of CD43, an important activation and function marker (Fig. 4 C). Finally, direct ex vivo cytotoxicity correlated to GzB expression and was

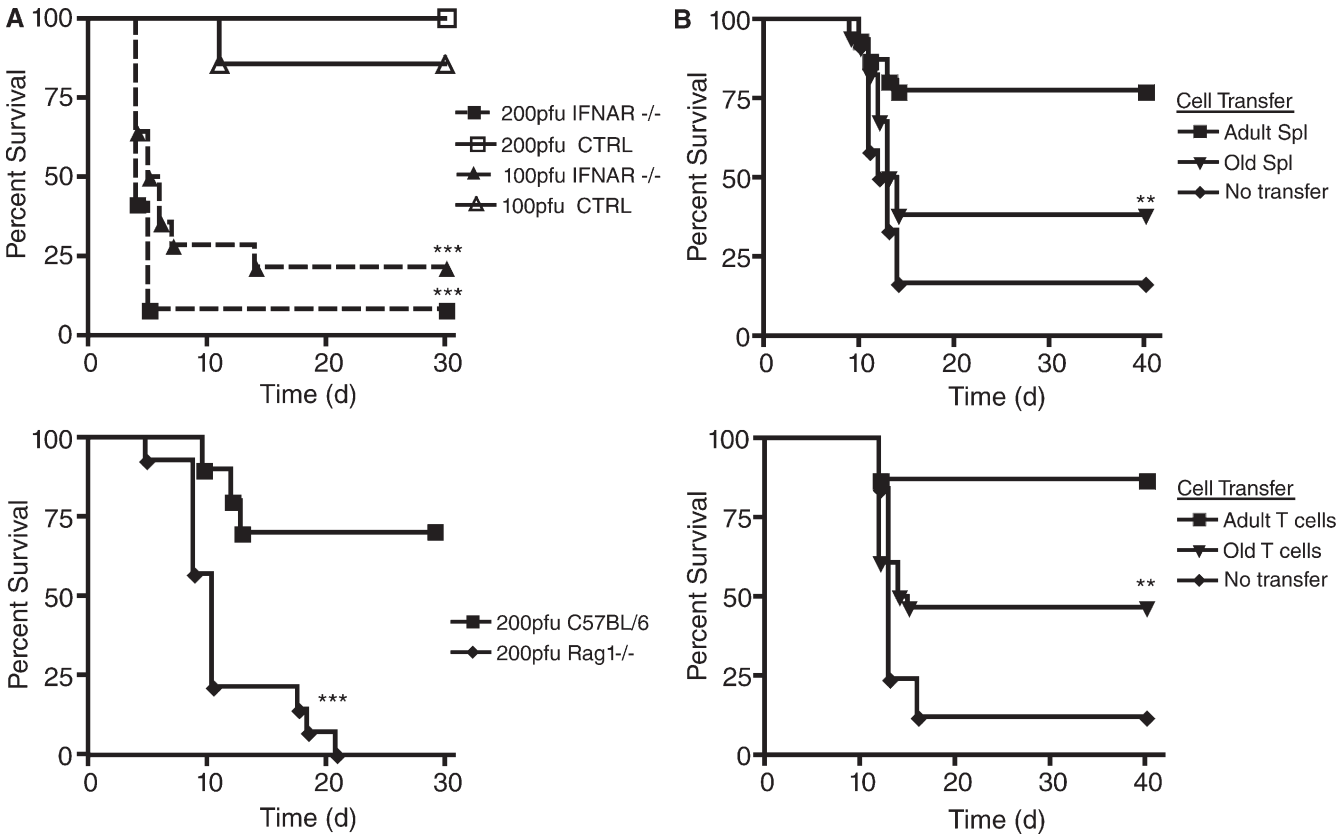


**Figure 2. Relative roles of innate and adaptive immunity and the importance of the age of T cells in resistance to WNV infection.** (A) $IFNAR^{-/-}$, $RAG1^{-/-}$, and WT mice were infected with the indicated WNV doses s.c., and MST and percentage of survival were scored. WNV caused rapid mortality (MST, 4.5 d at 200 PFU) with a very low rate of survival in $IFNAR^{-/-}$ mice compared with WT controls ($P < 0.0001$ at 200 PFU). WNV infection also caused significant mortality in $RAG\text{-}1^{-/-}$ mice compared with WT controls ($P < 0.0001$) but MST was similar to that of WT mice (MST, 13 d at 200 PFU). Data were compiled from two independent experiments. (B) Adoptive transfer of lymphocytes from old and adult mice reveals age-related defects in adaptive immunity. Splenocytes ($10^7$/animal) from adult mice protect $RAG\text{-}1^{-/-}$ significantly better than splenocytes from old mice (top; MST, 14.5 at 100 PFU; $P = 0.0025$). Transfer of purified T cells (CD4 and CD8; $5 \times 10^6$/mouse) reveals defects in old T cells, which were inferior at protecting $RAG1^{-/-}$ mice compared with the purified T cells from adult mice (bottom; 100 PFU; $P < 0.03$). Compared with $RAG1^{-/-}$ mice with no transfer, old splenocytes conferred no significant protection, whereas old T cells showed some protection ($P = 0.04$). **, $P = 0.01$–$0.001$; ***, $P < 0.001$. Results are representative of two comparable experiments.

essentially nonexistent in CD8 T cells from old mice compared with adult counterparts (Fig. 4 D).

These findings were consistent in every experiment performed. Sometimes not all the parameters would reach statistical significance when smaller animal groups were used, but at least three out of four observations were always significant. Similarly, these trends and significant differences were confirmed with the CD8 epitope $ENV_{347}$ and also in $CD4^+$ cells using the immunodominant $CD4^+$ T cell epitopes ($ENV_{641}$ and $NS3_{1616+2066}$) by measuring frequency and magnitude of the IFN-γ (Fig. S4) and other responses (not depicted). Based on the prior descriptions of signaling defects in CD4 T cells (Garcia and Miller, 2003; for review see Miller, 1996), we expected that some of the stated problems in old T cells may trace to inferior ability of old T cells to process antigenic stimulus. However, experiments examining peptide sensitivity of WNV-specific CD8 and CD4 T cells showed superimposable sensitivity of old and adult cells at the peak of the response (Fig. S5). We conclude that old mice exhibit profound quantitative and qualitative defects in mobilizing fully developed effector T cells but that these defects do not extend to all aspects of antigen recognition and activation.

### Mechanisms of T cell–mediated protection against WNV disease and their failure in old mice

We next wanted to determine whether the observed reduction in IFN-γ secretion and/or GzB expression were relevant for protection against WNV in vivo. To that effect, we performed two types of adoptive transfer experiments using $RAG1^{-/-}$ mice as recipients. First, we transferred total T cells from WT, IFN-$\gamma^{-/-}$, or perforin$^{-/-}$ donors into $RAG1^{-/-}$ recipients, which showed that T cells defective in IFN-γ or perforin provide negligible, if any, protection as compared with animals which received no cells at all (Fig. 5 A). This confirms and extends prior results on the importance of these molecules in anti-WNV protection (Engle and Diamond, 2003; Wang et al., 2003) and stresses the critical role of their expression in T cells. However, because old T cells have a reduction but not a complete absence of these molecules, we tested their antiviral ability in vivo by separately transferring highly purified (< 0.5% cross-contamination) CD4 and CD8 T cells from old or adult naive donors into adult $RAG1^{-/-}$ recipients. These results convincingly showed that either adult CD4 or adult CD8 T cells were sufficient to confer significant protection to $RAG1^{-/-}$ mice against primary WNV infection in the absence of other components of the adaptive immune system (Fig. 5 B). More importantly, neither the $CD4^+$ nor the $CD8^+$ T cells from the old mice were able to confer any protection upon $RAG1^{-/-}$ mice over the level seen in the absence of transfer (Fig. 5 B), although the combination of the two old T cell subsets did show some synergy, affording a low level of protection (Fig. 2 B, bottom).

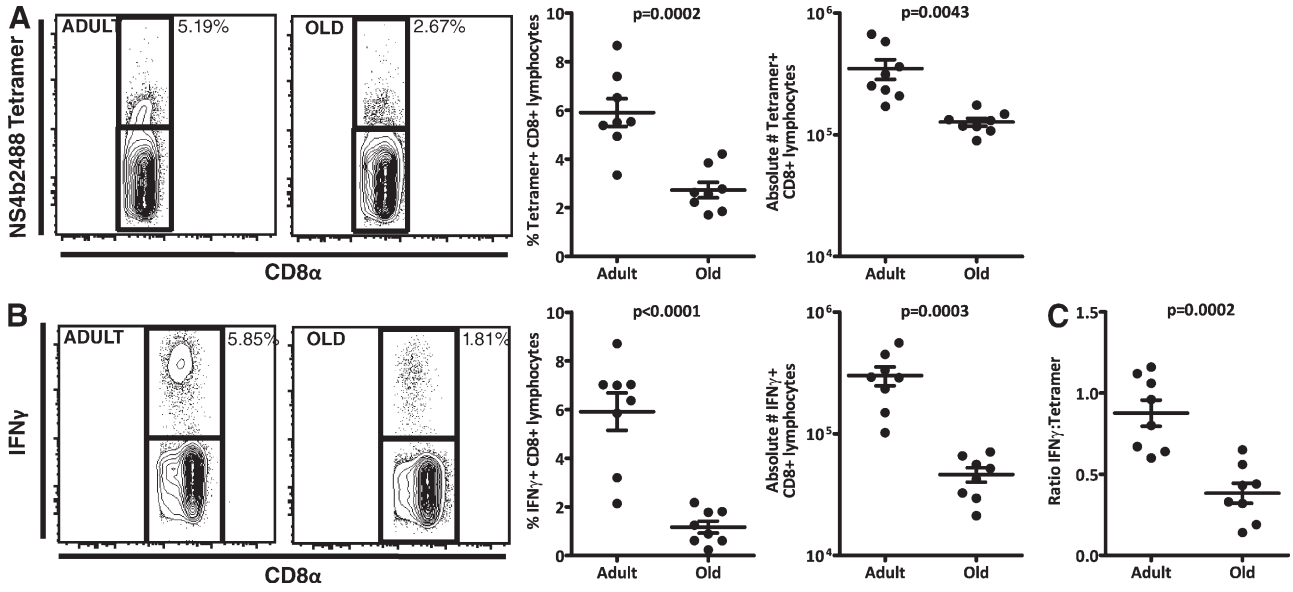


**Figure 3. Functional quantitative defects in T cell activation in response to WNV infection.** Infection was as in Fig. 1 A. (A) $CD8^+$ T cells derived from spleens of old and adult mice were harvested on day 8 and analyzed for the proportion (left and middle left, representative examples; middle right, aggregate analysis of relative numbers) and absolute numbers (right, absolute numbers) of cells expressing the $CD8^+$ NS4b-2488:$K^{b+}$ phenotype. Dots denote individual mice ($n = 8$) and are representative of four experiments. (B) Same as A, except that measurements show relative representation and absolute numbers of $CD8^+$ IFN-$\gamma^+$ cells in response to the immunodominant NS4b-2488 CD8 epitope by ICCS (Brien et al., 2007) at the peak of the immune response (day 8). Old mice mobilized significantly fewer numbers of IFN-γ–producing WNV-specific $CD8^+$ T cells than their adult counterparts. Results were compiled from three independent experiments. (C) Ratio between IFN-$\gamma^+$ and $Tet^+$ $CD8^+$ cells shows that many fewer Ag-specific T cells are functionally able to manufacture this cytokine in the old mice. Groups, representation, and graphics are as in A, and results are representative of two experiments. Horizontal bars indicate mean values of the unpaired Student's *t* test. Error bars represent SEM.

### Too little and too weak: insufficient numbers of ineffective effector T cells accumulate in the brains of WNV-infected old mice

If our conclusions were correct, we would expect that they would hold at the level of key organs targeted by WNV. Alternatively, it was also possible that there may be defects in migration and localization of virus-specific T cells to the old brain. To address this issue, we examined the localization and composition of leukocyte infiltrate in the brains of WNV-infected old and adult mice. It is of interest that immunohistochemical examination revealed that in old mice, like in the adult animals, $CD3^+$ cells readily infiltrated the brain parenchyma on days 7 (not depicted) and 12 (Fig. 6 A), suggesting that there was no major difference in migration and homing. In contrast, the composition of the infiltrate was both qualitatively and quantitatively different (Fig. 6, B and C). The large (activated) mononuclear cell gate (Fig. 6 B) contained nearly 3× fewer cells in old mice compared with adult counterparts (3.1 vs. 9.1%, $P < 0.0005$). Moreover, within that gate, the percentage of CD4 and CD8 cells was also about twofold lower with old age (5.6 vs. 11.4%, $P < 0.013$, and 14.1 vs. 24.3%,

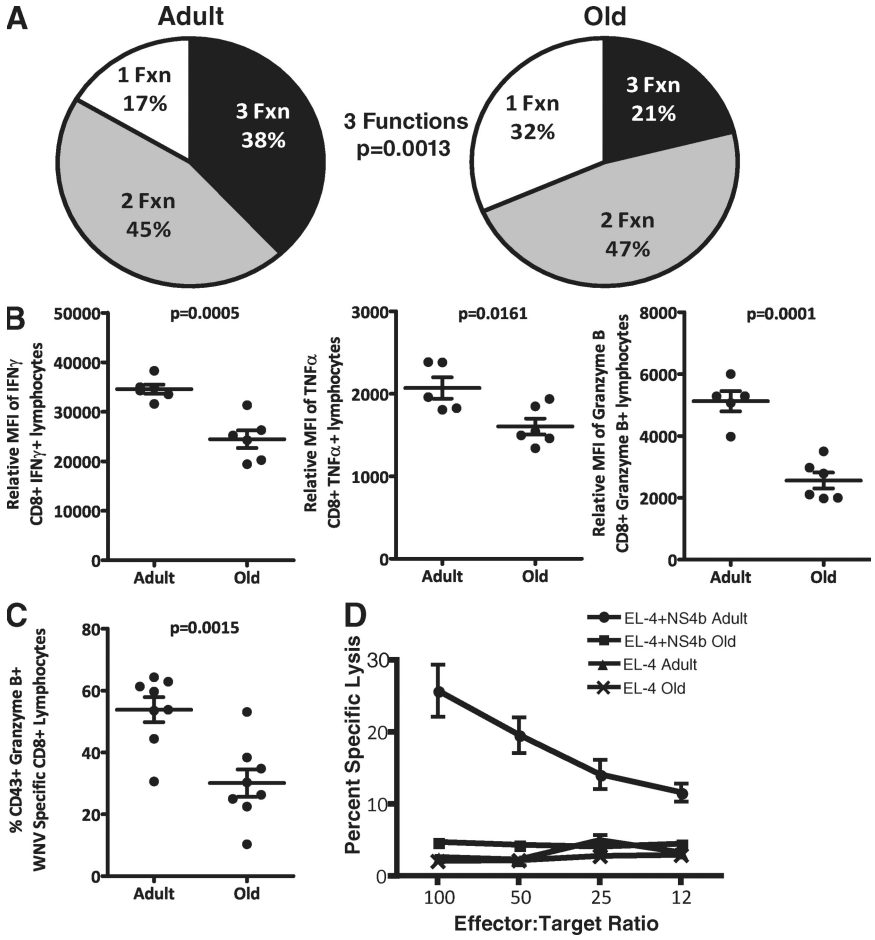


**Figure 4. Quality of WNV-specific responses in old mice is impaired at several levels.** For all panels, animals were infected with 1,000 PFU WNV 385–99 and were analyzed on day 8, unless otherwise indicated. (A) At the peak of infection, splenic CD8 cells were stimulated with class I–restricted peptides and analyzed for the expression of IFN-γ, TNF, and GzB, and results were plotted to denote percentages of cells exhibiting all three molecules (3 Fxn), two molecules (2 Fxn), or a single molecule (1 Fxn). Results depict eight animals per group and are representative of two such experiments. (B) Relative intensity of expression of IFN-γ, TNF, and GzB among $CD8^+$ cells in response to 6 h of NS4b-2488 peptide stimulation as described in Materials and methods. After gating on $CD8^+$ T cells, normalized mean fluorescent intensity (MFI) was obtained by subtracting the mean fluorescent intensity of the negative cell population from that of the positive cells. Data depict five adult and six old individual mice, representative of four experiments, and are shown as in Fig. 1 A. (C) Expression of CD43 on WNV-specific CD8 cells from adult and old mice was analyzed on day 8 after infection by selective gating and is expressed as percentage of $Tet^+CD8^+$ cells that are $CD43^+$, shown for eight animals per group and representative of three such experiments. Horizontal bars in B and C indicate mean values of the unpaired Student's *t* test. (D) Ex vivo cytotoxic activity of adult and old $CD8^+$ T cells demonstrates major age-related functional defects. Adult and old mice were infected s.c. with WNV at a dose (1,000 PFU) that caused decreased survival of old mice compared with adult animals. $CD8^+$ T cells from adult mice exhibited stronger cytotoxic activity than $CD8^+$ T cells from old mice when assayed directly ex vivo 7 d after infection in a 6-h standard $^{51}Cr$-release assay. Peptide-coated $NS4b_{2488}$ ($10^{-6}M$) EL-4 cells were used as targets. Values for five mice per group with standard deviations, representative of two experiments, are shown. P-values are depicted above the graphs.

$P < 0.037$ for CD4 and CD8 cells, respectively; Fig. 6 C), leading to a >5× reduction in total CD8 and CD4 cells in old WNV-infected brains. Among these already diminished numbers, the content and quality of virus-specific cells was further reduced. Therefore, the percentage of NS4b-specific tetramer$^+$ CD8 cells specific for WNV was reduced by another twofold (18.6 vs. 38.8%, $P < 0.0012$; Fig. 6 D), with another reduction in GzB-producing cells (trend but not significant at 3.3 vs. 6.7%, $P < 0.06$; not depicted). Most importantly, when all the reductions were taken into account, compared with the levels found in adult mice (taken as 100%), our analysis suggests that old brains contain at least 12.5× fewer and up to 20× fewer Tet$^+$ GzB$^+$ CD8 T cells (Fig. 6 D) compared with adult brains at the peak of infection.

Two important conclusions can be drawn from these results. First, migration to the site of major virus-induced tissue damage is not drastically affected in T cells of old animals. Second, although old T cells apparently arrived to the site of infection, they were not able to differentiate into effector T cells either systemically or locally in numbers sufficient to ensure control of neurovirulence. Therefore, our results identify defects in generation of sufficient number and quality of effector antiviral T cells as the key phenomenon underlying age-related susceptibility to WNV in this model, providing targets for potential therapeutic manipulation.

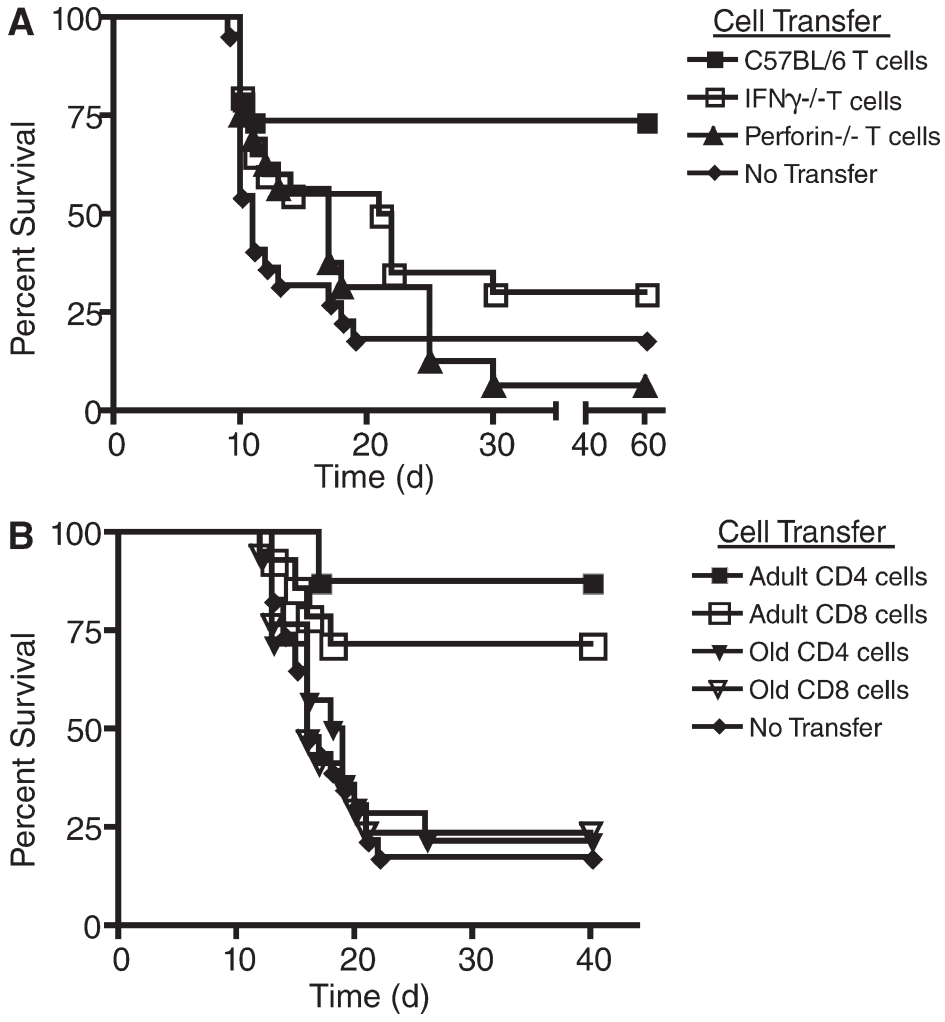


**Figure 5. Inability of aged CD4 and CD8 T cells to protect RAG-1$^{-/-}$ mice against lethal WNV infection.** (A) Purified CD8 and CD4 T cells from C57BL/6 (closed squares), IFN$\gamma^{-/-}$ (open squares), or perforin$^{-/-}$ (closed triangles) mice were transferred into RAG-1$^{-/-}$ mice, which were then infected with 200 PFU WNV and scored for survival. WT T cells exhibited significantly enhanced protection when compared with RAG-1$^{-/-}$ mice with no transfer (closed circles, $P < 0.0006$) or RAG-1$^{-/-}$ mice receiving perforin$^{-/-}$ ($P < 0.003$) or IFN-$\gamma^{-/-}$ T cells ($P < 0.01$). (B) Young RAG-1$^{-/-}$ mice received no cells (closed diamonds) or received highly purified adult (squares) or old (inverted triangles) CD4 (closed symbols) or CD8 (open symbols) T cells ($5 \times 10^6$ cells/mouse). Engraftment was verified after 24 h, with animals infected with 200 PFU WNV, and survival was scored thereafter. Adoptive transfer of old CD4 or CD8 T cells failed to confer any protection to RAG-1–deficient hosts, whereas transfer of adult CD4 ($P < 0.0001$) or CD8 ($P < 0.01$) T cells afforded a high degree of protection. Both parts of the figure were reproduced in three separate experiments.

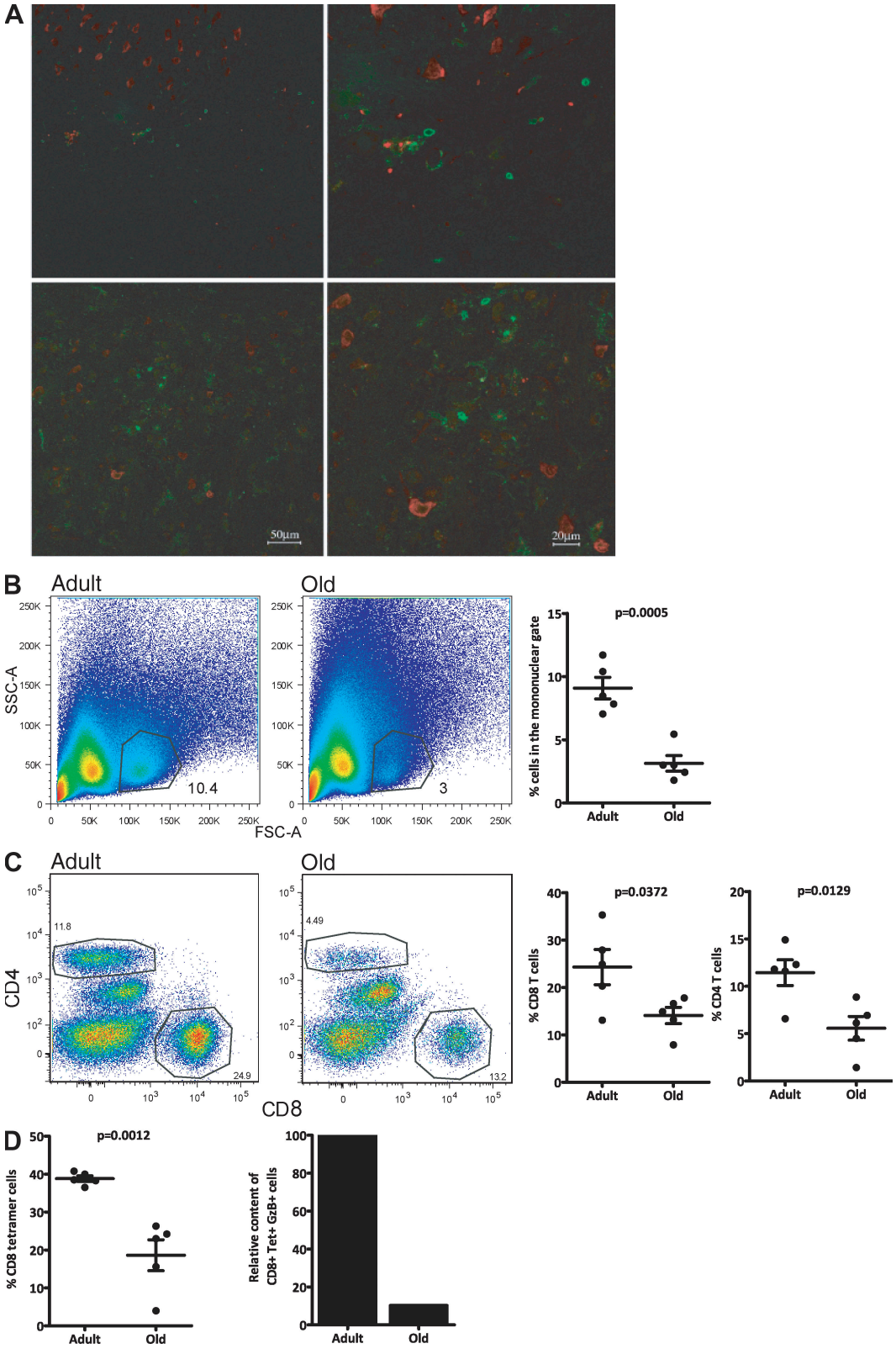


**Figure 6. Analysis of brain infiltrates from WNV-infected adult and old mice.** The brains of old and adult C57BL/6 mice were harvested 12 d after infection with 1,000 PFU WNV 385–99 s.c., sectioned, and costained for CD3 (green) and WNV (red). (A) Representative adult (top) and old (bottom) brains are shown at medium (left) and high (right) power. CD3 T cells are stained green and WNV-infected neurons are red. Bar: (left) 50 µm; (right) 20 µm. (B) Brains of 10 old and 10 adult mice harvested 10 d after infection with WNV. To generate five independent samples per group, with sufficient cell

## DISCUSSION

Our results show that specific age-related defects in T cell immunity, affecting both CD8 and CD4 T cells, underlie the susceptibility of old mice to WNV. This is in contrast to some of the other viral infections, such as Vaccinia, where no defects in GzB expression directly ex vivo can be detected (unpublished data), or influenza, where there is no reproducible age related decrease in survival in mice (Bender et al., 1995; Gardner, 2005) and where the loss of CD8 T cells was not accompanied by impaired viral resistance (Eichelberger et al., 1991), but it is similar to impaired lytic function of CD8 T cells against HSV-1 in old mice (Messaoudi et al., 2004). Numerous innate (IFN-I, complement, and innate IgM antibody) and adaptive (B cells and CD4 and CD8 T cells) mechanisms were implicated in anti-WNV resistance in adult mice (Diamond, 2005) but their relative importance and primary roles still remain incompletely mapped. Our results show that both T cell subsets play important and independent antiviral roles in adult mice, although there is evidence that they could also synergize (e.g., Fig. 2) against the virus. Adult CD4 and CD8 T cells were both able to secrete IFN-γ (results presented in this study) and to kill infected target cells (Brien et al., 2007, 2008; Purtha et al., 2007), which are critically important anti-WNV effector mechanisms (Shrestha et al., 2006; Sitati and Diamond, 2006; Fig. 5 A), and both functions were impaired with aging (Figs. 3 and 4). Moreover, adult T cells were polyfunctional and exhibited significantly more robust per cell responses at the level of effector molecule mobilization but did not exhibit higher TCR sensitivity (Fig. 4, Fig. S4, and Fig. S5). Of major importance are the results from adoptive transfers into RAG1$^{-/-}$ mice, where adult, but not old, CD4 or CD8 T cells could confer significant anti-WNV protection. Because these animals possess functional (and young) innate and reticuloendothelial system components and only lack B, T, and NK-T cells, our results reveal that the defects in transferred aged T cells are cell autonomous in nature and are not precipitated by the aging of accessory and/or antigen-presenting cells. Consistent with this finding, examination of brain T cell infiltrates revealed normal migration in old mice but insufficient accumulation of differentiated effector T cells, which were 10–20× less numerous in the brains of old mice. This would suggest that the use of T cell rejuvenation therapies to remedy this condition may offer the best chance for therapeutic intervention. In this case, thymic reawakening and production of additional cohorts of Ag-specific T cells by the use of a combination of stem cell intervention to circumvent the block in lymphopoiesis and the stromal rejuvenation using KGF, androgen blockade, or IL-7 treatment (for reviews see Linton and Dorshkind, 2004; Nikolich-Žugich, 2005) would appear to provide the best approach.

However, it will also be important to understand other, perhaps more subtle, potential defects in innate and adaptive immunity. Thus, although our results strongly suggest that there are no major age-related defects in early virus control by the innate immune system, they do not exclude the possibility of correction of T cell defects by providing enhanced activation of innate immunity by cytokine manipulation (Haynes et al., 1999, 2004) and/or antigen presentation. Indeed, such approaches may provide more practical means for therapeutic intervention compared with T cell rejuvenation, which still remains to be effectively customized for mass utilization. However, one could imagine providing key cytokines or costimulatory molecules in repeated rounds of antigen-driven T cell and B cell expansion to enhance and improve effector T cell generation. It is our belief that such intervention may be most effective to remedy the defects described in this paper. Overall, given the multitude of defects that affect an aging immune system, it seems prudent to elucidate specific and key defects affecting resistance to each pathogen and then rationally target the identified defects for intervention.

## MATERIALS AND METHODS

**Mice.** Old (18–22 mo) and adult (4–6 mo) C57BL/6 (B6) mice were purchased from the National Institute of Aging breeding colony (Harlan). C57BL/6 RAG1$^{-/-}$, 129, C57BL/6 perforin$^{-/-}$, and C57BL/6 IFN-γ$^{-/-}$ mice were purchased from The Jackson Laboratory and bred at the Oregon Health & Science University (OHSU) West Campus vivarium. IFNAR$^{-/-}$ mice were a gift of A. Hill (OHSU, Beaverton, OR). All animals were housed and bred under specific pathogen-free conditions at OHSU and experiments conducted under the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee approvals in accordance with all applicable federal, state, and local regulations. All WNV experiments were completed within a United States Department of Agriculture (USDA)–inspected Biosafety Level three facility.

**Virus, peptides, and cell lines.** WNV strains NY99, 31A, and 385–99 were used and all virus strains yielded similar results. WNV strains NY99 and 385–99 were gifts of R. Tesh (University of Texas Medical Branch, Austin, TX). Strain 31A was provided by the USDA. Peptides NS3 $_{1616}$, NS3 $_{2066}$, and NS4b $_{2488\text{-}2496}$ (21st Century Biochemicals) were diluted in 10% $H_2O$ and 90% DMSO and stored at −80°C. Vero, MC57g, and EL-4 cell lines were mycoplasma negative and were cultured under aseptic conditions with DME (VERO and MC57g) or RPMI (EL-4) supplemented with antibiotics and 5% fetal calf serum. MC57gs were infected using an MOI of 10 for 30 h before use for $^{51}$Cr assays. MC57gs were infected using an MOI of 10 for 30 h before use for $^{51}$Cr assays.

numbers for analysis, pools of two brains each were made and cells isolated using percoll gradient. The representation of cells using mononuclear/lymphocyte gate in representative adult (left) and old (middle) mice are shown. The aggregate analysis of cell percentages in this gate are shown on the right, illustrating that there are significantly fewer cells in the lymphocyte gate in old mice. Comparison is based on at least 5 × $10^5$ collected events/sample. (C) Representation (left) and aggregate analysis (right) of the percentage of CD4 and CD8 T cells within the brain of old versus adult mice as determined by flow cytometry. Significant differences were seen in representation of these cell subsets as well. (D, left) Representation of CD8 NS4b-2488 tetramer$^+$ T cells among all CD8 T cells in the brains of old mice is further reduced compared with adults (P = 0.00372). (D, right) Illustration of cumulative effects of reduced representation of total lymphocytes, CD8 cells, and CD8 NS4b-2488 Tet$^+$/GrB$^+$ T cells in the brains of old mice compared with adults (taken as 100%) reveal an ∼12× age-related difference. Experiment is representative of three independent experiments. Horizontal bars indicate mean values of the unpaired Student's *t* test. Error bars represent SEM.

**Flow cytofluorometric analysis and intracellular cytokine staining.** Cytokine-producing T cells were detected using the Cytofix-Cytoperm kit (BD). Single-cell splenocyte suspension was depleted of red blood cells and was incubated with 1 μM of peptide or infected with WNV in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich) for 6 h at 37°C. After 6 h, the cells were washed and blocked with Fc block (anti–mouse FcγRI/III; BD) and incubated overnight in the presence of a saturating dose of surface antibodies against CD8, CD3, CD4, CD11a, CD43 (clone 1B11), CD44, and CD62L (BD, eBioscience, and BioLegend). After washing, the cells were fixed and permeabilized, and intracellular antibodies (anti–IFN-γ or anti–IL-2; eBioscience) were added for 30 min. The samples were then washed and analyzed using either a FACSCalibur or LSR II cytometer (BD) instrument. GzB (clone gb12; Invitrogen) intracellular staining was completed directly ex vivo with no stimulation.

**IFN-I bioassay.** Serum levels of IFN-I were measured using a bioassay. Type I IFN standards (National Institute of Allergy and Infectious Diseases international standard) and experimental sample mouse serum were serially diluted twofold down a flat-bottom 96-well tissue culture–treated plate in complete media, 10% FBS, Pen/Strep, and DME. IFN-responsive L929 cells were plated at $5 \times 10^4$ cells/well, incubated overnight with the serum, and media aspirated, and media containing 5 PFU VSV Indiana was added to each well, except for control wells. 24 h later, media was aspirated, the plate washed twice with PBS, and the monolayer fixed with 5% formaldehyde, incubated for 10 min, and stained with 0.05% crystal violet for 10 min. Washed monolayers were allowed to dry, and 100% methanol was used to elute the dye. Absorbance is measured at 595 nm on an ELISA plate reader (Invitrogen). Experimental samples are compared with the standard curve to determine endpoint values.

**Infection and CTL analysis.** Mice were infected i.p. with 0.16–20 PFU of WNV virus or s.c. with 20–1,200 PFU per mouse as denoted in the figure legends. At indicated times after infection, lymphocytes were isolated and used for direct flow cytometry analysis, direct ex vivo restimulation for cytokine production, and direct ex vivo $^{51}Cr$ assay. Percentage of specific lysis was calculated as $[(E-S)/(M-S)] \times 100$, where E equals the counts per minute released from targets incubated with lymphocytes, S equals the counts per minute released from target cells incubated with no lymphocytes, and M equals the counts per minute released from cells after lysis with 1% Nonidet P40 (Affymetrix).

**Lymphocyte purification and adoptive transfer.** Old and adult T cells from corresponding mouse populations were enriched by positive selection of CD4 and/or CD8 T cells using MACS separation (Miltenyi Biotec) in accordance with manufacturer's protocol. Purity of obtained cells was 90–95% $CD8^+/CD4^+$ as determined by flow cytometry, and the opposite subset and B cells were present at <0.5% in all transferred populations. $2–10 \times 10^6$ cells were injected i.v. and engraftment success was evaluated by flow cytometry 24 h later at the time of infection.

**Survival experiments.** Animals were challenged with no other manipulations or 24 h after cell transfer with indicated doses and routes of WNV. 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 d, at which point the experiment was discontinued. Data are shown as percentage of survival at the termination of the experiment.

**Determination of viral burden.** Animals were sacrificed and liver, spleen, brain, and kidney were removed and homogenized in RPMI using a bead-beater-96 (BioSpec). Samples were spun at 2,000 rpm for 10 min at 4°C and aliquots of each sample were stored at −80°C. Viral titer was determined by plaque assay by serially diluting sample onto Vero cells. After co-culture of the virus with the cells for 2 h, agarose overlay was added. 2 d after the initial overlay, cells were overlayed with additional agarose containing Neutral red (0.2%). Plaques where then counted to determine viral load.

**Statistical analyses.** Fisher's exact test and Log-rank test were used to analyze results from survival experiments. Statistical significance of viral titer observed between groups was analyzed using a Mann Whitney *U* test. Other tests are as indicated. All calculations were performed using Prism software (GraphPad Software, Inc.).

**Online supplemental material.** Fig. S1 shows survival of adult and old BALB/c mice infected with WNV. Fig. S2 shows viral titer in the organs of adult and old mice at indicated days after administration of a WNV dose lethal to old but not adult mice. Fig. S3 shows IFN-I activity in the serum of old and adult mice. Fig. S4 shows the response of adult and old CD4 T cells to class II MHC-restricted WNV peptide epitopes. Fig. S5 shows that peptide sensitivity of 8-d-old and adult CD8 and CD4 T cells is largely superimposable. Table S1 shows that old mice exhibit increased WNV mortality regardless of route of injection or viral isolate. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090222/DC1.

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