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The Depletion of NK Cells Prevents T Cell Exhaustion to Efficiently Control Disseminating Virus Infection

Kevin D. Cook* and Jason K. Whitmire*‡

NK cells have well-established functions in immune defense against virus infections and cancer through their cytolytic activity and production of cytokines. In this study, we examined the frequency of NK cells and their influence on T cell responses in mice given variants of lymphocytic choriomeningitis virus that cause acute or persisting infection. We found increased frequencies of circulating NK cells during disseminating infection compared with uninfected or acutely infected mice. Consistent with recent reports, we observed that the depletion of NK cells in mice with disseminated infection increased peak numbers of virus-specific cytotoxic CD8+ T cells and resulted in the rapid resolution of disseminated infection. Additionally, we show that NK cell depletion sustained T cell responses across time and protected against T cell exhaustion. The positive effects of NK cell depletion on T cell responses only occurred when NK cells were depleted within the first 2 d of infection. We find that the improved CD8+ T cell response correlated with an enhanced ability of APCs from NK cell–depleted mice to stimulate T cell proliferation, independently when the signals from the activating receptors outweigh those of activating and inhibitory receptors, and cytotoxicity is initiated.

IFN-α/β, are potent activators of NK cells. Dendritic cells (DCs) are important in activating NK cells through direct interactions and the production of NK-activating cytokines. Intravital imaging shows that DCs and NK cells interact in lymph nodes in vivo, and in vitro analyses demonstrate that DCs directly activate NK cells (6–9). These NK–DC interactions are regulated by the Nkp30–activating receptor, DNAX accessory molecule-1, and TNF-α, as well as the transpresentation of IL-15 by DCs (10–13). Thus, DCs are often a critical cell type in the activation of NK cell responses.

NK–DC interactions also impact the functions of DCs. NK cells promote DC activity by inducing their maturation, including the upregulation of costimulatory molecules, and increasing DC production of IL-12 (6, 8, 14). However, NK cells can also directly lyse DCs or decrease their Ag presentation functions (10, 15–17). Additionally, indirect effects through NK cell–mediated lowering of the viral load can impact DC frequency (18). Therefore, the effect of the NK–DC interactions on DCs is context dependent and can be positive or negative.

As with their effect on DCs, the effect of NK cells on T cell responses can also be positive or negative. Recent data show virus-specific CD4+ and CD8+ T cell responses are negatively regulated by NK cells through perforin-dependent mechanisms (19–21). Additionally, the elimination of certain surface molecules, including Qa-1 on T cells or 2B4 on NK cells, enhances NK cell–mediated regulation of T cell responses, presumably through direct lysis of activated T cells by NK cells (22, 23). Other studies have implicated NKG2D receptor signaling in the lysis of activated T cells (24–26). In addition to direct lysis, NK cell acquisition of MHC class II molecules following DC interactions has been shown to downregulate CD4 T cell responses (27). NK cells produce IL-10 and TGF-β, which have negative effects on T cell activation (28–30). However, NK cells also produce cytokines, such as IFN-γ, that enhance T cell responses (31, 32). In addition to these mechanisms of NK cell regulation of T cell responses, the effects of NK cells on DCs will subsequently impact T cell activation. Thus, NK cell functions span innate immune defense and primary adaptive immune responses to infection.

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Abbreviations used in this article: DC, dendritic cell; DP, double-positive; ICCS, intracellular cytokine staining; KLRG-1, killer cell lectin-like receptor G1; LCMV, lymphocytic choriomeningitis virus; MCMV, mouse cytomegalovirus; PD-1, programmed cell death-1.

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During chronic lymphocytic choriomeningitis virus (LCMV) infection, there is a generalized immune suppression mediated by impaired Ag presentation and immune-mediated destruction of DCs (33–35). In addition to the decrease in DC number, there is also a loss in their ability to stimulate an allogeneic MLR as early as 3 d postinfection (36). Later during persisting virus infections in mice and man, virus-specific T cells undergo significant decline in number, whereas those that remain are retained in a dysfunctional state (37). These populations of exhausted virus-specific CD8+ T cells are characterized by poor cytokine output, cytolytic activity, and impaired proliferative capacity (38). The dysfunctional (exhausted) cells are actively inhibited by signals transmitted through surface receptors programmed cell death-1 (PD-1), Lag3, Tim-3, CD160, and 2B4 and by the cytokine IL-10 (38, 39). Given the regulatory functions of NK cells described above, it is plausible that NK cell activity may directly influence virus-specific T cell responses or contribute to the loss in DC number or effectiveness and affect viral control during a persisting virus infection.

In this study, we examined the role of NK cells in mice that were either acutely infected with LCMV-Armstrong or given a disseminating infection with LCMV-Clone13. We found that the depletion of NK1.1+ cells in acutely infected mice minimally impacted the rate of viral clearance. Thus, NK cells contribute to the T cell exhaustion that emerges during persisting virus infection.

Materials and Methods

Mice and virus

C57BL/6 mice were purchased from Jackson ImmunoResearch Laboratory. P14 TCR-transgenic mice specific for the LCMV epitope GP34-41 (40) were crossed to C57BL/6.Thyl.1 mice to generate TCR-transgenic Thy1.1+ mice. The depletion of NK cells was accomplished by i.p. administration of two doses of 75 μg NK1.1-PK136 (BioXCell) on successive days; control mice were administered either two doses of 75 μg rat IgG2a isotype control or PBS. Armstrong and Clone13 strains of LCMV were prepared and quantitated as described previously (41). Mice were infected by i.v. administration of 2 × 10^6 PFU Clone13 or Armstrong or i.p. administration of 2 × 10^5 PFU Armstrong. Infectious LCMV in serum, liver, lung, and kidney was quantified by plaque assay on Vero cell monolayers as described previously (41). All mouse experiments were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Flow cytometry

Spleen cells were stained directly ex vivo with Abs against CD8 (53-6.7), CD4 (RM4-5), NK1.1 (PK136), CD49b (DX5), CD44 (IM7), CD62L (MEL-14), CD3 (17A2), CD11c (N418), CD11b (M1/70), Siglec H (551), plasmacytoid DC Ag-1 (927), PD-1 (RMP1-30), Lag-3 (C9B7W), CD244 (m2B4[66]) and KLRG1, killer cell lectin-like receptor G1 (KLRG-1) (2F1/KLRG1), CD127 (LG.3A10), and Thy1.1 (eBioscience; HIS5.1) Abs. Uninfected mice had nearly equal populations of CD11blow, double-positive (DP), and CD27low cells. To determine if this increase in NK cell activation was associated with enhanced frequency of KLRG-1+ NK cells, KLRG-1 was also increased on blood NK cells shortly after Armstrong and Clone13 infections, and the enhanced frequency of KLRG-1+ NK cells remained higher than in the Armstrong group. The frequency of NK cells in Clone13-infected mice eventually returned to preinfection levels, whereas NK cell frequencies in Armstrong-infected mice were reduced below this level (Fig. 1B).

In vitro T cell stimulation

APCs were isolated from spleens of mice that were lightly smashed in Petri dishes and then incubated at 37°C for 30 min in media supplemented with 1 mg/ml collagenase I (Calbiochem) and 10 μg/ml DNase I (Sigma-Aldrich) before being forced through a mesh filter. P14 T cells were isolated from spleens of P14-transgenic mice and labeled with 2 μM CFSE. The APCs and T cells were mixed at a ratio of 20:1 and incubated for 5 d before measuring CFSE dilution by flow cytometry.

Statistics

Statistical analyses were performed using Prism software (GraphPad), employing an unpaired two-tailed Student t test to evaluate the significance of differences between groups.

Results

NK cells are more active during chronic virus infection than acute infection

C57BL/6 mice that are infected with LCMV-Armstrong (Armstrong) develop an acute infection that is resolved within 1 wk by expanded populations of virus-specific CD8+ T cells. In contrast, mice given LCMV-Clone13 (Clone13), a variant that replicates rapidly and spreads widely, develop a persisting infection that lasts for 2 to 3 mo. Although much energy has been focused on understanding the T cell response to these viruses, a full characterization of the NK cell response is lacking. Therefore, we followed NK cell frequencies in the spleen and blood at different times postinfection. The NK cell number in the spleen decreased 2-fold by day 8 in acutely infected mice and began declining by day 3 in Clone13-infected mice and reached a 4-fold decrease by day 8 (Fig. 1A). In contrast to the spleen, NK cell frequencies increased in the blood after Clone13 infection at 7 d postinfection (Fig. 1B), whereas the frequency of NK cells in the blood after acute infection did not change compared with uninfected mice. At days 14 and 21 postinfection, the frequency of NK cells in the blood of the Clone13 group remained higher than in the Armstrong group. The frequency of NK cells in Clone13-infected mice eventually returned to preinfection levels, whereas NK cell frequencies in Armstrong-infected mice were reduced below this level (Fig. 1B).

To determine if this increase in NK cell activation was associated with an increase in maturation, we measured the expression level of granzyme B, a mediator of cytotoxicity. During an acute infection, the granzyme B level rapidly increased during an acute infection, the granzyme B level rapidly increased by day 3 but then decreased to near baseline levels afterward (Fig. 1C, 1D). The level of granzyme B expression also peaked at day 3 during a Clone13 infection, but the level was higher than during an Armstrong infection (Fig. 1C, 1D). Additionally, the increase in granzyme B expression level was maintained for a longer amount of time during chronic infection, through day 14 (Fig. 1C, 1D). Therefore, if this increase in NK cell activation was associated with an increase in maturation, we measured the expression of CD11b and CD27 on NK cells (42). Uninfected mice had nearly equal populations of CD11b^low, double-positive (DP), and CD27low NK cells, and both Armstrong and Clone13 infections induced maturation of a majority of the splenic NK cells into the CD27low subset by day 7 postinfection (Fig. 1E). An additional marker of NK cell maturation, KLRG-1 (43), was also increased on blood NK cells shortly after Armstrong and Clone13 infections, and the enhanced frequency of KLRG-1+ NK cells was maintained for a longer time in Clone13-infected mice (Fig. 1F). Thus, there is a correlation between the persistence of infection and the frequency, activation status, and maturity of NK cells for multiple weeks.

NK cells are capable of either promoting or inhibiting adaptive immune responses to infection. Although some data indicate that NK cells promote T cell responses, multiple recent papers have suggested that NK cells limit T cell responses by inducing perforin-
mained the number of NK1.1+DX5+ NKp46+ splenic NK cells at the indicated days is depicted as mean ± SEM, with n = 4–13 over five experiments for Armstrong and n = 12–16 over six experiments for Clone13. The significant differences between the Armstrong- and Clone13-infected mice relative to the uninfected samples are indicated. (B) The frequency of NK cells within PBLs was measured in mice bled repeatedly over time and is depicted as mean ± SEM, with n = 3–11 over four experiments for Armstrong and n = 6–10 over four experiments for Clone13. The asterisks indicate differences between Armstrong and Clone13 at each time. (C and D) NK cells isolated from the blood were intracellularly stained for granzyme B. (C) The histograms show examples of the granzyme B levels in NK cells from uninfected (shaded), Armstrong-infected (thin line), and Clone13-infected (thick line) mice at various time points postinfection. (D) The line graph shows the geometric mean fluorescence intensity (gMFI) of granzyme B in NK cells from each group over time, depicted as mean ± SEM, with n = 4 for each group from two experiments. (E) NK cells isolated from the spleen at day 7 pi were stained for CD11b and CD27; the numbers indicate the frequency of NK cells within each quadrant. The dot plots are representative of three mice from one experiment. (F) NK cells isolated from the blood were stained for KLRG-1. The dot plots are representative of three mice from one experiment. Significant differences between Armstrong and Clone13 at each time are indicated by asterisks: *p < 0.05, **p < 0.01.

mediated cell death of activated T cells or APCs (18–21, 23, 44). To determine what effect NK cells have on T cell responses during acute and chronic viral infection, we depleted NK cells using the NK1.1 (PK136) Ab. The Ab treatment specifically eliminated NK cells quickly and was durable (Supplemental Fig. 1). We considered that NK1.1 expression might be induced on non-NK cells during infection. However, we found that NK1.1 expression was restricted to DX5+ NK cells (Supplemental Fig. 1A) and <1.5% of T cells, B cells, regulatory T cells, or DCs (Supplemental Fig. 1C), indicating that NK1.1 Ab treatment is unlikely to deplete potential targets of LCMV infection (33). We confirmed that the Ab treatment did not change the number of CD4+Foxp3+ regulatory T cells before infection (Supplemental Fig. 1D).

**NK cells limit early CD8+ T cell responses to a disseminating virus infection**

To further understand how NK cells affect a defined population of virus-specific CD8+ T cells after an acute or chronic virus infection, we adoptively transferred a small number of naive LCMV<sub>GP33-41</sub>-specific TCR-transgenic P14 cells prior to infection. The mice were subsequently given the NK1.1 or control Ab, and the P14 cell response was analyzed by flow cytometry at day 8 of the infection. For uninfected control mice, much larger numbers of P14 cells were given to facilitate their detection; in the absence of infection, these P14 cells retained their naive phenotype (CD62L<sup>+</sup>, CD44<sup>lo</sup>) following the adoptive transfer (data not shown). The mice that were treated with NK1.1 but left uninfected had similar frequencies of P14 cells in the spleen compared with control-treated recipients, indicating that NK cell depletion does not affect the engraftment of P14 cells (Fig. 2A). The control and NK1.1-treated mice that were given Armstrong showed a vigorous expansion of the P14 and endogenous CD8+ T cells with little difference between the two groups (Fig. 2A and data not shown). As expected, in the control-treated mice, there was a lower level of expansion by the P14 cells in Clone13 compared with Armstrong-infected mice (Fig. 2A). However, the Clone13-infected mice that were depleted of their NK cells showed a pronounced increase in the frequency of P14 cells (Fig. 2A). Based on the total cell counts, NK cell depletion resulted in a modest increase in P14 cells in Armstrong-infected mice and a 3-fold increase in the Clone13-infected mice (Fig. 2B), which became similar in magnitude to the number seen in acutely infected mice.

The ability of virus-specific CD8+ T cells to express cytokine upon exposure to peptide was examined by ICCS followed by flow cytometry analysis. There was no detectable IFN-γ or TNF expression by the P14 cells in uninfected mice; however, by day 8 post-Armstrong infection, >70% of the P14 cells expressed IFN-γ and TNF when exposed to cognate peptide (Fig. 2C). In Clone13-infected mice, only 20% of the P14 cells produced both cytokines, consistent with cells that are exhausted (38), whereas the depletion of NK cells increased the frequency of IFN-γ<sup>+</sup>TNF<sup>+</sup> DP P14 cells (Fig. 2C). In both Armstrong- and Clone13-infected mice, the depletion of NK cells increased the total number of IFN-γ<sup>+</sup>, TNF<sup>+</sup> cells, but the effect was greatly exaggerated in the Clone13-infected mice (Fig. 2D). The ~10-fold increase in IFN-γ<sup>+</sup>TNF<sup>+</sup>, as well as IFN-γ<sup>+</sup>IL-2<sup>+</sup>, P14 cells seen after NK depletion in Clone13-infected mice resulted in cell numbers matching those seen in the Armstrong-infected mice. NK cell depletion also increased the frequency and number of endogenous polyclonal virus-specific CD8+ T cells by 4–15-fold in Clone13-infected mice (Supplemental Fig. 2), indicating that NK cell depletion enhances multiple populations of cells and is not unique to the P14 cells or the GP<sub>33-41</sub> specificity.

Because NK cell depletion leads to greater numbers of virus-specific CD8+ T cells that are capable of producing multiple cytokines that broadly enhance immune system activation (IFN-γ, TNF) and drive T cell memory (IFN-γ, IL-2) at the peak of the response, we considered that NK cell depletion might have lasting effects on virus-specific T cell abundance. Indeed, the percentage of P14 cells in Clone13-infected mice was enhanced by 4-fold at day 14 in mice depleted of NK cells (Fig. 2E). After day 14, the P14 response contracted in the Clone13-infected mice, but the NK cell–depleted mice continued to have 11-fold higher frequencies of P14 cells than control-treated mice at day 28. These data show that the depletion of NK cells results in vigorous CD8+ T cell
endogenous CD4+ T cells in uninfected mice express the naive (CD62L+/CD44+) surface phenotype, whereas a high proportion in Armstrong infected mice express the activated (CD62L−/CD44+) surface phenotype, with no differences seen in either condition following the depletion of NK cells (Fig. 2F). The frequency of activated CD4+ T cells in control-treated Clone13-infected mice resembled uninfected mice; however, the depletion of NK cells increased the percentage of activated CD4 cells to a level similar to that in Armstrong-infected mice (Fig. 2F). To confirm that the global effect on CD4 activation accurately reflects the LCMV-specific CD4+ T cell response, we measured the frequency of CD4+ T cells specific for LCMV by ICCS after stimulation with the immunodominant LCMV Gp33−41 peptide. The depletion of NK cells had very little effect in Armstrong-infected mice, yet the number of LCMV-specific IFN-γ−producing CD4+ T cells was significantly enhanced in the absence of NK cells in Clone13-infected mice (Fig. 2G). These data indicate that both the CD4+ and CD8+ T cell responses are significantly improved by the depletion of NK cells during chronic LCMV infection.

**NK cell depletion reduces the expression of T cell exhaustion markers**

During persisting virus infections, virus-specific T cells are held in an inactive state due to their expression of inhibitory surface molecules, including PD-1, Lag-3, and 2B4, and interactions with corresponding ligands on APCs (45). Given the data in Fig. 2 showing reduced frequencies of virus-specific cytokine-producing CD8+ T cells during Clone13 infection and the well-characterized exhaustion phenotype observed with this infection, we next examined whether NK cell depletion affected the expression of these exhaustion markers on CD8+ T cells. The P14 CD8+ T cells did not express detectable levels of PD-1 or Lag-3 in uninfected mice (Fig. 3A). At day 8 postinfection, only a small population of the P14 cells expressed these markers in Armstrong-infected mice, but 80% of the P14 cells expressed PD-1 at this time in Clone13-infected mice, and 30% were DP for PD-1 and Lag-3 (Fig. 3A, 3B), consistent with a significant functional exhaustion. Minimal levels of 2B4 were expressed on the P14 cells in uninfected or day 8 Armstrong-infected mice; however, half of the P14 cells expressed 2B4 in control-treated Clone13-infected mice (Fig. 3B).

The depletion of NK cells prior to Clone13 infection significantly reduced the percentage of P14 cells expressing PD-1, Lag-3, and 2B4 (Fig. 3A, 3B). Thus, NK cell depletion reduces the percentage of virus-specific CD8+ T cells that express the inhibitory receptors associated with T cell exhaustion early postinfection.

T cell exhaustion is typically analyzed several weeks postinfection. To evaluate if NK cell depletion had durable effects on T cell exhaustion, sets of mice were infected with Clone13, and, across several weeks, PBLs were isolated, and the P14 cells were stained for exhaustion markers. The percentage of cells that expressed PD-1, Lag-3, and 2B4 were consistently reduced in Clone13-infected mice that were depleted of NK cells (Fig. 3C), and the per-cell level of PD-1, Lag-3, and 2B4 on these cells was reduced as indicated by geometric mean fluorescence intensity (data not shown). The ability of the P14 cells to produce cytokines was determined 4 wk postinfection. Few of the P14 cells from Clone13-infected mice were able to produce both IFN-γ with TNF or IL-2 at day 29, which is consistent with the exhausted phenotype (Fig. 3D). However, elevated percentages of P14 cells from NK-depleted mice vigorously produced multiple cytokines at this time (Fig. 3D). Moreover, the overall number of P14 cells capable of producing these cytokines was enhanced >55-fold in mice depleted of NK cells compared with control-treated mice (Fig. 3E). These data indicate that NK cell depletion limits the for-

**Figure 2.** NK cell depletion improves early T cell responses to chronic virus infection. A total of 2 × 10⁶ P14 T cells from Thy1.1+ mice were transferred to Thy1.2+ B6 mice; the mice were treated with NK1.1 or control Ab at days −2 and −3 and then infected with LCMV-Armstrong or Clone13. Uninfected mice were given 2 × 10⁶ P14 T cells to facilitate the detection of P14 cells. (A) The dot plots identify P14 T cells in the splenens from uninfected mice or mice 8 d postinfection (pi); the numbers indicate the frequency of CD4+ Thy1.1+ P14 cells among all splenens. (B) The total number of CD4+Thy1.1+ splenic P14 cells at day 8 pi, depicted as mean ± SEM, with n = 3 from one experiment for Armstrong and n = 11–14 over five experiments for Clone13. (C) An example of IFN-γ and TNF production by splenic P14 cells at day 8 pi after ex vivo stimulation with LCMV peptide Gp33−41; the numbers above each plot indicate the percentage of IFN-γ+TNF+ and IFN-γ+TNF− cells among P14 cells. (D) The total number of IFN-γ+TNF− (top panel) or IFN-γ−IL-2+ (bottom panel) splenic P14 cells at day 8 pi, depicted as mean ± SEM, with n = 3 from one experiment for Armstrong and n = 11–14 over five experiments for Clone13. (E) The frequency of P14 cells among blood leukocytes in mice that were bled repeatedly following infection with LCMV-C1one13 (mean ± SEM; n = 6–10 from four experiments). (F) CD4+ T cells from the spleen were stained for exhaustion markers CD62L and CD44. The numbers indicate the percentage of CD4+ cells in each quadrant. (G) The total number of IFN-γ+ splenic CD4 cells at day 8 pi, as measured by ICCS, depicted as mean ± SEM, n = 6 for Armstrong across two experiments and n = 11–14 across five experiments for Clone13. The asterisks indicate significant differences between control and NK1.1-treated groups: **p < 0.01, ***p < 0.001.

responses that are sustained across time during persisting infection.

It has been suggested that the effect of NK cell depletion on CD8+ T cell activity during a chronic viral infection is due to a defect in the CD4 response caused by direct NK cell–mediated killing of activated CD4+ T cells (20). To test this hypothesis, we measured the endogenous CD4+ T cell responses during acute and chronic infections after NK cell depletion. The majority of
viral clearance

NK cells negatively regulate APC stimulatory capacity and viral clearance

The brief period when NK cell depletion affects CD8+ T cell responses suggests that NK cells target DCs, which are responsible for initiating T cell stimulation. Consistent with this idea, NK cells directly lyse DCs during mouse cytomegalovirus (MCMV) infection to restrict subsequent T cell activation (44). Therefore, we measured the number of DCs after Clone13 infection in the presence or absence of NK cells. The total number of splenic DCs (Fig. 5A), as well as the CD11b+ C, CD8+, CD4+, and pDC subsets (Fig. 5B–E), rapidly decreased in the first few days after Clone13 infection. However, there was no difference between the control- and NK1.1-treated mice. Additionally, the DC expression levels of the stimulatory receptors MHC class I/II, the costimulatory receptors B7-1/2, and the inhibitory receptors PD ligand-1/2 were not altered by NK cell depletion (Supplemental Fig. 3). Taken together, these data indicate that NK cells do not impact the overall number or surface stimulatory profile of DCs.

The previous data do not account for NK cell effects on the presentation of LCMV-specific epitopes; therefore, we developed an in vitro stimulation assay to test whether APC stimulation of T cell proliferation is altered by the presence or absence of NK cells. Splenocytes from NK-depleted or control-treated Clone13-infected mice were assessed for their capacity to stimulate CFSE-labeled naive P14 cells. In control-treated mice, the highest level of CFSE dilution occurred using APCs from day 1 postinfection, whereas cells from uninfected mice did not stimulate proliferation in the absence of added peptide (Fig. 6). The stimulatory capacity of the splenic APCs underwent a significant decline by days 2 and 3 postinfection (Fig. 6), which parallels the decrease in the number of DCs shown in Fig. 5. In NK cell-
depleted mice, APCs from uninfected mice stimulated minimal P14 cell division, and APCs from day 1 induced strong proliferation, similar to that induced by splenocytes from the control-treated mice at those times (Fig. 6). However, APCs isolated on days 2 and 3 of infection from NK cell–depleted mice stimulated a significantly higher level of CFSE dilution compared with day 2 and 3 cells from control-treated mice (Fig. 6). The inclusion of cognate peptide to the cultures induced maximal levels of T cell division, regardless of which APC population was used (Fig. 6B), which suggests that APCs from each day are potentially capable of promoting T cell proliferation, but NK cells limit the stimulation of T cells by LCMV-infected APCs. These data indicate that the ability of APCs from infected mice to stimulate LCMV-specific T cell proliferation is sustained when NK cells are depleted.

NK cells limit early acute virus infection by killing virus-infected target cells; however, our data indicate that NK cells restrict the ability of APCs to stimulate T cell proliferation (Fig. 6) and decrease the magnitude and function of virus-specific CD8+ T cells during a highly disseminated infection (Figs. 2, 3). To determine if these effects alter viral clearance, the viral loads were measured in control or NK cell–depleted mice at different times after Clone13 infection. The viral titers in the lung, liver, and kidney at day 3 postinfection (Fig. 7), as well as at days 1 and 2 (data not shown), were overlapping between the control and NK-depleted mice. This indicates that the differences observed in the in vitro stimulation assay (Fig. 6) were not caused by major changes in the viral load. Along this line, NK depletion had no effect on the initial Armstrong titers at day 3 or viral clearance by day 8 (data not shown), consistent with earlier reports (46, 47). At day 8 of Clone13 infection, there remained high viral loads in all mice, but there was a significant reduction in the NK cell–depleted mice in the liver, lung, and kidney compared with the control-treated mice (Fig. 7). By day 29, all of the control-treated mice continued to harbor 10^4–10^5 PFU/g tissue of virus; however, in all of the tissues measured, at least seven out of nine mice treated with NK1.1 reduced viral loads to near or below detection, which represents a 100–10,000-fold reduction in viral burden (Fig. 7). These findings show that the depletion of NK cells before infection does not impact the early replication of LCMV; however, there is a dramatic enhancement in the subsequent antiviral CD8+ T cell response that accelerates the control of this disseminating virus infection.

Discussion
Overall, these results show that NK cells negatively regulate adaptive T cell responses to persisting virus infection; T cell activity is significantly increased by NK cell depletion during persistent infection, whereas it is only marginally affected during acute infection. We also detect an increase in the stimulatory capacity of APCs from infected NK cell–depleted mice. We propose that NK cell depletion enhances APC functions to improve subsequent T cell responses and control of the infection.

The interactions between NK cells and DCs can have either positive or negative effects on the subsequent priming of T cell responses. These interactions have been shown to activate NK cells, alter NK and DC cytokine production, increase DC maturation, decrease Ag presentation, or result in DC lysis. The outcome of these interactions can be influenced by the differentiation status of the cells or the NK–DC ratio, as well as the type of infection or strain of virus (6, 48). For example, during Smith strain MCMV infection, NK cells promote CD8 T cell responses by limiting the
viral load and preserving the DC frequency (18); however, during K181-Perth MCMV infection, NK cells limit the ability of T cells to control a chronic infection by reducing the availability of infected APCs for T cell priming (44). Our data indicate that NK cell depletion did not increase either the DC number or their expression of MHC and B7 molecules or decrease expression of PD ligand molecules (Fig. 5, Supplemental Fig. 3). However, the ability of these APCs to stimulate CD8 T cell proliferation was severely blunted by the presence of NK cells (Fig. 6), and preliminary experiments indicate that NK cells also reduce the ability of APCs to stimulate CD4 T cell proliferation (data not shown).

The main APC population responsible for stimulating T cell proliferation early postinfection appears to be CD11c+CD8+ DCs (data not shown), and we are currently investigating whether NK cell depletion specifically affects this population of cells. The ability of NK cells to alter the APC stimulatory capacity could be mediated by multiple different mechanisms. Perhaps NK cells reduce the production of cytokines by APCs that either favor T cell stimulation, such as IL-12, or increase those that inhibit T cell proliferation, such as IL-10. Additionally, the trans-presentation of IL-15 by DCs is crucial for both NK and T cell responses (11, 49). The removal of NK cells may increase the availability of IL-15 (or perhaps IL-2 and IL-7) to support T cell accumulation. Another possibility is that even though the overall level of surface MHC class I expression is not affected by NK cell

**FIGURE 5.** NK cell depletion does not affect the number of splenic DCs. Adult B6 mice were infected with LCMV-Clone13. At days 0, 1, 2, and 3 postinfection (pi), splenocytes were harvested, stained for DC cell makers, and analyzed by flow cytometry. The dot plots shows examples of the staining used to identify CD11c+CD3+ DCs (A), CD11b+ DCs (B), CD8+ DCs (C), CD4+ DCs (D), and pDCs (E). Samples (A) and (E) depict all spleen cells, whereas samples (B), (C), and (D) were gated on CD11c+CD3+ DCs. The bar graphs display the total number of the indicated DC subsets and are depicted as mean + SEM; n = 3–8 for each group over three experiments.

**FIGURE 6.** APCs from infected mice depleted of NK cells show improved capacity to stimulate T cell proliferation. Naive P14 T cells from Thy1.1+ mice were stained with CFSE and incubated with splenocytes from day 0, 1, 2, or 3 LCMV-Clone13–infected B6 mice at a ratio of 1:20. The splenocytes were from mice that had been treated with NK1.1 or control Ab at days −2 and −3 before infection. (A) The histograms are gated on the CD8+Thy1.1+ P14 cells and show their dilution of CFSE on day 5 of the in vitro culture. The numbers indicate the percentage of P14 cells that had divided more than once. (B) The bar graphs display the percentage of the P14 cell population that had divided in the absence of exogenous peptide and is depicted as mean + SEM, n = 3–5 for each group over three experiments. The short lines above each bar indicate the percentage of divided cells after addition of 1 μM of LCMV peptide GP33-41 at the beginning of the culture. The asterisk indicates a significant difference (p < 0.05) between control and NK1.1-treated samples. *p < 0.05.

**FIGURE 7.** The depletion of NK cells improves viral clearance. A total of 2 × 10^6 P14 T cells from Thy1.1+ mice were transferred to Thy1.2+ B6 mice; the mice were treated with NK1.1 or control Ab at days −2 and −3 and then infected with LCMV-Clone13. The level of virus infection was determined by plaque assay from liver, lung, and kidney tissues (A–C) isolated from mice harvested on days 3, 8, or 29 postinfection, and serum samples isolated from mice (D) bled repeatedly over time. The solid line shows the average titers for each group; the dashed line represents the limit of detection. Significant differences between control and NK1.1-treated samples are indicated: *p < 0.05, **p < 0.01 [n = 7–11 over four experiments in (A)–(C); n = 3–5 over two experiments in (D)].
depletion, the proportion of MHC molecules bearing LCMV epitopes could be altered to better stimulate T cells. Further experiments are needed to distinguish between these possibilities.

In addition to the ability of NK cells to indirectly regulate T cell responses through effects on APCs (Fig. 6), NK cells can also directly impact T cell activity. Activated T cells can be direct targets of NK-mediated lysis (24). CD4+ T cells are crucial for sustaining CD8+ T cell responses during chronic virus infection (50), and some evidence indicates that NK cells focus perforin-mediated cytolysis against activated CD4 T cells, which ultimately causes defects in the CD8+ T cell response to LCMV (20). Although NK cells are capable of killing activated CD8+ T cells, this appears to be prevented by the interaction between CD48 on the T cells and 2B4 on the NK cells (23). We see greatly enhanced numbers of activated CD4+ T cells and LCMV-specific cytokine-positive CD4+ T cells when NK cells are depleted prior to infection (Fig. 2), consistent with the idea that activated CD4 T cells are direct targets of NK cell–mediated cytotoxicity. However, we believe that this is not the sole mechanism for NK cell regulation of the CD8 response. Our results (Fig. 4) show that delaying NK cell depletion until 2 d postinfection enhances CD4 T cell activation and cytokine production, yet the CD8 T cell response is not rescued in the presence of greater numbers of virus-specific CD4+ T cells. These data indicate that NK cell effects on CD8 T cells can occur independently of NK cell effects on CD4 T cells.

A hallmark of persistent viral infections is the establishment of the exhausted T cell phenotype, wherein T cells are maintained in a state of inactivity. Typically, this is thought to occur late during infection after the T cells have been repeatedly exposed to Ag. Surprisingly, the effect of NK cells on CD8+ T cell responses occurs before day 2, which is before endogenous virus-specific T cells commence cell division and become detectable (51, 52). This is consistent with NK cells impairing APC-mediated induction and early propagation of the T cell response and implies that the exhaustion phenotype can be programmed early during infection. Therefore, NK cells can promote T cell exhaustion by modifying the APCs that initiate T cell responses.

NK cells may have evolved dual functions to protect the host during infection: directly fighting against infection on the one hand (48) and controlling detrimental immune-mediated pathogenesis on the other hand. In contrast to the expected antiviral role of NK cells during virus infection, the removal of NK cells had no effect on the initial levels of LCMV replication. However, NK cell restriction of the CD8 T cell response permitted lengthy chronic infection (Fig. 7). Interestingly, the extent of NK-mediated regulation of CD8+ T cells varied by the chronicity of the virus: T cell activity was significantly increased by NK cell depletion during persistent infection, whereas it was only marginally affected during acute infection. This ability of NK cells to limit T cell activity may function to thwart immunopathology when infections are widely disseminating. If unchecked, the continuous production of viral Ags during persistent infection would stimulate T cells to destroy vital tissues. Therefore, the balance between NK cells directly fighting virus infection versus limiting antiviral T cell responses can be crucial in determining the survival of the host.

In summary, we confirm that the removal of NK cells greatly increases the quantity and the cytokine output of the CD8 T cell response (20, 21). Additionally, we show that these effects on CD8+ T cells are long lasting and establish that NK cells contribute to T cell exhaustion. Our data demonstrate that the major effect of NK cells on CD8+ T cell responses occurs within the first 2 d of infection. The NK cell–mediated regulation of CD8 cells is accomplished by a combination of effects, and we reveal that a major mechanism involves limiting the ability of APCs to stimulate T cell proliferation. This ability of NK cells to diminish T cell responses implies that the elimination of NK cells may be improving the success of vaccination, whereas promoting NK cell activity may be beneficial in protecting against immune-mediated disorders.

Disclosures

The authors have no financial conflicts of interest.

References


