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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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<sub>CD8+</sub> 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<sub>CD8+</sub> 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<sub>CD8+</sub> 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<sub>CD8+</sub> cells, virus titers 7 dpi are higher than those in the absence of B cells or Abs (unpublished data), even though T<sub>CD8+</sub> cells do not require T<sub>CD8+</sub> 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 (~6 mo) and was completely lost when the mice reached 14 mo of age (~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 a ~10<sup>3</sup>-fold increase in virus titers in the spleen and liver 7 dpi as compared with young (~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, ~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<sub>CD8+</sub> 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<sub>CD8+</sub> cell responses are essential for resistance to mousepox (Fang and Sigal, 2006), we compared the T<sub>CD8+</sub> cell responses to ECTV in young and aged B6 mice. We found that the T<sub>CD8+</sub> 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<sub>CD8+</sub> cells expressing granzyme B (GzB) and IFN-γ 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<sub>CD8+</sub> 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 [Tscharke 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<sub>CD8+</sub> cell response to WT ECTV.

Aged mice mount normal T<sub>DB4</sub>, 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<sub>CD8+</sub> cell response in aged mice,
Because in vivo ECTV α166 and VACV replicate and disseminate much less efficiently than WT ECTV, we hypothesized that the failure of aged mice to mount TCD8+ 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 µg 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 CD8+ cell responses comparable to those of young mice, indicating that uncontrolled viral replication is the major reason for the failure of the TCD8+ cell response of aged mice to WT ECTV (Fig. 3, E and F).

The defective TCD8+ 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 TCD8+ 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 TCD8+ 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 TCD8+ cells from young and aged
Figure 3. Aged mice mount normal T<sub>CD8</sub> 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 Δ166 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-γ and GzB, followed by flow cytometry. Column graphs indicate the mean ± SD for the calculated absolute number of T<sub>CD8</sub> cells in the spleen that expressed GzB (left) or IFN-γ (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 ± SD of the calculated absolute numbers and representative plots of CD8<sup>+</sup>K<sup>-</sup>-TSYKFESV<sup>+</sup> cells in the spleen. Data correspond to the mean ± SD of six individual mice from two independent experiments. (C) As in A but infected with 10<sup>6</sup> PFU VACV i.p. (D) As in B but infected with VACV i.p. Data correspond to the mean ± 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 µg cidofovir (A+C) i.p. (F) Splenocytes from mice treated as in E were analyzed as in B. Data correspond to the mean ± SD of six individual mice from two experiments.
donor mice responded to WT ECTV with similar efficiency in the young environment, as revealed by activation determined by IFN-γ 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 TCD8+ cells from young B6-CD45.1 mice responded as poorly as the recipients’ endogenous TCD8+ cells in aged B6 mice (Fig. 4D), and a similar result was obtained with reciprocal transfer of TCD8+ 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
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-γ (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 the TCD8+ 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.

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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 Δ166 (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 Δ166 is highly attenuated in aged mice (not depicted) this data also indicates that a fully functional NK cell response is necessary for resistance to high 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 LNs 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 Δ166 (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<sup>+</sup> 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 the indicated NK population in the blood that was CD62L+. 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+ cells as the NK cells matured. As we showed before, NKG2D expression significantly increased the proportion of NKG2D+ cells in all maturation stages to >90%. In addition, most IFN-γ–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.
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 (NKY, 0.2 µM CFSE) and aged (NKA, 4 µM CFSE) mice into young hosts (Fig. 8 A, top), more NKY cells migrated to the D-LN, as compared with the ND-LN of infected mice (or the LN of uninfected control recipients). In contrast, fewer NKA 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 NKY cells in the D-LN were of R3 phenotype and R2 in ND-LN or the LN of uninfected mice, whereas the NKA cells were mostly R2 regardless of the LN or infection status (Fig. 8 A, middle and bottom). The almost complete absence of mature R3 NKA cells in the D-LN of infected mice could be a result of defective migration of R3 NKA cells from the blood to the D-LN or of the R2 NKY cells, but not the R2 NKY cells, differentiating into R3 cells after migration into the D-LN. To test whether NKY cells differentiate in the

Figure 8. The mature R3 NK cells from aged mice are intrinsically defective in their ability to home to the D-LN. (A) Purified NKY and NKA 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-CD3- NK. The bar graph on the right shows mean ± SD for the ratio in D-LN versus ND-LN for NKY and NKA cells. Top middle panels are gated on CFSE- host NK cells. Bottom middle panels are gated on transferred NKY cells. Bottom panels are gated on transferred NKY cells. Data are representative of three similar experiments. (B) R1-R3 NKY 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 NKY cells before sorting. For each type of LN, the plots on the left were gated on NK1.1-CD3- NK cells and the box shows the transferred cells. For each type of LN, the plots on the right are gated on 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.
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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\(^{Y}\) cells migrated to the D-LN of aged mice, we investigated whether adoptive transfer of NK\(^{Y}\) cells could protect aged mice from mousepox. For this purpose, we transferred \(\sim 5 \times 10^6\) NK\(^{Y}\) cells, NK-depleted spleen cells from young donors (NK\(^{A}\)), or T\(_{CD8}\)Y cells into aged hosts and challenged them with ECTV. Strikingly, the aged mice that received NK\(^{Y}\) cells, but not those which received T\(_{CD8}\)Y cells or NK\(^{A}\) cells, were significantly protected from mousepox (Fig. 9 C). In addition, aged mice that received NK\(^{Y}\) cells had significantly decreased virus spread to liver and spleen 5 dpi as compared with aged T\(_{CD8}\)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>CD8+</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% CO2.

**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 5 d. 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. Titters 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 titters 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 ECTVΔ166, mice were infected in the left footpad with 25 µl PBS containing 3 × 10^6 pfu ECTV or ECTVΔ166. When indicated, mice were given 600 µg cidofovir/mouse i.p. 2 dpi. VACV was inoculated via the i.p. route with 500 µl PBS containing 5 × 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 LN were obtained from the indicated mice and labeled with 4 µM CFSE (Invitrogen), and a total of 1 × 10^6 cells in 0.5 ml PBS was inoculated i.v. into the recipient mice according to published procedures (Fang and Sigal, 2005, 2010; Xu et al., 2007; Fang et al., 2008). In brief, TCD8 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 × 10^6 cells each) were inoculated into the recipient mice. For NK cell transfer experiments, NK cells were purified from spleens and LN 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 FACS Vantage SE flow cytometer (BD) to separate the three populations. When indicated, purified NK cells were labeled with 4 µM CFSE or 0.2 µM CFSE, and 3–5 × 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% NH4Cl, cells were washed and 10^6 cultured at 37°C in 96-well plates in the presence of 2 × 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) at 37°C for 1 h in media containing 10 µg/ml brefeldin A, made into single cell suspensions. The cells were then stained for cell surface molecules, fixed, and permeabilized 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 LN 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 DNsase at 37°C for 1 h, and subsequently stained with FITC-conjugated anti-BrdU mAb.

To determine NK cell responses in LN, intact organs were incubated at 37°C for 1 h in media containing 100 µg/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 reagents were used: anti-CD4 (RM4-5; BD), anti-CD8a (53–6.7; BD), anti-CD3ε (145–2C11; BD), anti-Thi.1 (H1S1; eBioscience), anti-CD45.1 (A20; eBioscience), anti-NK1.1 (PK136; BD), anti-Brdu (PBR-1; BD), anti–IFN-γ (clone XMG1.2; BD), anti-CD11b (M1/70; eBioscience), anti-CD27 (LG3A10; 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 TSYYKFSV-specific TCD8 cells, H-2Kd Ig recombinant fusion protein (Dmter-X; BD) was incubated with synthetic TSYYKFSV (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 LN 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 ± SEM (SEM).

Online supplemental material. Fig. S1 shows that the defective T CD8+ 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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