

# Age-dependent susceptibility to a viral disease due to decreased natural killer cell numbers and trafficking

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**Although it is well known that aged hosts are generally more susceptible to viral diseases than the young, specific dysfunctions of the immune system directly responsible for this increased susceptibility have yet to be identified. We show that mice genetically resistant to mousepox (the mouse parallel of human smallpox) lose resistance at mid-age. Surprisingly, this loss of resistance is not a result of intrinsically defective T cell responses. Instead, the primary reason for the loss of resistance results from a decreased number of total and mature natural killer (NK) cells in the blood and an intrinsic impairment in their ability to migrate to the lymph node draining the site of infection, which is essential to curb systemic virus spread. Hence, our work links the age-dependent increase in susceptibility to a viral disease to a specific defect of NK cells, opening the possibility of exploring treatments to improve NK cell function in the aged with the goal of enhancing their resistance to viral diseases.**

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Abbreviations used: Ab, antibody; D-LN, draining LN; dpi, days post infection; ECTV, Ectromelia virus; GzB, granzyme B; ND-LN, non-D-LN; OPV, *Orthopoxvirus*; VACV, *vaccinia virus*.

Much of the work to date on resistance to viral disease during aging has compared immunological fitness of the young and aged by looking at their immune responses to vaccination, by challenging vaccinated mice with viruses, or by comparing susceptibility to suboptimal viral doses. These experimental models, however, do not reproduce the situation of increased susceptibility to viral disease after a primary infection (Murasko and Jiang, 2005). The genus *Orthopoxvirus* (OPV) includes viruses important to human health such as variola virus (the agent of smallpox), monkeypoxvirus, and the vaccine species *vaccinia virus* (VACV). Ectromelia virus (ECTV) is an OPV of the laboratory mouse. ECTV naturally penetrates the body through microabrasions in the skin of the footpad and rapidly becomes systemic by spreading to the blood via the lymphatic system (Esteban and Buller, 2005), a mechanism of spread which is common not only to pathogenic OPVs but also to many animal and human pathogens of diverse genii (Fields et al., 2007). After footpad inoculation of outbred mice and many inbred strains, such as BALB/c, ECTV rapidly spreads through the regional LNs to the blood to reach the liver and spleen. The uncontrolled massive replication in the liver results in a fatal disease: mousepox, the mouse parallel of human smallpox.

Human smallpox, a disease caused by the OPV variola virus, penetrated through the upper or lower respiratory tract to spread to the blood by way of the regional LNs (Fenner et al., 1988). The C57BL/6 (B6) and 129 mouse strains, known as naturally resistant to mousepox, also become infected but are able to control spread to and replication in the liver by rapidly mounting effective innate and adaptive immune responses that, together, control the virus (Esteban and Buller, 2005). Natural resistance to mousepox requires Type I and II IFNs (Jacoby et al., 1989; Karupiah et al., 1993; Xu et al., 2008), macrophages (Pang and Blanden, 1976; Tsuru et al., 1983; Karupiah et al., 1996), NK cells (Jacoby et al., 1989; Delano and Brownstein, 1995; Parker et al., 2007; Fang et al., 2008), CD8<sup>+</sup> T (T<sub>CD8+</sub>) cells and CD4<sup>+</sup> T (T<sub>CD4+</sub>) cells (Kees and Blanden, 1977; Karupiah et al., 1996; Fang and Sigal, 2006), and B cells (Fang and Sigal, 2005; Chaudhri et al., 2006; Panchanathan et al., 2008) but not NKT cells (Parker et al., 2007). The roles of the different components of the immune system are

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complementary. For example, NK cells rapidly migrate to the LN draining the primary site of infection (draining LN [D-LN]), peaking 2 d post infection (dpi). This process is important to curb virus spread before 3 dpi (Fang et al., 2008). In contrast, the  $T_{CD8+}$  cell response peaks in the D-LN 5 dpi and in the spleen 7 dpi. Still, death in the absence of NK cells or  $T_{CD8+}$  cells occurs 7–9 dpi. In contrast, death in the absence of B cells or antibodies (Abs) occurs much later, indicating that their major role is the long-term control of the virus (Fang and Sigal, 2005).  $T_{CD4+}$  cells are essential to provide help to B cells but may also have some important direct effector functions that participate in the early control of the virus because in the absence of  $T_{CD4+}$  cells, virus titers 7 dpi are higher than those in the absence of B cells or Abs (unpublished data), even though  $T_{CD8+}$  cells do not require  $T_{CD4+}$  cell help (Fang and Sigal, 2006).

## RESULTS

### B6 mice gradually lose their natural resistance to mousepox as they age

It is known that 56-wk-old outbred mice are more susceptible than 8-wk-old animals to the attenuated Hampstead ECTV strain (Fenner, 1949b). To test whether age can affect natural resistance to mousepox, we infected B6 mice of both sexes and increasing age with WT ECTV (virulent Moscow strain, herein referred to as ECTV) in the footpad and found that the resistance started to wane at a relatively early age ( $\sim 6$  mo) and was completely lost when the mice reached 14 mo of age ( $\sim 60\%$  of life span, hence mid-age; Fig. 1 A). Consistent with these results, mid-aged B6 mice (14–18 mo, herein referred to as aged for simplicity) demonstrated the massive depletion of splenocytes that is typical of acute mousepox in susceptible strains such as BALB/c mice, as opposed to the increased cellularity that is characteristic of WT ECTV-infected young B6 mice (Fig. 1 B). Moreover, aged B6 mice had an  $\sim 10^3$ -fold increase in virus titers in the spleen and liver 7 dpi as compared with young ( $\sim 2$  mo old) B6 mice (Fig. 1 C). Although these experiments were performed with 3,000 PFU ECTV, we also observed 100% mortality in aged B6 mice infected

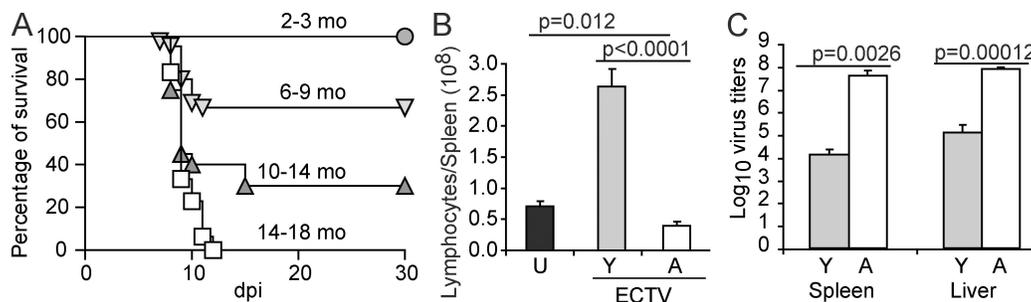
with 30 PFU (the minimal dose that we tested), suggesting that aged B6 mice are as susceptible to mousepox as young BALB/c mice (LD50,  $\sim 1$  PFU). From these results, we conclude that aged B6 mice lose the capacity to control ECTV replication and spread, resulting in lethal mousepox. Thus, infection of aged B6 mice with ECTV can serve as a tractable model to understand the mechanism involved in the increased susceptibility of the aged to viral diseases.

### Severely reduced $T_{CD8+}$ cell response to WT ECTV in aged mice

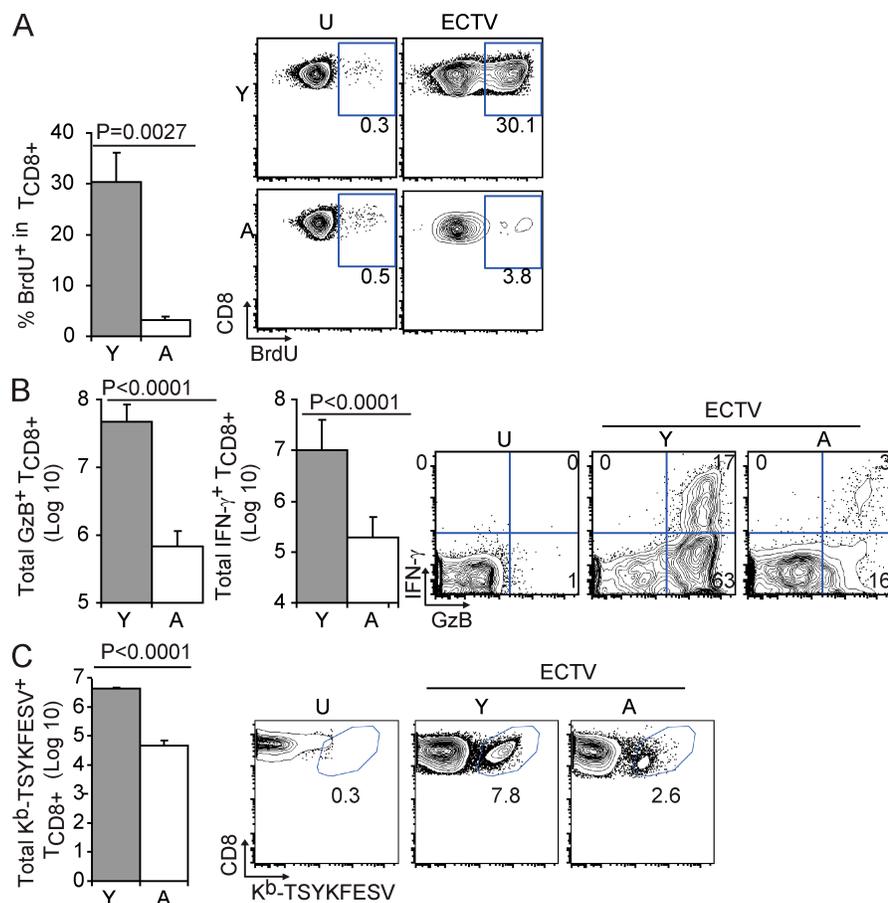
Because aging has been associated with poor antiviral T cell responses (Woodland and Blackman, 2006; Yager et al., 2008; Maue et al., 2009) and strong  $T_{CD8+}$  cell responses are essential for resistance to mousepox (Fang and Sigal, 2006), we compared the  $T_{CD8+}$  cell responses to ECTV in young and aged B6 mice. We found that the  $T_{CD8+}$  cells of aged mice proliferated poorly in vivo as measured by BrdU incorporation in the popliteal D-LN 5 dpi (Fig. 2 A) or in the spleens 7 dpi (not depicted). Moreover, aged mice infected with ECTV had a very significant decrease in the relative and absolute numbers of  $T_{CD8+}$  cells expressing granzyme B (GzB) and IFN- $\gamma$  after ex vivo restimulation for 5 h with infected cells when measured 5 dpi in the D-LN (not depicted) and 7 dpi in the spleen (Fig. 2 B). In agreement with this finding, aged mice also had a dramatic decrease in the relative and absolute numbers of  $T_{CD8+}$  cells restricted to the immunodominant determinant TSYKFESV (corresponding to aa 19–26 of the ECTV EVM158 protein as well as to aa 20–27 of the VACV B8R protein [Tschärke et al., 2005]) when determined 5 dpi in the D-LN (not depicted) and 7 dpi in the spleen (Fig. 2 C) using TSYKFESV-loaded H-2K<sup>b</sup> dimers (Dimer-X). Thus, aged mice lose the capacity to mount a strong  $T_{CD8+}$  cell response to WT ECTV.

### Aged mice mount normal $T_{CD8+}$ cell responses to nonvirulent OPVs or WT ECTV when its replication is curtailed

Although the experiments in the previous section demonstrated a severely defective  $T_{CD8+}$  cell response in aged mice,



**Figure 1. B6 mice gradually lose their natural resistance to mousepox as they age.** (A) B6 mice of the indicated ages were infected with WT ECTV in the footpad and survival was determined. 2–3 mo ( $n = 50$ ) versus 6–9 mo ( $n = 44$ ),  $P = 0.0205$ ; 2–3 mo versus 10–14 mo ( $n = 19$ ),  $P = 0.0001$ ; 2–3 mo versus 14–18 mo ( $n = 47$ ),  $P < 0.0001$ . Animals of both sexes were used. (B) Young (Y) and aged (A) B6 mice were infected with WT ECTV, euthanized 7 dpi, and the number of live cells/spleen was determined by trypan blue exclusion. Results for uninfected controls (U) are shown for comparison. Data correspond to the mean  $\pm$  SD of pooled organs of two to three mice per group from three individual experiments. (C) Young and aged B6 mice were infected with WT ECTV and euthanized 7 dpi, and virus titers in spleens and livers were determined by plaque assay. Data correspond to the mean  $\pm$  SD of pooled organs of two to three mice per group from three individual experiments.



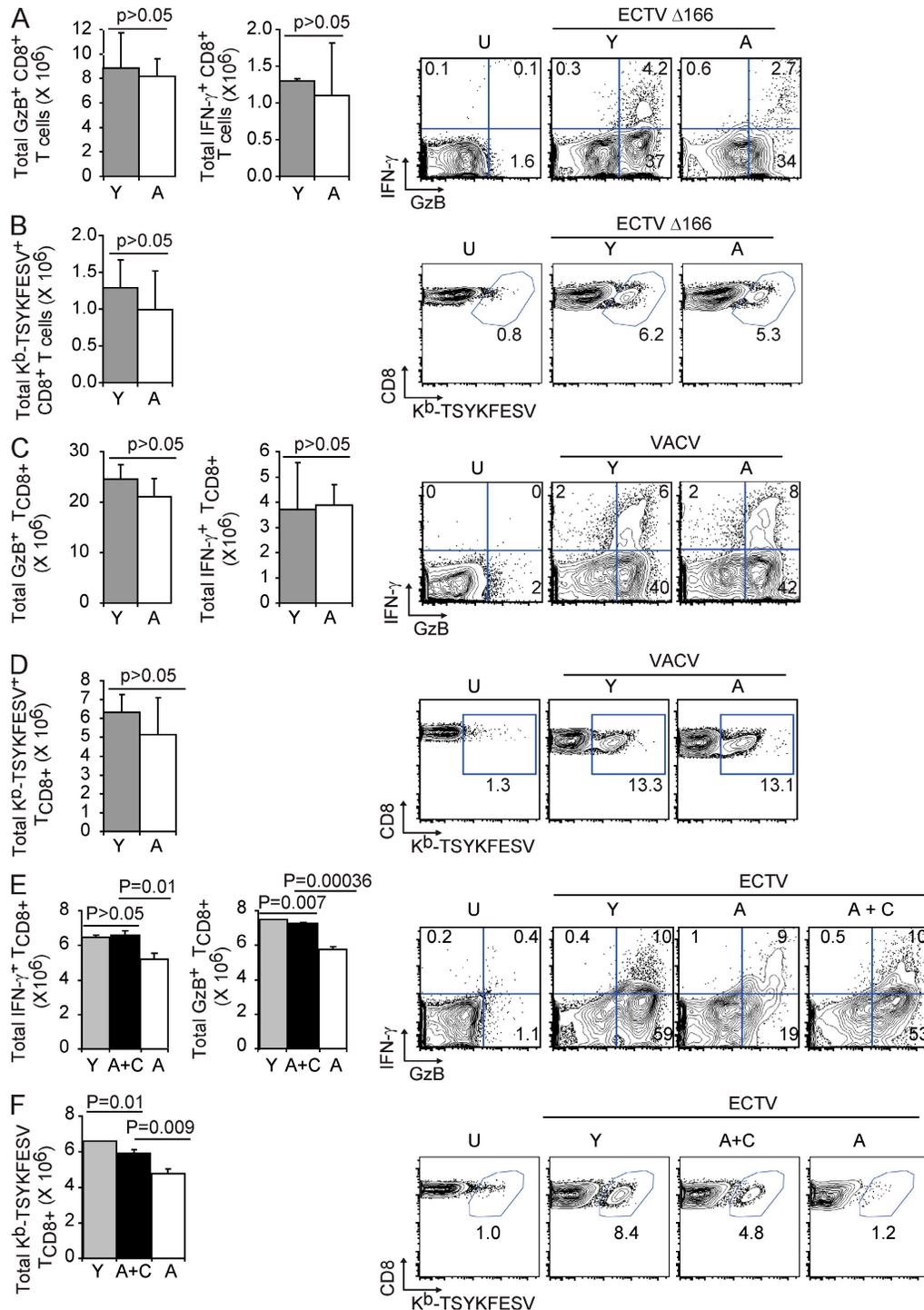
**Figure 2. Severely reduced T cell response to WT ECTV in aged mice.** (A) Young (Y) and aged (A) B6 mice were infected with 3,000 PFU WT ECTV. 5 dpi they were inoculated i.p. with 2 mg BrdU and euthanized 3 h later. D-LNs were collected, made into single cell suspensions, and stained for surface CD3 and CD8 and for intracellular BrdU. Column graphs indicate the percentage of T<sub>CD8</sub><sup>+</sup> cells in the D-LN that incorporated BrdU. Representative flow cytometry plots are shown together with those from uninfected controls (U) for comparison. Data are representative of three independent experiments. (B) Young and aged B6 mice were infected with 3,000 PFU WT ECTV and euthanized 7 dpi. Splenocytes were counted and restimulated with infected cells for 6 h with the addition of brefeldin A during the last hour. Next, the cells were stained for surface CD3 and CD8 and for intracellular IFN- $\gamma$  and GzB, followed by flow cytometry. Column graphs indicate the calculated absolute number of T<sub>CD8</sub><sup>+</sup> cells in the spleen that expressed GzB (left) or IFN- $\gamma$  (right). Representative flow cytometry plots are shown together with a plot corresponding to an uninfected control mouse for comparison. (C) As in B but showing the calculated absolute numbers and representative plots of CD8<sup>+</sup>K<sup>b</sup>-TSYKFESV<sup>+</sup> cells in the spleen. Data correspond to the mean  $\pm$  SD of pooled organs of two to three mice per group from five individual experiments.

they did not indicate whether the defect was T cell intrinsic. We therefore performed experiments to determine whether the T<sub>CD8</sub><sup>+</sup> cell response of aged mice was also impaired when mice were infected with nonlethal viruses. For this purpose, we infected young and aged mice with highly attenuated ECTV  $\Delta$ 166 in the footpad. This ECTV mutant was made avirulent by deletion of EVM166, the gene encoding the Type I IFN binding protein (Xu et al., 2008). Strikingly, the T<sub>CD8</sub><sup>+</sup> cell responses of aged and young mice to ECTV  $\Delta$ 166 were similar as determined by IFN- $\gamma$ , GzB, and K<sup>b</sup>-TSYKFESV dimer staining (Fig. 3, A and B). As an additional approach to compare the T<sub>CD8</sub><sup>+</sup> cell responses of young and aged mice to poorly pathogenic viruses, we infected mice i.p. with VACV (WR strain), which is only mildly pathogenic in mice but shares most of the T<sub>CD8</sub><sup>+</sup> cell determinants of ECTV including TSYKFESV (Tschärke et al., 2005). VACV was inoculated i.p. because it is a well established route of infection that we and others have frequently used to induce anti-VACV responses (Selin et al., 1998; Sigal et al., 1998, 1999; Basta et al., 2002; Shen et al., 2002; Quigley et al., 2008; Xu et al., 2010). Similar to the results with ECTV  $\Delta$ 166, aged mice mounted T<sub>CD8</sub><sup>+</sup> cell responses to VACV that were comparable to those of young mice (Fig. 3, C and D). It is important to stress that neither ECTV  $\Delta$ 166 nor VACV were lethal or produced overt disease in aged B6 mice (unpublished data).

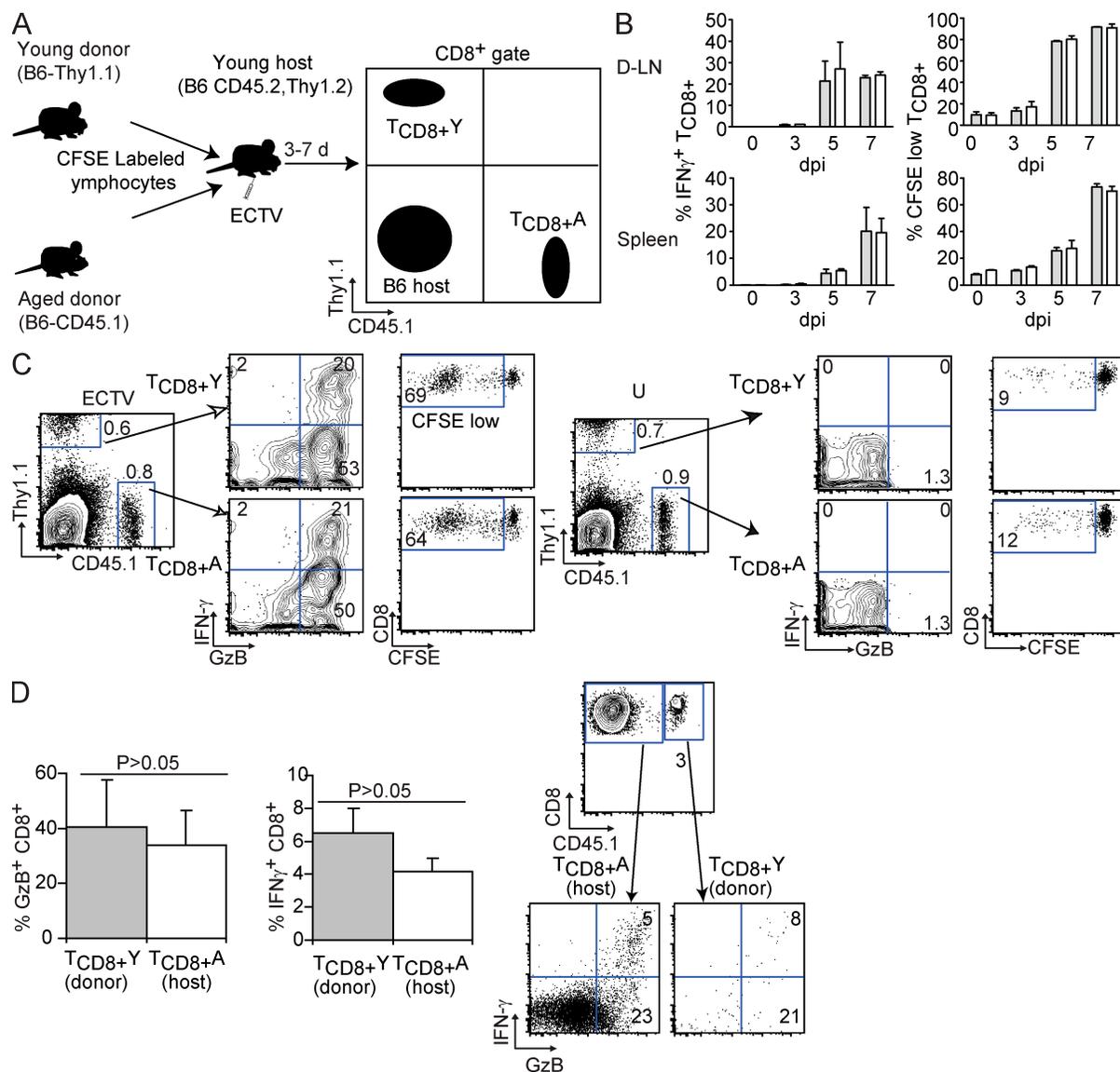
Because in vivo ECTV  $\Delta$ 166 and VACV replicate and disseminate much less efficiently than WT ECTV, we hypothesized that the failure of aged mice to mount T<sub>CD8</sub><sup>+</sup> cell responses to WT ECTV was the rapid and excessive replication of the WT virus. To test this hypothesis, we infected aged mice with WT ECTV and left them untreated as before or treated them 2 dpi with a single dose of 600  $\mu$ g cidofovir, a drug which inhibits OPV replication (Neyts and De Clercq, 1993; Bray et al., 2000; Smee et al., 2001) and prevents mousepox in aged mice (unpublished data). Remarkably, cidofovir-treated aged mice mounted T<sub>CD8</sub><sup>+</sup> cell responses comparable to those of young mice, indicating that uncontrolled viral replication is the major reason for the failure of the T<sub>CD8</sub><sup>+</sup> cell response of aged mice to WT ECTV (Fig. 3, E and F).

### The defective T<sub>CD8</sub><sup>+</sup> cell response of aged mice to ECTV is not T cell intrinsic

The data in the previous section strongly suggested that the inability of aged mice to mount a T<sub>CD8</sub><sup>+</sup> cell response to WT ECTV was T cell extrinsic. To formally test for this possibility, we compared the proliferation and activation of congenically marked CFSE-labeled T<sub>CD8</sub><sup>+</sup> cells from young B6-Thy1.1 and aged B6-CD45.1 mice after adoptive transfer into young B6 hosts (Th1.2 and CD45.2) as schematized in Fig. 4 A. We found that at various dpi, the T<sub>CD8</sub><sup>+</sup> cells from young and aged



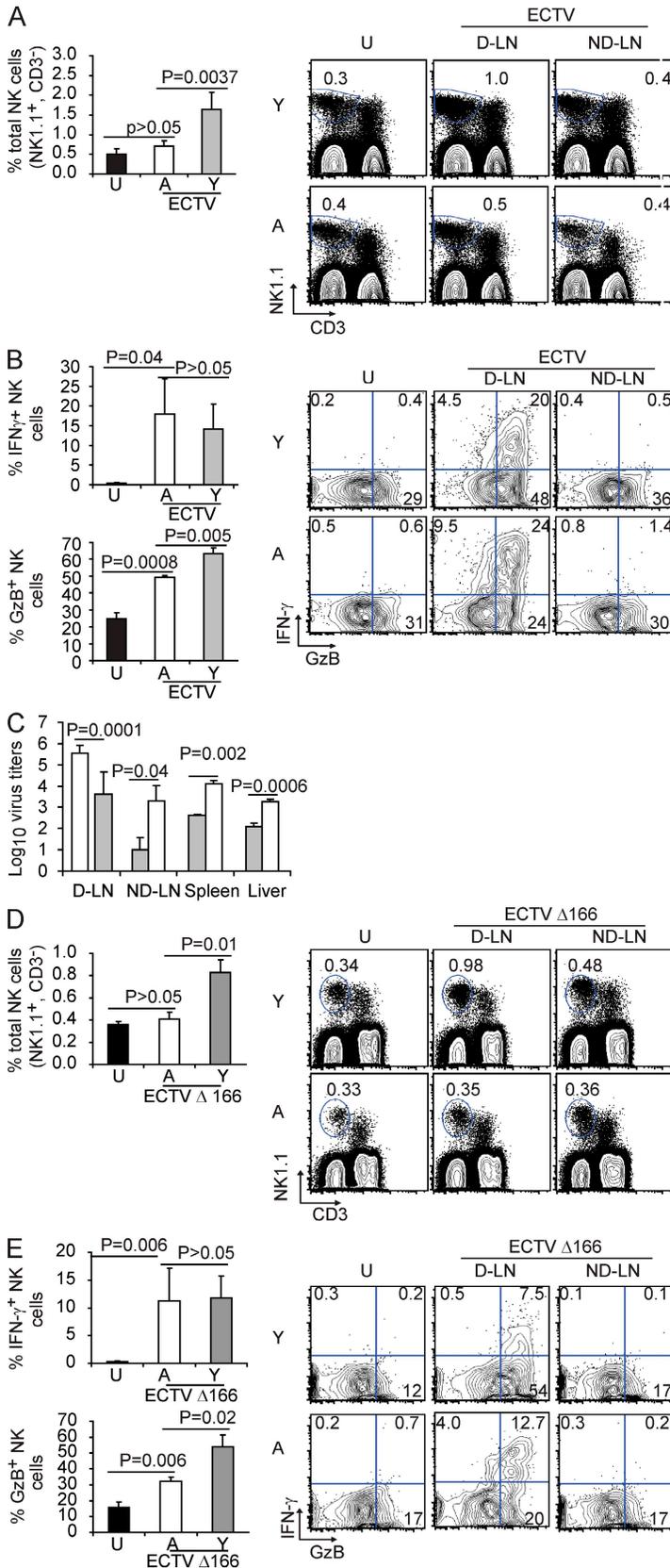
**Figure 3. Aged mice mount normal  $T_{CD8+}$  cell responses to nonvirulent OPVs or WT ECTV when its replication is curtailed.** (A) Young (Y) and aged (A) B6 mice were infected with 3,000 PFU ECTV  $\Delta 166$  and euthanized 7 dpi. Splenocytes were counted, restimulated with infected cells for 6 h with the addition of brefeldin A during the last hour, and stained for surface CD3 and CD8 and for intracellular IFN- $\gamma$  and GzB, followed by flow cytometry. Column graphs indicate the mean  $\pm$  SD for the calculated absolute number of  $T_{CD8+}$  cells in the spleen that expressed GzB (left) or IFN- $\gamma$  (right). Representative flow cytometry plots are shown together with a plot corresponding to an uninfected control (U) mouse for comparison. (B) As in A but showing the mean  $\pm$  SD of the calculated absolute numbers and representative plots of  $CD8+K^b$ -TSYKFESV $^+$  cells in the spleen. Data correspond to the mean  $\pm$  SD of six individual mice from two independent experiments. (C) As in A but infected with  $10^6$  PFU VACV i.p. (D) As in B but infected with VACV i.p. Data correspond to the mean  $\pm$  SD of six individual mice from three experiments. (E) As in A but infected with WT ECTV and, when indicated, aged mice were treated 2 dpi with 600  $\mu$ g cidofovir (A+C) i.p. (F) Splenocytes from mice treated as in E were analyzed as in B. Data correspond to the mean  $\pm$  SD of six individual mice from two experiments.



**Figure 4. The defective T<sub>CD8+</sub> cell response of aged mice to ECTV is not T cell intrinsic.** (A) Experimental design for the adoptive transfer of CFSE-labeled lymphocytes from young (B6-Thy1.1) and aged (B6-CD45.1) donor mice into young B6 (B6 CD45.2-Thy1.2) hosts followed by ECTV infection and flow cytometry analysis gating on CD8<sup>+</sup> cells. (B) B6 mice, as in A, were euthanized on the indicated dpi. Cells from the D-LN and spleen were restimulated ex vivo for 5 h with infected cells in the presence of brefeldin A and stained for surface CD3, CD8, Thy1.1, and CD45.1 and for intracellular IFN- $\gamma$  and GzB, followed by flow cytometry. Top graphs show the data for the D-LN and lower graphs for the spleen. Columns show the percentage of T<sub>CD8+</sub>Y (gray bars) or T<sub>CD8+</sub>A (white bars) cells that produced IFN- $\gamma$  (left) or that proliferated as indicated by CFSE dilution (right). Data correspond to the mean  $\pm$  SD of pooled organs of three mice per group from three individual experiments. (C) Representative plots from B corresponding to spleens of individual mice 7 dpi. Plots corresponding to an uninfected control mouse are also shown for comparison. Plots were gated on CD8<sup>+</sup> cells. (D) Aged B6 mice were adoptively transferred with lymphocytes from young B6-CD45.1 and infected with ECTV in the footpad. 7 dpi, the host (T<sub>CD8+</sub>A) and donor (T<sub>CD8+</sub>Y) T<sub>CD8+</sub> cell responses were determined in spleens. Representative plots are shown. Data correspond to the mean  $\pm$  SD of pooled organs of three mice per group from three individual experiments.

donor mice responded to WT ECTV with similar efficiency in the young environment, as revealed by activation determined by IFN- $\gamma$  and GzB staining and proliferation detected by CFSE dilution (Fig. 4, B and C). Similar results were obtained with the reciprocal transfer of aged B6-Thy1.1 and young B6-CD45.1 into young B6 hosts (Fig. S1). Conversely, the T<sub>CD8+</sub> cells from young B6-CD45.1 mice responded as

poorly as the recipients' endogenous T<sub>CD8+</sub> cells in aged B6 mice (Fig. 4 D), and a similar result was obtained with reciprocal transfer of T<sub>CD8+</sub> cells from young B6 mice into aged B6-CD45.1 mice (not depicted). It is of note that, similar to B6 mice, congenic B6-Thy1.1 and B6-CD45.1 mice are resistant to mousepox when young and susceptible when aged (unpublished data). Collectively, these data demonstrate that

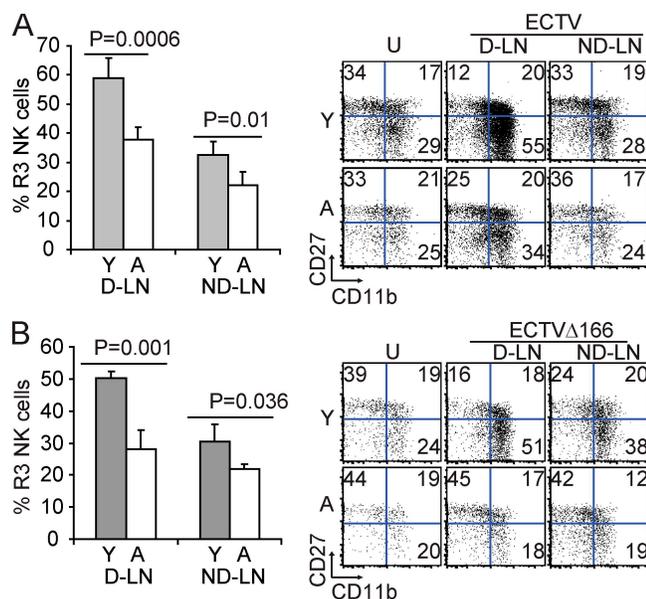


**Figure 5. Deficient NK cell response to WT and attenuated ECTV in aged mice.** (A) Young (Y) and aged (A) mice were infected with 3,000 PFU WT ECTV or left uninfected (U) and euthanized 2 dpi. D-LNs were made into single cell suspensions, incubated with brefeldin A for 1–2 h, and stained for surface NK1.1 and CD3 and for intracellular IFN- $\gamma$  and GzB. Column graph indicates mean  $\pm$  SD for the proportion of NK cells identified as NK1.1+ and CD3-. Representative flow cytometry plots are also shown. (B) The samples in A were gated on NK cells and analyzed for expression of IFN- $\gamma$  and GzB. Data correspond to the means  $\pm$  SD for the proportion of NK cells expressing IFN- $\gamma$  or GzB. Representative flow cytometry plots are also shown. Data are representative of at least five independent experiments. (C) Young and aged mice were infected with 3,000 PFU WT ECTV and euthanized 3 dpi. Virus titers in the indicated organs were determined by plaque assay. Gray bars, young mice; white bars, aged mice. Data correspond to the mean  $\pm$  SD of six individual mice from two individual experiments. (D) As in A, but the mice were infected with ECTV  $\Delta$ 166. (E) As in B, except for the mice infected with ECTV  $\Delta$ 166 as in D. Data are representative of five independent experiments.

the T<sub>CD8+</sub> cells of aged mice are capable of responding to ECTV but that extrinsic factors in the aged environment affect their response to rapidly replicating WT ECTV. However, this defect is lost during infections with viruses that replicate and disseminate less efficiently in vivo or when viral replication of the WT ECTV is purposely curtailed.

**Deficient NK cell response to WT and attenuated ECTV in aged mice**

The data in the previous section suggested that the defect in the T cell responses of aged mice to WT ECTV could be a result of inefficient early control of virus replication and spread. We have previously shown that after footpad infection with ECTV, NK cells increase their numbers in the popliteal D-LN of young B6 mice 2 dpi. This process is essential to curb early systemic virus spread through efferent lymphatics and for resistance to mousepox (Fang et al., 2008). We therefore hypothesized that a defective NK cell response in aged mice could lead to the inefficient control of early replication and spread of the WT ECTV. Thus, we analyzed the NK cell responses to ECTV 2 dpi and found that, in sharp contrast to young control mice, the NK cells did not increase in the D-LN of aged mice relative to the inguinal non-D-LN (ND-LN) or the LNs of uninfected controls (Fig. 5 A). Moreover, fewer NK cells expressed GzB in the D-LNs of aged mice as compared with the D-LNs of young mice, although no differences were found in the proportion of cells that expressed IFN- $\gamma$  (Fig. 5 B). Experiments at later times showed that the proportion of NK cells in the D-LN of aged mice increased 3 dpi but significantly less than in young mice (unpublished data). Moreover, the total cell counts in



**Figure 6. Mature NK cells fail to accumulate in the D-LN of aged mice.** (A) Young and aged mice were infected with 3,000 PFU WT ECTV and euthanized 2 dpi. D-LN and ND-LN were made into single cell suspension and surface stained for CD3, NK1.1, CD11b, and CD27. Column graphs show the means  $\pm$  SD for the proportion of cells within the NK1.1<sup>+</sup>CD3<sup>-</sup> population (NK cells) that were CD11b<sup>+</sup> and CD27<sup>-</sup> (mature R3 cells). Gray columns, young mice; white columns, aged mice. Representative flow cytometry plots are shown with those from uninfected control mice for comparison. (B) As in A, but the mice were infected with ECTV  $\Delta$ 166. Data correspond to three experiments with three mice/group.

the D-LNs of aged mice were lower than in young mice (unpublished data). Also consistent with a defective NK cell response (Fang et al., 2008), 3 dpi the ND-LNs, livers, and spleens from aged mice had significantly higher virus titers than those from young mice (Fig. 5 C). In additional experiments, we found that the NK cell response was still decreased in aged mice infected with ECTV  $\Delta$ 166 (Fig. 5, D and E), indicating that, unlike the T<sub>CD8+</sub> cell response, the defect of the NK cell response was independent of the virulence of the virus. Importantly, because ECTV  $\Delta$ 166 is highly attenuated in aged mice (not depicted) this data also indicates that a fully functional NK cell response is necessary for resistance to highly virulent but not to attenuated viruses.

#### Mature NK cells fail to accumulate in the D-LN of aged mice

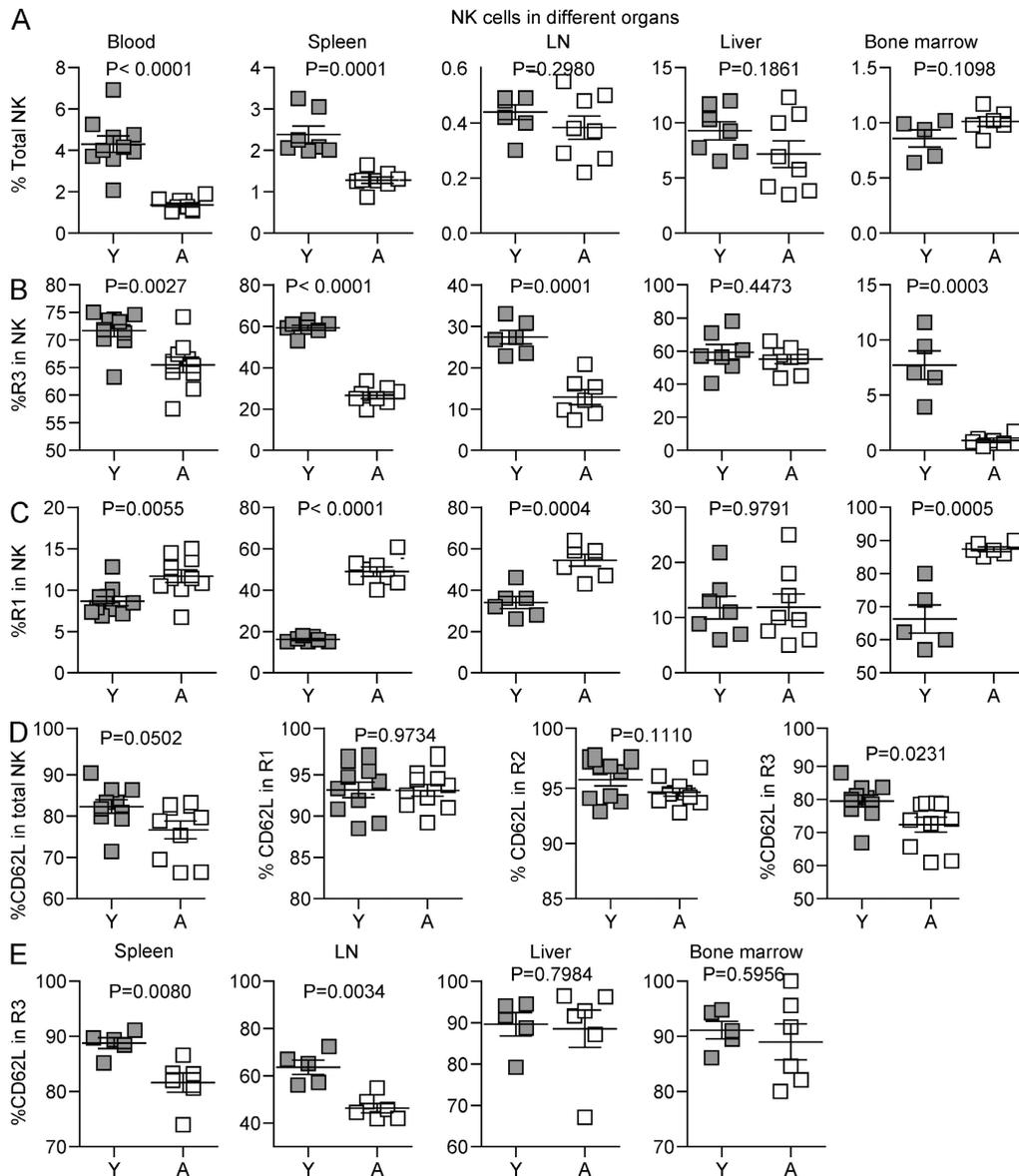
NK cells are subdivided into three functionally distinct maturation compartments according to their surface expression of CD27 and CD11b. R1 NK cells (CD27<sup>+</sup> and CD11b<sup>-</sup>) are the less mature, R2 NK cells (CD27<sup>+</sup> and CD11b<sup>+</sup>) are intermediate, and R3 NK cells (CD27<sup>-</sup> and CD11b<sup>+</sup>) are the most mature (Hayakawa and Smyth, 2006) and the most cytolytic (Chiossone et al., 2009). We have previously shown that the increase in the proportion of NK cells and most of the GzB expression in the D-LN of ECTV-infected mice occurs mostly in the mature R3 population (Fang et al., 2008). When we compared the proportion of R1-R3 NK cells in

LN of uninfected and infected (2 dpi) aged and young mice, we found significantly fewer R3 cells in the D-LN of aged mice infected with WT ECTV (Fig. 6 A) or ECTV  $\Delta$ 166 (Fig. 6 B). Hence, mature R3 NK cells fail to accumulate in the D-LN of aged mice, which may allow early virus spread resulting in lethal mousepox.

#### Alterations in number and phenotype of NK cells in various organs of aged mice

We have previously shown that the increase of NK cells in the D-LN 2 dpi is a result of recruitment and not proliferation (Fang et al., 2008). When we compared numbers of NK cells in the blood of young and aged naive mice (Fig. 7 A), we found that their proportion was significantly diminished in aged mice, resulting in a decrease in absolute numbers because the white blood cell counts were similar in young and aged mice (not depicted). In addition, we found that the relative (and absolute) number of total NK cells was also significantly reduced in the spleen of aged mice. In contrast, the relative numbers of total NK cells remained the same in LNs, liver, and bone marrow (Fig. 7 A). The reduced total number of NK cells in the blood and spleen of aged mice strongly suggested that the cause of decreased accumulation of NK cells in the D-LN might be an insufficient amount of NK cells capable of migrating from the blood. However, in addition to this quantitative total NK cell defect, aged mice had a significant decrease in the proportion R3 NK cells in the blood, spleen, and LNs but not in the liver. The deficit in R3 cells was even more pronounced in the bone marrow, where R3 NK cells were almost absent in aged mice (Fig. 7 B). This suggested that the reduced number of NK and R3 NK cells in the blood, spleen, and LNs may reflect a generalized defect in NK cell maturation in the bone marrow of aged mice. All the changes in the R3 populations were accompanied by a reciprocal increase in the immature R1 NK cells (Fig. 7 C). No major changes were seen in the R2 NK cells (unpublished data).

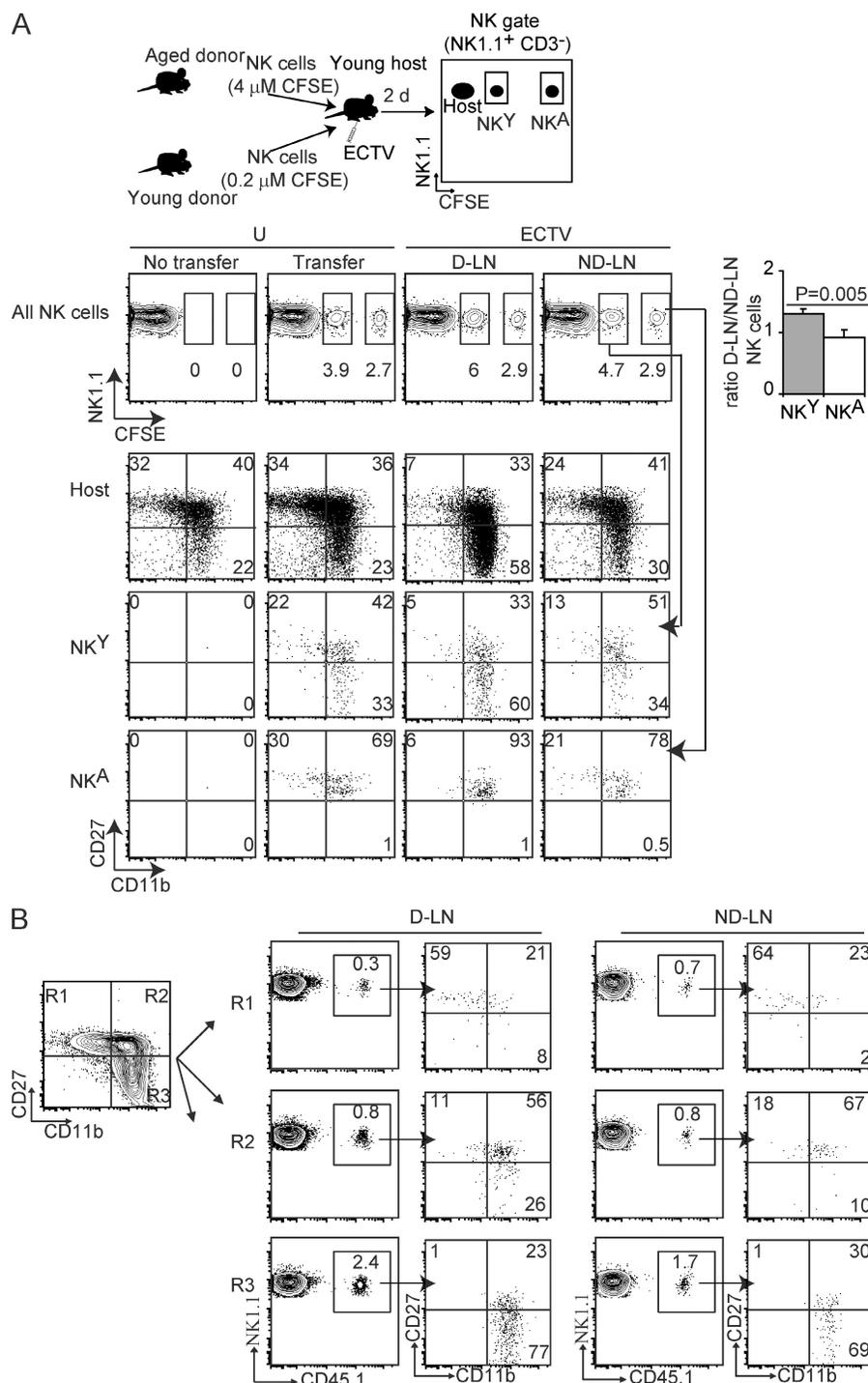
It has also been shown that NK cells rapidly migrate from the blood to inflamed LNs by means of CD62L (L-selectin) interacting with L-selectin ligands on high endothelial venules (Chen et al., 2005). Therefore, we compared CD62L expression in blood NK cells from young and aged naive mice. When total NK cells were analyzed, there was a trend but not significant reduction in the proportion of CD62L<sup>+</sup> cells in aged mice. However, when analyzed by maturation stages, the proportion of R3, but not of R1 or R2, NK cells in the blood of aged mice that expressed CD62L was significantly reduced (Fig. 7 D). CD62L was also decreased in the R3 NK cells from the spleen and LNs but not from liver and bone marrow (Fig. 7 E). No differences were found in the expression of other molecules known to affect lymphocyte homing to LNs or sites of infection, such as CCR2, CCR7, and CXCR3 as determined by flow cytometry. In addition, microarray analysis of expressed genes in blood R3 NK cells from young and aged mice did not provide any leads to identify additional molecular deficits in the aged R3 NK cell population (unpublished data).



**Figure 7. Alterations in number and phenotype of NK cells in various organs of aged mice.** (A) Cells from the indicated organs from young and aged naive mice were stained for surface CD3, NK1.1, CD27, CD11b, and CD62L and analyzed by flow cytometry. For blood, mononuclear cells were isolated by ficoll-hypaque centrifugation. For the liver, mononuclear cells were isolated by centrifugation in a Percoll gradient. Data are representative of two to four similar experiments. P-values are for two-tailed Student's *t* tests. (B) As in A, but displaying the proportion of R3 cells. (C) As in A, but displaying the proportion of R1 cells in the total NK cell population. (D) As in A, but displaying the proportion of the indicated NK population in the blood that was CD62L<sup>+</sup>. (E) As in A, but displaying the proportion of R3 NK cells in the indicated organs that was CD62L<sup>+</sup>. P-values are for two-tailed Student's *t* tests. For all panels, the horizontal bars indicate the mean and the vertical bars the SEM.

Because we previously found that NKG2D is important for resistance to mousepox (Fang et al., 2008), we also compared NKG2D expression in the R1-R3 NK cells in D-LN, spleen, and liver of uninfected and infected young and aged mice (2 dpi for the LN and 5 dpi for liver and spleen). We found that most NK cells expressed NKG2D but with significantly decreasing proportion of NKG2D<sup>+</sup> cells as the NK cells matured. As we showed before for all NK cells (Fang et al., 2008), infection significantly increased the proportion of NKG2D<sup>+</sup> cells in all maturation stages to >90%. In addition,

most IFN- $\gamma$ -producing cells coexpressed NKG2D. However, no significant differences were found between young and aged mice in the levels of NKG2D expression whether infected or not (Fig. S2). Thus, although NKG2D is an important factor for the resistance of young B6 mice to mousepox (Fang et al., 2008), aged mice are not different from young mice in terms of NKG2D expression under normal conditions or during infection. Whether the up-regulation of NKG2D is important for the resistance of young B6 mice to mousepox remains to be explored.



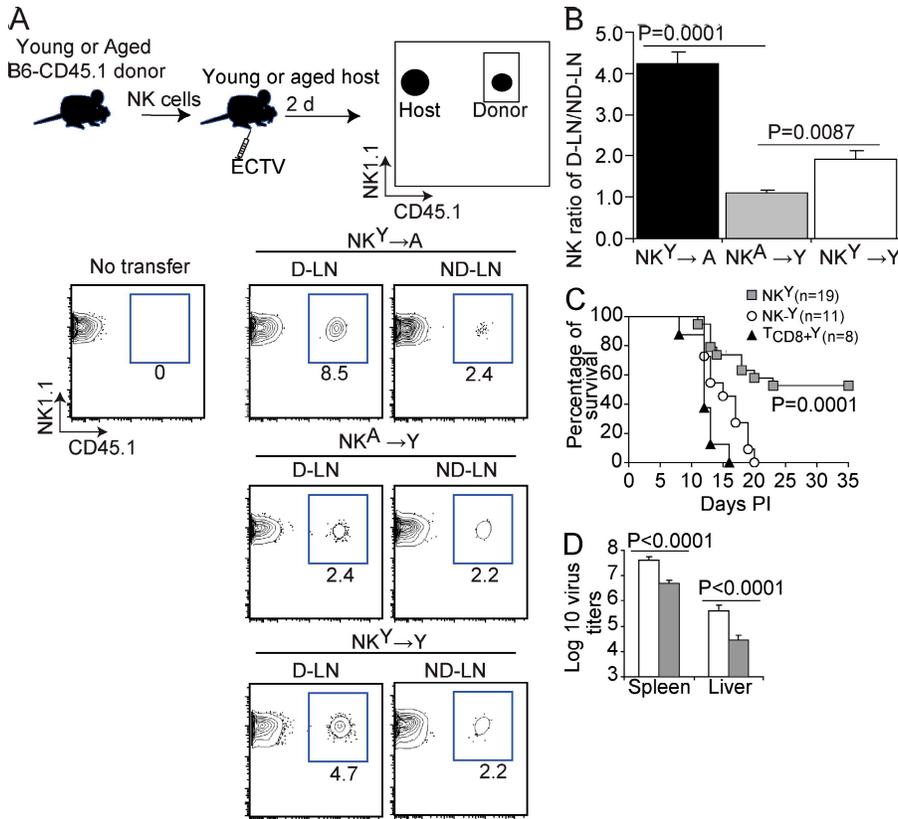
**Figure 8. The mature R3 NK cells from aged mice are intrinsically defective in their ability to home to the D-LN.** (A) Purified NK<sup>A</sup> and NK<sup>Y</sup> cells were labeled with 4 μM and 0.2 μM CFSE (to allow detection of both populations in the same mouse) and cotransferred in equal numbers into young B6 mice. 1 d later, the mice were infected with ECTV and the NK cell responses determined on 2 dpi as indicated at the top of the figure. The top shows representative flow cytometry data gated on the NK1.1<sup>+</sup>CD3<sup>-</sup> NK. The bar graph on the right shows mean ± SD for the ratio in D-LN versus ND-LN for NK<sup>Y</sup> and NK<sup>A</sup> cells. Top middle panels are gated on CFSE<sup>-</sup> host NK cells. Bottom middle panels are gated on transferred NK<sup>Y</sup> cells. Bottom panels are gated on transferred NK<sup>A</sup> cells. Data are representative of three similar experiments. (B) R1-R3 NK<sup>Y</sup> cells from B6-CD45.1 mice were sorted with a flow cytometer (left) and adoptively transferred into young B6 (CD45.2) mice. The next day, the mice were infected with 3,000 PFU ECTV and the migration of the transferred cells to D-LN and ND-LN was determined 2 dpi. The left plot shows the NK<sup>Y</sup> cells before sorting. For each type of LN, the plots on the left were gated on NK1.1<sup>+</sup>CD3<sup>-</sup> NK cells and the box shows the transferred cells. For each type of LN, the top, middle, and bottom panels are for mice transferred with R1, R2, and R3 cells, respectively. Note the relative increase of R3 cells in the D-LN as compared with the ND-LN. Data are representative of three similar experiments.

(NK<sup>A</sup>, 4 μM CFSE) mice into young hosts (Fig. 8 A, top), more NK<sup>Y</sup> cells migrated to the D-LN, as compared with the ND-LN of infected mice (or the LNs of uninfected control recipients). In contrast, fewer NK<sup>A</sup> cells migrated to the LNs in general, and there was no difference in the proportion that migrated to the D-LN as compared with the ND-LN of infected mice (or the LN of uninfected mice; Fig. 8 A, top and bar graph). Interestingly, the majority of NK<sup>Y</sup> cells in the D-LN were of R3 phenotype and R2 in ND-LN or the LN of uninfected mice, whereas

### The mature R3 NK cells from aged mice are intrinsically defective in their ability to home to the D-LN

The data in the previous section strongly suggested that in addition to reduced numbers in the blood, the defective recruitment of NK cells to the D-LNs of aged mice is partly a result of intrinsic NK cell trafficking defects. This was indeed the case because when we cotransferred equal numbers of CFSE-labeled NK cells from young (NK<sup>Y</sup>, 0.2 μM CFSE) and aged

the NK<sup>A</sup> cells were mostly R2 regardless of the LN or infection status (Fig. 8 A, middle and bottom). The almost complete absence of mature R3 NK<sup>A</sup> cells in the D-LN of infected mice could be a result of defective migration of R3 NK<sup>A</sup> cells from the blood to the D-LN or of the R2 NK<sup>Y</sup> cells, but not the R2 NK<sup>A</sup> cells, differentiating into R3 cells after migration into the D-LN. To test whether NK<sup>Y</sup> cells differentiate in the



**Figure 9. The NK cells from young mice preferentially migrate to the D-LN to reduce virus spread and protect aged mice from mousepox.** (A) NK<sup>Y</sup> and NK<sup>A</sup> from B6-CD45.1 mice were transferred into aged or young B6 (CD45.2) mice. The next day, the mice were infected with 3,000 PFU ECTV and the transferred NK cells were determined 2 dpi in the LNs. Data are representative of three experiments. (B) Ratio of NK<sup>Y</sup> and NK<sup>A</sup> cells that migrate to the D-LN versus the ND-LN in young and aged mice. (C) Aged B6 mice were inoculated with 6–8 × 10<sup>6</sup> NK<sup>Y</sup> cells, T<sub>CD8+</sub>Y cells, or NK cell-depleted splenocytes from young mice (NK<sup>-Y</sup>) and infected with 50 PFU ECTV. Mice were observed daily for signs of disease and death. Data combines four individual experiments. Statistical analysis was obtained with the Log-Rank test. (D) Aged B6 mice were inoculated with 5 × 10<sup>6</sup> purified T<sub>CD8+</sub>Y cells (white columns) or NK<sup>Y</sup> cells (gray columns) and infected with 50 PFU ECTV. Virus titers in these were determined in the indicated organs 5 dpi. Data correspond to the mean ± SD of five individual mice per group.

D-LN of infected mice, we purified R1, R2, and R3 NK<sup>Y</sup> cells from B6-CD45.1 congenic mice and transferred them to a young B6 host that was infected with ECTV 1 d later. 2 dpi, most of the NK<sup>Y</sup> cells maintained their input phenotype rather than differentiating into other subpopulations in the D-LN of the young infected host (Fig. 8 B). Thus, the lack of mature R3 NK<sup>A</sup> cells in the D-LN observed in Fig. 8 A was a result of the intrinsic defective migration of the R3 NK<sup>A</sup> cells rather than deficient differentiation from less mature NK cells within the D-LN.

**The NK cells from young mice preferentially migrate to the D-LN to reduce virus spread and protect aged mice from mousepox**

We next tested whether the aged environment could affect the migration of NK cells. For this purpose, we transferred purified NK<sup>Y</sup> or NK<sup>A</sup> cells from CD45.1 mice into aged or young hosts and determined their migration to the D-LN and ND-LN 2 dpi. We found that the transferred NK<sup>Y</sup>, but not NK<sup>A</sup>, cells preferentially migrated to the D-LN of aged mice, reaching a proportion that was higher than that in the D-LN of young recipient mice (Fig. 9, A and B), most likely reflecting decreased competition for space with the endogenous NK cells. These results also excluded environmental defects in the recruitment of mature NK cells into the aged D-LNs, such as decreased expression of CD62L ligands by the high endothelial venules or defective chemokine production. Therefore, from these experiments we conclude that fully mature endogenous

NK cells fail to migrate to the D-LN of aged mice in response to ECTV infection as a result of the compounded effects of decreased numbers in the blood and an intrinsic trafficking defect that may include decreased expression of CD62L.

Given that NK<sup>Y</sup> cells migrated to the D-LN of aged mice, we investigated whether adoptive transfer of NK<sup>Y</sup> cells could protect aged mice from mousepox. For this purpose, we transferred ~5 × 10<sup>6</sup> NK<sup>Y</sup> cells, NK-depleted spleen cells from young donors (NK<sup>-Y</sup>), or T<sub>CD8+</sub>Y cells into aged hosts and challenged them with ECTV. Strikingly, the aged mice that received NK<sup>Y</sup> cells, but not those which received T<sub>CD8+</sub>Y cells or NK<sup>-Y</sup> splenocytes, were significantly protected from mousepox (Fig. 9 C). In addition, aged mice that received NK<sup>Y</sup> cells had significantly decreased virus spread to liver and spleen 5 dpi as compared with aged T<sub>CD8+</sub>Y cell recipients (Fig. 9 D), aged recipients of NK cell-depleted splenocytes from young mice, or aged mice that did not receive cells (not depicted).

**DISCUSSION**

The work presented in this paper demonstrates for the first time that mice genetically resistant to mousepox when young become highly susceptible as they age. It is of interest that complete loss of resistance begins when mice are of mid-age rather than old, which is similar to what was observed during smallpox infections in humans (Fenner et al., 1988). More importantly, our work also reveals that the reason for this loss of resistance is the inability to control early virus spread as a consequence of a significant decrease in the migration of mature NK cells to the D-LN. This deficiency appears to be the

result of a decreased absolute number of NK cells in the blood of aged mice, the key R3 population in particular, and to additional defects that impair their migration, such as decreased CD62L expression. Interestingly, we also found a very significant reduction in the R3 NK population in the bone marrow of aged mice, suggesting that a generalized deficiency in NK cell maturation in the bone marrow may be the cause for the decrease in R3 cells in the blood and other organs such as the spleen. Furthermore, our demonstration that NK cells obtained from young mice, but not other cells, can preferentially migrate to the D-LN of aged mice and protect them from mousepox indicates that the decreased numbers and trafficking defects of NK is the major reason for the susceptibility of aged mice to mousepox. It is relevant to note that we performed the reconstitution experiments with NK cells purified with an anti-CD45b Ab (DX5). Even though CD45b is also present in NKT cells, it is known that these cells are dispensable for resistance to mousepox (Parker et al., 2007). Thus, the interpretation of our results is strongly supported. To our knowledge this is the first description of a specific age-related immune dysfunction resulting in increased susceptibility to viral disease.

Interestingly, the inability of NK cells to control early virus spread is also the major reason for the susceptibility of the DBA/2J strain to mousepox (Jacoby et al., 1989; Delano and Brownstein, 1995) and probably the BALB/c strain. However, the specific mechanisms are quite distinct because in these strains, NK cells migrate to the D-LN but appear unable to exert their killing function (unpublished results). Even though we also found a defective T cell response to WT ECTV in aged mice, we were surprised to find that this defect is T cell extrinsic. This seemingly contradicts a body of literature indicating intrinsic defects in the T<sub>CD8+</sub> cells of aged mice such as decreased signaling, proliferation, and cytokine production and a skewed and diminished T cell repertoire. These T cell defects have been linked to a slower clearance of influenza virus from the lungs (Haynes et al., 2002; Haynes and Swain, 2006; Ely et al., 2007; Yager et al., 2008). Because the loss of resistance to mousepox emerges at a relatively early age, we performed our experiments in mid-aged rather than very old mice, and this could account for our different results. It is also possible that OPVs exert a stronger antigenic stimulus than other antigens or viruses making any T cell-intrinsic defect nonapparent during OPV infections. In support of this view and in contrast to infection with influenza virus, primary T<sub>CD8+</sub> cell responses to OPVs do not require T<sub>CD4+</sub> cell help and can occur in the absence of CD28 co-stimulation (Fang and Sigal, 2006). It is also of interest that our finding that T<sub>CD8+</sub> cells in aged mice (which have dysfunctional NK cells) can respond strongly to poorly pathogenic OPV or when the replication of the WT virus is curtailed indicates that the role of NK cells in this type of infection is not to activate antigen-presenting cells (Gerosa et al., 2002; Walzer et al., 2005) or to provide for a source of cytokines for effective T<sub>CD8+</sub> cell activation (Zingoni et al., 2005). As we previously discussed (Fang et al., 2008), our

work here also suggests that the increased virus loads resulting from the NK cell-deficient migration results in an overwhelming antigenic stimulus for the T cells or in their death as a consequence of infection.

In summary, our results demonstrate that aged B6 mice have a deficit in the ability of their mature NK cells to migrate to the D-LN, resulting in increased early virus replication and spread and susceptibility to an acute viral disease. Our results strongly suggest that the deficit in NK cell migration to the D-LN is the result of an impaired and deficient maturation of NK cells in aged mice. Many infectious diseases important for human health spread systemically via LNs. Thus, it is possible that a defective NK cell response may be responsible for the increased susceptibility of older people to at least some infectious diseases. It would now be important to determine whether there is a defect in the maturation of NK cells in aged humans and whether this may result in increased susceptibility to viral diseases. If so, therapies could be devised to favor the maturation of NK cells as a way to increase resistance to infectious diseases.

#### MATERIALS AND METHODS

**Tissue culture.** The DC line DC2.4 was a gift from K. Rock (University of Massachusetts Medical Center, Worcester, MA). BSC-1 cells were obtained from American Type Culture Collection. As standard tissue culture medium, we used RPMI 10 that consisted of RPMI 1640 tissue culture medium (Invitrogen) supplemented with 10% FCS (Sigma-Aldrich), 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen), 10 mM HEPES buffer (Invitrogen), and 0.05 mM 2-ME (Sigma-Aldrich). For determination of virus titers, RPMI 2.5 (like RPMI 10 but with 2.5% FCS) was used instead. When required, 10 U/ml IL-2 was added to RPMI 10 (RPMI 10-IL2). All cells were grown at 37°C in 5% CO<sub>2</sub>.

**Viruses.** Initial stocks of VACV Western Reserve were obtained from B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD) and amplified in HeLa S3 cells as previously described (Earl et al., 2001). In brief, HeLa S3 cells in T150 flasks were infected with 0.1 PFU/cell VACV. After 3 or 4 d, cells were collected, resuspended in PBS, frozen and thawed three times, and stored in aliquots at -80°C as virus stock. Virus titers in VACV stocks were determined by plaque assays on confluent BSC-1 cells using 10-fold serial dilutions of the stocks in 0.5 ml RPMI 2.5 in 6-well plates (2 wells/dilution) for 1 h. 2 ml of fresh RPMI 2.5 was added and the cells were incubated at 37°C for 3 d (VACV). Next, the media was aspirated and the cells were fixed and stained for 10 min with 0.1% crystal violet in 20% ethanol. The fix/stain solution was subsequently aspirated, the cells were air-dried, the plaques were counted, and the plaque-forming units per milliliter in stocks were calculated accordingly.

Initial stocks of the WT ECTV Moscow (Fenner, 1949a; Chen et al., 1992) were obtained from American Type Culture Collection. ECTV Δ166 is a mutant of WT ECTV with the gene encoding for EVM166 disrupted with the coding sequence for GFP (Xu et al., 2008). New stocks of ECTV (WT and Δ166) were expanded in BSC-1 cells infected with 0.1 PFU/cell. Titers in stocks were determined as for VACV but the plates were incubated for 5 d. For the determination of virus titers in spleens or LNs, the organs were removed from experimental mice at the indicated days after footpad infection, made into a single cell suspension between two frosted slides, and resuspended in 10 ml of complete RPMI medium. 1 ml of the cell suspensions was frozen and thawed three times and titers were determined in 10-fold serial dilutions of the cell lysates as in the previous paragraph. Virus titers were calculated as PFU/spleen or PFU/LN. To determine the virus titers in liver, a portion of the liver was weighed and homogenized in medium using a TissueLyser (QIAGEN). The virus titers were calculated as PFU/100 mg of liver.

**Mice and infections.** The experimental protocols involving animals were approved by the Fox Chase Cancer Center Institutional Animal Care and Use Committee. Young mice were purchased from Taconic when they were 8–10 wk of age and rested at least a week before use in experiments. Most aged mice were purchased from Taconic and aged at FCCC, but in some experiments they were purchased aged from the National Institute of Aging. Unless indicated, for WT ECTV or ECTV $\Delta$ 166, mice were infected in the left footpad with 25  $\mu$ l PBS containing  $3 \times 10^3$  pfu ECTV or ECTV $\Delta$ 166. When indicated, mice were given 600  $\mu$ g cidofovir/mouse i.p. 2 dpi. VACV was inoculated via the i.p. route with 500  $\mu$ l PBS containing  $5 \times 10^6$  pfu virus. After infections, mice were observed daily for signs of disease (lethargy, ruffled hair, weight loss, skin rash, and eye secretions) and imminent death (unresponsiveness to touch and lack of voluntary movements).

**Adoptive transfer of lymphocytes.** In some cases, total lymphocytes of spleen and LNs were obtained from the indicated mice and labeled with 4  $\mu$ M CFSE (Invitrogen), and a total of  $1 \times 10^7$  cells in 0.5 ml PBS was inoculated i.v. into the recipient mice according to published procedures (Fang and Sigal, 2006). When indicated, T<sub>CD8+</sub> cells were purified using anti-CD8 microbeads (Miltenyi Biotec) and an AutoMACS magnetic cell sorter (Miltenyi Biotec) according to manufacturer's instructions. For co-transfer experiments, equal numbers of the indicated lymphocytes ( $1 \times 10^7$  cells each) were inoculated into the recipient mice. For NK cell transfer experiments, NK cells were purified from spleens and LNs using anti-CD49b-conjugated microbeads (positive selection) or mouse NK cell isolation kit (isolation of untouched NK cells) and a manual LS column (Miltenyi Biotec) according to the manufacturer's instructions. For adoptive transfer of R1, R2, and R3 NK populations, purified NK cells were stained with surface CD27 and CD11b for 30 min at 4°C and then sorted with a FACSVantage SE flow cytometer (BD) to separate the three populations. When indicated, purified NK cells were labeled with 4  $\mu$ M CFSE or 0.2  $\mu$ M CFSE, and  $3\text{--}5 \times 10^6$  NK cells were inoculated into recipient mice.

**Flow cytometry.** Detection of T and NK cell responses was performed as described previously (Fang and Sigal, 2005, 2010; Xu et al., 2007; Fang et al., 2008). In brief, for T cell responses, lymphocytes were obtained from mice at different dpi and made into single cell suspensions. After osmotic lysis of red blood cells with 0.84% NH<sub>4</sub>-Cl, cells were washed and  $10^6$  cultured at 37°C in 96-well plates in the presence of  $2 \times 10^5$  VACV-infected DC2.4 cells or uninfected DC2.4 cells as control. (Stimulation with VACV- or ECTV-infected DC2.4 cells produces similar results when measuring anti-ECTV responses. For simplicity, we used cells restimulated with VACV in all experiments.) After 5 h, brefeldin A (Sigma-Aldrich) was added to block the secretory pathway and allow for the accumulation of cytokines inside the cells. After an additional 1.5-h incubation, Ab 2.4G2 (anti-Fc $\gamma$  II/III receptor; American Type Culture Collection) was added to block nonspecific binding of labeled Ab to Fc receptors. The cells were then stained for cell surface molecules, fixed, permeabilized, and stained for intracellular molecules using the Cytofix/Cytoperm kit (BD) according to the manufacturer's instructions. For BrdU incorporation, mice were injected with 2 mg BrdU i.p. at the indicated dpi. 3 h later, spleens and LNs were removed and made into single cell suspensions. The cells were then stained for cell surface molecules, fixed, and permeabilized using the Cytofix/Cytoperm kit, incubated with DNase at 37°C for 1 h, and subsequently stained with FITC-conjugated anti-BrdU mAb.

To determine NK cell responses in LNs, intact organs were incubated at 37°C for 1 h in media containing 10  $\mu$ g/ml brefeldin A, made into single cell suspensions, stained, and analyzed as described in the previous paragraph. At least 100,000 cells were analyzed by flow cytometry at the Fox Chase Cell Sorting Facility using an LSR II system (BD). The following Ab and staining reagents were used: anti-CD4 (RM4-5; BD), anti-CD8 $\alpha$  (53-6.7; BD), anti-CD3e (145-2C11; BD), anti-Th1.1 (HIS51; eBioscience), anti-CD45.1 (A20; eBioscience), anti-NK1.1 (PK136; BD), anti-BrdU (PBR-1; BD), anti-IFN- $\gamma$  (clone XMG1.2; BD), anti-CD11b (M1/70; eBioscience), anti-CD27 (LG 3A10; BD), anti-CD62L (MEL 14; BD), anti-NKG2D (C7; BD),

and APC-labeled anti-human GzB (Invitrogen) that cross-reacts with mouse GzB (Wolint et al., 2004). For TSYKFESV-specific T<sub>CD8+</sub> cells, H-2K<sup>b</sup>:Ig recombinant fusion protein (Dimer-X; BD) was incubated with synthetic TSYKFESV (GenScript) and used as recommended by the manufacturer. Stained cells were analyzed by flow cytometry at the Fox Chase Cell Sorting Facility using an LSR II system.

**Data displayed and statistical analysis.** Unless indicated, all displayed data correspond to one representative experiment of at least three similar experiments with groups of three to five mice. In most instances, the LNs from the mice in a group were pooled, whereas the spleens were analyzed individually. Statistical analysis was performed using Excel (Microsoft) or Prism (GraphPad Software, Inc.) software. For survival studies, P-values were obtained using the Log-rank (Mantel-Cox) test. All other statistical analyses were performed using an unpaired two-tailed Student's *t* test. When applicable, data are displayed with mean  $\pm$  SEM (SEM).

**Online supplemental material.** Fig. S1 shows that the defective T<sub>CD8+</sub> cell response of aged mice to ECTV is not T cell intrinsic. Fig. S2 shows that aged mice are not different from young mice in terms of NKG2D expression under normal conditions or during infection. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20100282/DC1>.

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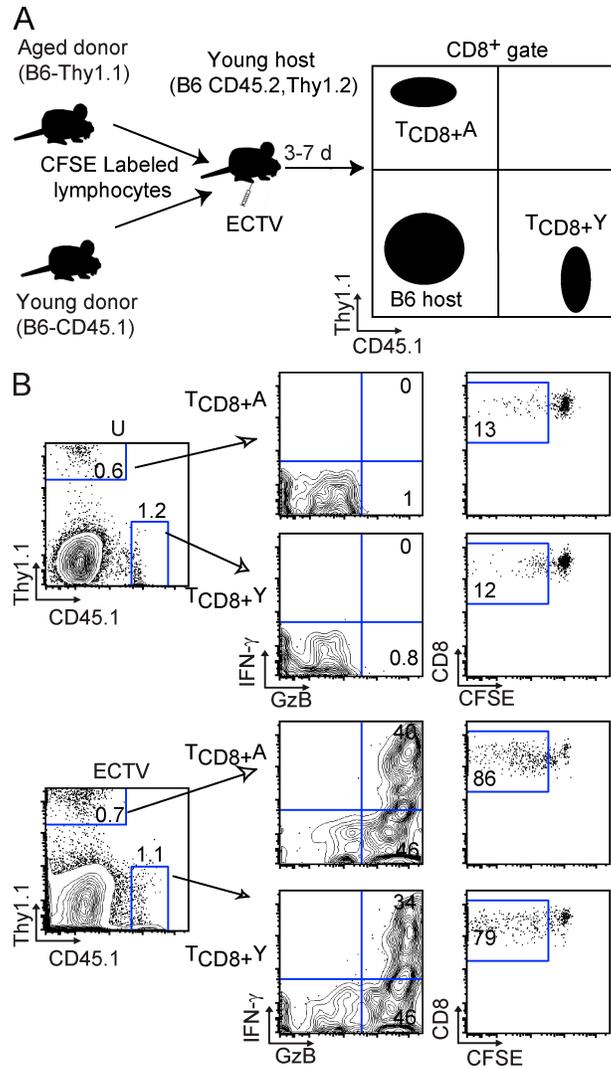
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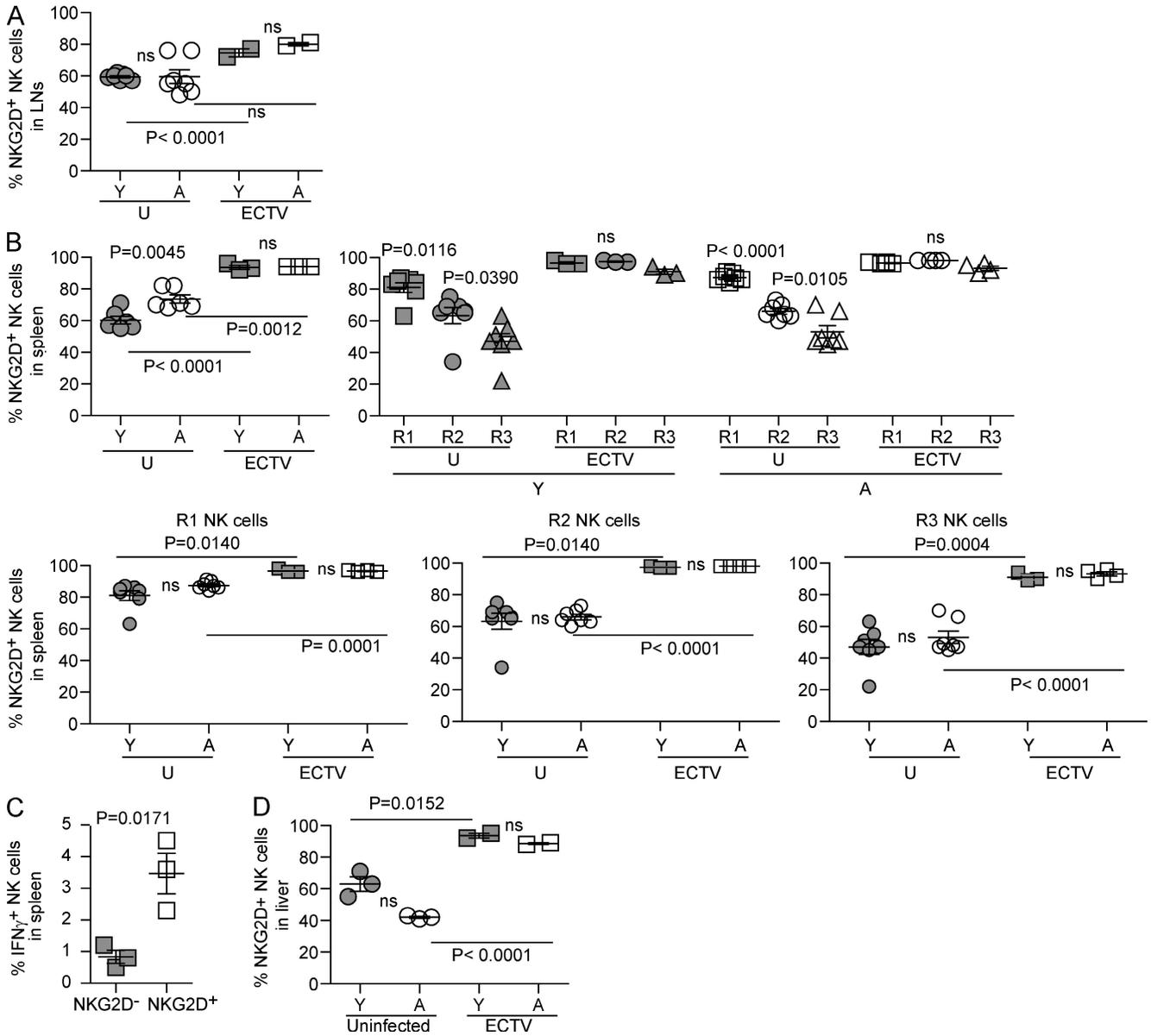
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SUPPLEMENTAL MATERIAL

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**Figure S1. The defective T<sub>CD8+</sub> cell response of aged mice to ECTV is not T cell intrinsic.** A reciprocal experiment to that depicted in Fig. 4 A and B is shown. (A) Experimental design for the adoptive transfer of CFSE-labeled lymphocytes from aged (B6- Thy1.1) and young (B6-CD45.1) donor mice into young B6 (B6 CD45.2-Thy1.2) hosts followed by ECTV infection and flow cytometry analysis gating on CD8<sup>+</sup> cells. (B) B6 mice, as in A, were euthanized on the indicated dpi. Cells from the D-LN and spleen were restimulated ex vivo for 5 h with infected cells in the presence of brefeldin A and stained for surface CD3, CD8, Thy1.1, and CD45.1, and for intracellular IFN- $\gamma$  and GzB, followed by flow cytometry. Representative plots correspond to spleens of individual mice 7 dpi. Plots corresponding to an uninfected control mouse are also shown for comparison. Plots were gated on CD8<sup>+</sup> cells. Data are representative of two independent experiments.



**Figure S2. Aged mice are not different from young mice in terms of NKG2D expression under normal conditions or during infection.** Young or aged mice were left uninfected or infected with WT ECTV. (A) 2-dpi groups of mice were euthanized, and the D-LNs were made into single cell suspensions and stained for CD3, NK1.1, and NKG2D. The proportion of CD3<sup>-</sup>NK1.1<sup>+</sup> cells that expressed NKG2D was determined by flow cytometry. Each symbol in the uninfected groups corresponds to individual mice. Each symbol in the infected groups corresponds to pooled D-LNs from two or three mice. Data are representative of two independent experiments. (B) 5-dpi groups of mice were euthanized, and their spleens were made into cell suspensions, stained for surface CD3, NK1.1, CD27, and CD11b, and analyzed by flow cytometry. The same data are analyzed in several ways to allow for the various comparisons. Top left, NKG2D in all NK cells young versus aged in uninfected or ECTV infected. Top right, R1 versus R2 versus R3 in uninfected or ECTV-infected young or aged mice. Bottom, NKG2D expression in R1 (left), R2 (center), or R3 (right) NK cells in young versus aged uninfected or ECTV-infected mice. (C) The NK cells in the young groups in B were analyzed for IFN- $\gamma$  expression by intracellular staining. (D) As in A, but with lymphocytes recovered from the livers by percoll gradient centrifugation 5 dpi. Data correspond to three to five individual mice from infected mice and six to eight individual naive mice from each group. In all panels, the horizontal bar represents the mean and the vertical bars the SEM.