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Weak vaccinia virus-induced NK cell regulation of CD4 T cells is associated with reduced NK cell differentiation and cytolytic activity

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Abstract

Natural killer (NK) cells control antiviral adaptive immune responses in mice during some virus infections, but the universality of this phenomenon remains unknown. Lymphocytic choriomeningitis virus (LCMV) infection of mice triggered potent cytotoxic activity of NK cells (NK^{LCMV}) against activated CD4 T cells, tumor cells, and allogeneic lymphocytes. In contrast, NK cells activated by vaccinia virus (VACV) infection (NK^{VACV}) exhibited weaker cytolytic activity against each of these target cells. Relative to NK^{LCMV} cells, NK^{VACV} cells exhibited a more immature ($CD11b^{-}CD27^{+}$) phenotype, and lower expression levels of the activation marker CD69, cytotoxic effector molecules (perforin, granzyme B), and the transcription factor IRF4. NK^{VACV} cells expressed higher levels of the inhibitory molecule NKG2A than NK^{LCMV} cells. Consistent with this apparent lethargy, NK^{Vacv} cells only weakly constrained VACV-specific CD4 T-cell responses. This suggests that NK cell regulation of adaptive immunity, while universal, may be limited with viruses that poorly activate NK cells.

Keywords

NK cells; CD4 T cells; LCMV; vaccinia virus; NKG2A; interferon; IRF4

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Introduction

Natural killer (NK) cells have been known to be cytotoxic to T cells and T cell lines since their initial discovery (Hansson et al., 1979; Nabel et al., 1981; Rabinovich et al., 2000), but recently there has been renewed interest in the capacity of NK cells to regulate adaptive immunity by targeting T cells during infection (Cook et al., 2015; Crouse et al., 2014; Gill et al., 2016; Lang et al., 2012; Lee et al., 2009; Rydyznski et al., 2015; Schuster et al., 2014; Sepulveda et al., 2015; Soderquest et al., 2011; Waggoner et al., 2011; 2014; Xu et al., 2014). Studies with lymphocytic choriomeningitis virus (LCMV) infections in mice showed that NK cells directly attack activated CD4 T ($CD4^{act}$) cells and lyse them in a perforin-dependent manner (Waggoner et al., 2011). This was shown by *in vivo* cytotoxicity assay analysis, wherein fluorescently-labeled splenocytes from LCMV-infected mice were transferred directly into other infected mice that were depleted, or not, of NK cells, and a selective NK cell-dependent loss of donor $CD4^{act}$ cells was detected 5 hours later. By virtue of this targeting of $CD4^{act}$ T cells, NK cells indirectly affected cytotoxic CD8 T lymphocyte (Waggoner et al., 2011) and germinal center B-cell responses (Rydyznski et al., 2015). Cytolytic NK cell regulation of T cells consequently altered the balance between viral clearance and persistence as well as that between protective immunity and damaging immune pathology (Waggoner et al., 2011).

Several studies have revealed the importance of NK-cell suppression of T cells in the LCMV (Cook et al., 2015; Cook and Whitmire, 2013; Crouse et al., 2014; Guo et al., 2016; Lang et al., 2012; Rydyznski et al., 2015; Su et al., 2001; Waggoner et al., 2011; Waggoner and Kumar, 2012; Waggoner et al., 2010; Xu et al., 2014) and murine cytomegalovirus (MCMV) systems (Andrews et al., 2010; Lee et al., 2009; Schuster et al., 2014; Su et al., 2001; Waggoner et al., 2011; Zamora et al., 2017), but work with other viruses has been more limited, such that the universality of this phenomenon is unclear. Our group previously used an *in vivo* cytotoxicity assay to demonstrate that activation of CD4 T cells during infection with several different viruses induced susceptibility of these cells to NK cell-mediated killing (Waggoner et al., 2011; 2010; Waggoner and Kumar, 2012). These viruses included LCMV, MCMV, mouse hepatitis virus (MHV), Pichinde virus (PICV), and vaccinia virus (VACV). Similarly, three viruses (LCMV, MHV, PICV) tested for their capability to induce NK cell killing were capable of stimulating this activity. In contrast, VACV infection failed to stimulate substantial NK cell lysis of activated $CD4^{act}$ cells in the *in vivo* assays (unpublished observations). This exception suggested that NK cell killing of $CD4^{act}$ cells might not be a universal phenomenon and that the explanation and possible significance of this should be examined. Here we question why VACV is a weak trigger for NK-cell killing of $CD4^{act}$ cells and whether NK cells have any impact on VACV-specific T cell responses. We characterize NK^{VACV} cells as being in a reduced state of activation and diminished cytolytic function. Nevertheless, these poorly activated NK cells still had a negative impact on VACV-specific CD4 T cell responses. For the purposes of this study, NK cells are defined by their expression of NK1.1 and the lack of CD3 expression.

Materials and methods

Virus strains and poly I:C treatment

The following virus strains were used with doses indicated in plague forming units (pfu)/mouse: lymphocytic choriomeningitis virus (LCMV) [Armstrong] 5×10^4 pfu; vaccinia virus (VACV) [Western Reserve] 2×10^6 pfu; mouse hepatitis virus (MHV) [A59] 8×10^5 pfu; and Pichinde virus (PICV) [AN3739] 1.5×10^7 pfu. Poly I:C was injected at a dose of 100 μg per mouse in HBSS. All infections and treatments were delivered by intraperitoneal injection.

Cell culture

YAC-1 cells were grown in RPMI (Gibco BRL) and L929 cells were grown in MEM (Gibco BRL). RPMI and MEM each were supplemented with 10% fetal calf serum (FCS), L-Glu (5 mM), and Penn-Strep (5 U/mL) at 37° C and 5% CO_2.

Antibodies

Fluorescently-labeled antibodies were purchased from: BD Biosciences - NK1.1 (PK136), CD3e (145-2C11), CD4 (RM4-5), CD8β (YTS156.7.7), CD44 (IM7), CD11b (M1/70), CD107a (H4A3), Ly49 A (A1), Ly49 C/I (5E6), Ly49 D (4E5), Ly49 F (HBF-719), Ly49 G2 (4D11); eBioscience – 2B4 (244F4), Ly49 H (3D10), NKp46 (29A1.4), NKG2D (CX5); BioLegend – CD43 (1B11), CD69 (H1.2F3), CD27 (LG.3A10) DNAM-1 (TX42.1), NKG2A (16a11), IFNAR1 (MAR1-5A3), PD-1 (J43), IRF4 (3E4); and Invitrogen - GzmB (MHGB05).

Peptides

All peptides were ordered from 21st Century Biochemicals based on previously reported sequences (Cornberg et al., 2010; Jing et al., 2005; Moutaftsi et al., 2007; Oseroff et al., 2005; 2008; Tscharke et al., 2005): $A3L_{270\text{-}277}$ (KSYNYMLL); $A11R_{198\text{-}206}$ (AIVNYANL); A18R49-63 (PKGFYASPSVKTSLV); $A20R_{233\text{-}247}$ (DNIFIPSVITKSGKK); $A47L_{138\text{-}146}$ (AAFEFINSL); $B2R_{46\text{-}60}$ (VKDKYMWCYSQVNKR); $B5R_{46\text{-}60}$ (FTCDQGYHSSDPNAV);$B8R_{20\text{-}27}$ (TSYKFESV); $D13L_{486\text{-}500}$ (PKIFFRPTTITANVS); $E9L_{179\text{-}193}$ (PSVFINPISHTSYCY); $F15L_{55\text{-}69}$ (TPRYIPSTSISSSNI); I1L7-21 (QLVFNSISARALKAY); and $K3L_{6\text{-}15}$ (YSLPNAGDVI).

Mouse strains and tissue harvesting

C57BL/6 mice were used for all cytotoxic and phenotypic NK cell studies. BALB/c mice were used only as donors for some in vivo cytotoxicity assays (as specified). C57BL/6 and BALB/c mouse strains were purchased from Jackson Laboratories and housed in the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS). All research was done under the guidance and approval of the UMMS Institutional Animal Use and Care Committee. Spleens were harvested from infected mice into RPMI media supplemented with 10% fetal calf serum (FCS), L-Glu (5 mM, Gibco), and Penn-Strep (5 U/mL, Gibco). Cells were gently pelleted by spinning at 800 × **g** for all centrifugation steps. Leukocyte preparations were made by treatment of bulk splenocytes

with 0.84% NH_4Cl in 10 mM Tris (pH 7.2) to lyse red blood cells followed by washing with HBSS containing FCS (10%) prior to antibody staining.

Flow cytometry

Splenocytes and peritoneal exudate cells were washed and rinsed in HBSS containing 2% FCS, and surface staining antibodies were applied in this same buffer. After washing, surface-stained cells were fixed for 5 minutes with Cytofix (BD Biosciences) followed by washing and storage in HBSS with 2% FCS. Cells stained for CD107a were incubated at 37 C and 5% CO_2 for 1 hour in RPMI with FCS, L-Glu, Penn-Strep and monensin (BD Bioscience). Nuclear staining of IRF4 made use of the PhosFlow (BD Biosciences) fixation and permeabilization reagents followed by washing and storage in HBSS containing FCS. Peptide stimulation of T cells for intracellular cytokine staining (ICCS) was performed using the Cytofix/CytoPerm kit (BD Biosciences) according to manufacturer's instructions. Granzyme B (GzmB) staining of NK cells was also performed using Cytofix/CytoPerm. Nuclear and cytoplasmic stains each preclude the use of the other and were always performed separately according to manufacturer's instructions, which also allow for the inclusion of surface stains.

Reagents and labeling dyes

Poly I:C (Invivogen) was dissolved in HBSS and stored in frozen aliquots at -20° C. Cell Trace Violet (Molecular Probes), DDAO far red (Molecular Probes), and CFSE were freshly dissolved in HBSS at 37° C before incubation with target leukocytes prior to *in vivo* cytotoxicity assays.

In vivo cytotoxicity assay

Targets were incubated with labeling dye in HBSS for 15 minutes in a 37° C water bath and thoroughly rinsed in HBSS before transferring to host mice by tail vein injection. Spleens were harvested from host mice after incubation for 5-6 hours. In assays measuring the killing of activated T cells, donors were LCMV-infected mice that had been previously depleted of NK cells by intraperitoneal injection of monoclonal antibody (mAb) against NK1.1 (PK136, Bio-XCell) at 25 μg/mouse. Donors were harvested at day 4 post-infection to ensure that $CD4^{act}$ and $CD8^{act}$ cells would be abundant among the transferred target cells. Activated T cells were identified by high CD43 and CD44 expression (Waggoner et al., 2011). In assays measuring the killing of BALB/c target leukocytes, spleens were harvested from naïve BALB/c mice (Brehm et al., 2005) and then leukocyte suspensions were prepared and labeled as described above.

Chromium release assay

YAC-1 target cells were labeled with $Na_2{}^{51}CrO_4$ (Perkin Elmer) for 1 hour, and then combined with lymphocytes from individual mice. Targets and effectors were incubated at 37° C and 5% CO_2 for 4 hours before centrifugation at 800 × **g** for 5 minutes. Supernatants were dispensed into Optiphase scintillation fluid (Perkin Elmer) and incubated overnight at room temperature to allow for passive mixing and resolution of sample turbidity prior to reading.

Trogocytosis assay

Leukocyte suspensions were prepared with room temperature buffers and media from spleens collected in room temperature RPMI containing FCS (10 %), L-Glu (5 mM), and Penn-Strep (5 U/mL). YAC-1 target cells were labeled as previously described (Daubeuf et al., 2006) using a stock solution of the fluorescently-labeled lipid SpDiI18C (Molecular Probes) dissolved in dimethyl formamide (Fisher) that was freshly diluted in Diluent C (Sigma-Aldrich) before labeling.

RNA isolation and RT-PCR

NK^{LCMV} and NK^{VACV} cells were sorted from day 3 post-infection bulk splenocytes using antibodies against NKp46 (PE, eBioscience), NK1.1 (APC, BD Biosciences), and CD3 (eFluor 450, eBioscience) sorted on a FACS Aria II (BD Biosciences) dedicated to biosafety level 2 plus (BSL 2+) usage. Fluorophores were chosen to minimize spectral overlap. Each pre-sort mixture was prepared from the pooled splenocytes of either 5 LCMV-infected, or 5 VACV-infected, C57BL/6 mice. Total RNA was purified from sorted splenocytes using the RNeasy Micro kit (Qiagen) and reverse transcribed into cDNA using random hexamer primers (Clontech). qPCR reactions were assembled with iQ SYBR Green Supermix (BioRad). Primers were ordered from Integrated DNA Technologies for perforin (Baars et al., 2005) (F: 5′-TGTGAACCCTAGGCCAGAGG-3′; R: 5′-GTGGAGCTGTTAAAGTTGCGG-3′), granzyme B (Revell et al., 2005) (F: 5′-ATCAAGGATCAGCAGCCTGA-3′; R: 5′-TGATGTCATTGGAGAATGTCT-3′), and the housekeeping gene HPRT (Baars et al., 2005) (F: 5′-GCAGTACAGCCCCAAAATGG-3′; R: 5′-AACAAAGTCTGGCCTGTATCCAA-3′) were used in separate reactions and run in triplicate on a CFX96 Real-Time PCR Machine (BioRad).

Data acquisition

Fluorescently-labeled cells were read by a LSR-II flow cytometer (BD Biosciences) and data were recorded in the FACS Diva suite followed by analysis in FlowJo. Samples from the ^{51}Cr-release assay were read by a Wallac Trilux 1450 MicroBeta for 1 minute/well.

Statistical analysis

Comparisons between NK^{LCMV} and NK^{VACV} cells used Student's t-test (unpaired, parametric) with Welch's Correction in the Prism analysis suite (GraphPad).

Results

NK cell-mediated killing of activated CD4 T cells is negligible during VACV infection

Experiments were designed to compare the cytolytic activity induced 3 days after infection of C57BL/6 mice with LCMV or VACV, where the former but not latter virus efficiently triggers NK cell killing of activated CD4 T ($CD4^{act}$) cell targets *in vivo*. Splenocytes from NK cell-depleted, LCMV-infected donor mice at day 4 p.i. were labeled with a fluorescent intracellular dye and then transferred by tail vein injection into LCMV- or VACV-infected hosts at day. Target splenocytes were taken from NK cell-depleted donor mice at day 4 in order to have a detectable number of activated CD4 T cell targets that had not already been

eliminated by NK cells. Furthermore, although LCMV-infected donors were used in this study, $CD4^{act}$ cells from VACV-infected donors are known to have a comparable susceptibility to killing by NK cells in this cytotoxicity assay (Waggoner et al., 2011). Harvesting splenocytes from NK cell-depleted LCMV-infected donors ensures that a sufficiently large number of vulnerable $CD4^{act}$ cells is obtained for use as common targets for both LCMV- and VACV-infected hosts. NK depletion of the donors also prevents killing of the desired $CD4^{act}$ targets by NK cells in the donor prior to harvest or a carryover of donor NK cells skewing the results of the assay.

The donor cells were transferred into infected hosts at day 3 p.i. because at that time point NK cells are highly active and are the only cytotoxic lymphocytes mounting a significant response (Welsh, 1978; Welsh et al., 1979). $CD4^{act}$ express high levels of CD43 and CD44, and the loss of CD4 T cells expressing these molecules was an indicator of cytotoxicity against them. This loss was previously shown to be dependent on perforin expression by the NK cells and associated with apoptosis of the T cells (Waggoner et al., 2011), thus indicating that the decrease in $CD4^{act}$ population size is due to the death of $CD43^{+}CD44^{hi}$ CD4 T cells rather than a downregulation of CD43 and CD44 on $CD4^{act}$ cells. Previous studies have also demonstrated that the loss of transplanted $CD4^{act}$ cells in infected host spleens cannot be accounted for by altered trafficking of $CD4^{act}$ cells to, and subsequent sequestration in, organs such as the lungs and liver (unpublished observations). Because $CD4^{act}$ cells were also lost in these host tissues when NK cells were not depleted, these data suggest that NK cell-mediated killing of $CD4^{act}$ cells was also occurring in the lungs and liver. Staining with Annexin V, to identify dying cells with exposed phosphatidylserine on the extracellular face of their plasma membranes, previously demonstrated that the percentage of Annexin V(+) staining $CD4^{act}$ cells decreased in the absence of NK cells, indicating that the $CD4^{act}$ cells in question were indeed dying disproportionately when NK cells were present (Waggoner et al., 2011). NK cells in this *in vivo* cytotoxicity assay have been shown to target $CD4^{act}$ cells specifically, but not B cells, CD8 T cells, or naïve CD4 T cells, thus allowing these cells and other donor leukocyte subpopulations to serve as internal controls whose percentages among lymphocytes are unaffected by NK cell-mediated cytotoxicity (Waggoner et al., 2011). The differences in percentages of remaining donor $CD4^{act}$ cells between the infected and uninfected hosts were used to calculate the percentage of $CD4^{act}$ cells killed.

The loss of donor $CD4^{act}$ cells was greater following transfer into mice infected with LCMV (mean 28%) as compared to recipient mice infected with VACV (mean 8%) in an *in vivo* cytotoxicity assay (Fig. 1A). Previously, LCMV-associated loss of $CD4^{act}$ cells was shown to be NK cell-dependent and required perforin (Waggoner et al., 2011). Here we observe that the percentages of splenic NK cells are reduced at day 3 of either infection relative to naïve (uninfected) control mice, but that the percentage of NK cells in VACV-infected mice was higher than that in LCMV-infected mice (Fig. 1B). These results argue that the poor killing of $CD4^{act}$ cells in VACV-infected mice was not due to virus-induced reductions in NK-cell frequencies.

VACV-induced NK cells demonstrate lower cytolytic activity than LCMV-induced NK cells

We first questioned whether the poor VACV-induced killing of $CD4^{act}$ cells reflected a selective defect or a more generalized dysfunction of NK^{VACV} cytolytic functionality. Compared to NK cells from mice infected with LCMV or injected with the interferon-inducer, poly I:C, NK cells isolated from VACV-infected mice were poor killers of YAC-1 lymphoma target cells in an *ex vivo* ^{51}Cr-release cytotoxicity assay (Fig. 1C). These results support the hypothesis that NK^{VACV} cells exhibit a more generalized cytotoxicity deficiency. As another correlate of NK cell-mediated cytotoxicity *in vivo*, day 3 LCMV- or VACV-infected C57BL/6 mice were injected with CFSE-labeled allogeneic BALB/c splenocytes bearing $H2^d$ ligands that trigger the activating receptor Ly49D on C57BL/6 NK cells (Nakamura and Seaman, 2001). In these experiments, target splenocytes from BALB/c donors were labeled with CFSE and co-administered with internal control C57BL/6 splenocytes, using sufficiently different concentrations of CFSE to allow resolution of the two donor populations during flow cytometry. BALB/c splenocytes were cleared more vigorously in LCMV-infected than in VACV-infected mice (Fig. 1D). The greater abundance of NK cells in the spleens of VACV-infected mice, compared to LCMV-infected mice, was another indicator that the NK^{VACV} cells have a lower cytotoxic capacity.

The studies comparing NK cell-mediated cytotoxicity against $CD4^{act}$ cells between LCMV- and VACV-infected hosts (Waggoner et al., 2011) set the parameters for how NK cells from these infections would be analyzed. Because these assays were performed in hosts at day 3 post-infection, this was chosen as the time when detailed comparisons of function and phenotype between NK^{LCMV} and NK^{VACV} would also be done.

The potency of NK cell-mediated cytotoxicity was compared between LCMV- and VACV-infected mice by measuring expression of the effector molecules granzyme B (GzmB) and perforin as indicators of cytotoxic potential. In this report, we define NK cells as NK1.1(+), CD3(-) lymphocytes and the term “prevalence” refers to the percentage of NK cells staining positive for a marker or receptor while “expression level” refers to the mean fluorescence intensity (MFI) associated with that marker or receptor among positive-staining cells. GzmB is closely associated with perforin-mediated cytotoxicity in NK cells (Bochan et al., 1995; Clement et al., 1990). During viral infections, NK cell-mediated cytotoxicity against $CD4^{act}$ cells depends on expression of the effector protein perforin (Waggoner et al., 2011). Among splenic NK cells, lower GzmB prevalence and expression levels, compared to NK^{lcmV} cells, were measured in NK^{VACV} cells at day 3 post-infection. These differences were apparent at both the mRNA and protein levels. Total RNA was harvested from NKp46(+) NK1.1(+) CD3(-) NK cells that were FACS-purified from the spleens of day 3 LCMV- and VACV-infected mice. Quantitative PCR (qPCR) of the cDNA synthesized from these RNA preparations revealed that in NK^{LCMV} cells, GzmB mRNA was approximately eight times more abundant than in NK^{VACV} cells ($p < 0.0001$) (Fig. 2A). The level of perforin mRNA was slightly higher in NK^{LCMV} cells by approximately 14% ($p < 0.05$) compared to NK^{VACV} cells (Fig. 2B). An overlay of histograms (representing NK cells from individual mice among the naïve, LCMV-infected, and VACV-infected cohorts) illustrates the relative distribution of GzmB-expressing NK cells and the level of GzmB expression on GzmB(+) NK cells (Fig. 2C). Statistical comparison of these three cohorts revealed that GzmB was

expressed in both a smaller percentage of NK cells from VACV-infected mice than from LCMV-infected mice (Figs. 2D) and at lower levels within the GzmB(+) NK^{VACV} cells (Fig. 2E) than for GzmB(+) NK^{LCMV} cells.

The delivery of cytotoxic granules during perforin-mediated cytolysis results in the surface exposure of CD107a, a component of lysosome-derived vesicle membranes (Olsen et al., 1990), at the surface of cytotoxic lymphocytes (Rubio et al., 2003). CD107a is required for efficient loading of perforin into lytic granules (Krzewski et al., 2013). CD107a that is deposited in the plasma membrane during the efflux of lytic granules will be endocytosed and recycled but accumulates to greater steady state surface densities on more cytolytically active NK cells and serves as a marker of degranulation (Aktas et al., 2009; Alter et al., 2004). CD107a-expressing splenic NK cells were more common during LCMV infection compared to infection with VACV (Fig. 2F) with no additional stimulus applied *in vitro* during the staining. Both infections induced NK cells with greater CD107a percentages than observed in uninfected mice (Fig. 2G), although variations in CD107a percentages for NK^{LCMV} cells among LCMV-infected mice prevented statistically significant differences between the LCMV- and VACV-infected cohorts. The steady state cell surface density of CD107a was lower in NK^{VACV} cells than in either naïve or NK^{LCMV} cells, which indicated weaker degranulation by NK^{VACV} cells (Fig. 2H). A previous study with human NK cells from whole blood stimulated *in vitro* by UV-inactivated VACV showed that VACV-stimulated NK cells contained lower percentages of CD107a(+) cells than controls receiving media without inactivated VACV (Moreira-Silva et al., 2014). Although these data appear contradictory to our own observations with regard to the percentages of CD107a(+) NK cells, they still indicate that exposure to VACV causes a decrease in the percentage of NK cells bearing CD107a on their surface. One explanation for this phenomenon could be that VACV exposure causes a decrease in the basal levels of vesicle trafficking present in naïve NK cells. The expression levels of CD107a on CD107a(+) NK cells were not provided in this study and thus could not be compared to our own results which are consistent with a VACV-mediated decrease in CD107a at the NK cell surface.

The term trogocytosis refers to the ability of cells to capture portions of surface membrane from other cells during close intercellular interactions. We used a trogocytosis assay previously developed for measuring T cell interactions with cultured dendritic cells (Daubeuf et al., 2006) and modified it to use YAC-1 cells as targets. NK^{VACV} cells assimilated less membrane material from labeled YAC-1 target cells than did NK cells from LCMV-infected mice (Fig. 2I). Both the percentages of trogocytosing NK cells and the amount of the SpDiI-18C YAC-1 membrane marker transferred onto NK cells were lower in NK^{VACV} cells than in NK^{LCMV} cells (Figs. 2J, 2K). The amount of SpDiI-18C transferred from the labeled YAC-1 cells to NK cells correlated positively with the level of GzmB expression in GzmB(+) NK cells when data from NK^{LCMV}, NK^{VACV}, and naïve NK cells were combined (Fig. 2L). These data are consistent with the relatively low killing potential of NK^{VACV} cells observed during *in vivo* and *ex vivo* cytotoxicity assays.

It seemed likely that the degree of trogocytosis in these NK cells might be related to their level of activation. CD69 is an activating C-lectin type receptor that serves as a reliable marker of NK cell activation. CD69 is poorly expressed on resting NK cells, but is

prominently expressed on activated NK cells (Lanier et al., 1988). There was a positive correlation between the level of CD69 on CD69(+) NK cells and the amount of SpDiI-18C-labeled target membrane incorporated by NK cells (Fig. 2M).

NK cells from VACV-infected mice have a lower activation state than their LCMV-induced counterparts

The lower levels of GzmB expression and trogocytosis among NK^{VACV} cells compared to NK^{LCMV} cells implied that NK^{VACV} cells might possess a lower activation state. As noted above, the level of CD69 expression varies among NK cells in accordance with the degree of SpDiI-18C absorption by NK cells from targets, and SpDiI-18C incorporation is lower among naïve NK cells and NK^{VACV} cells. The percentage of NK^{VACV} cells expressing CD69 was substantially lower than the corresponding percentage of NK^{LCMV} cells (Figs. 3A, B). The CD69 expression level on CD69(+) NK cells was also much lower in NK^{VACV} cells compared to NK^{LCMV} cells (Fig. 3C). The percentage of CD69(+) NK cells and the CD69 expression level correlated positively with the cytolytic effector molecule GzmB (Fig. 3D, E). The weak expression of CD69 and GzmB in NK^{VACV} cells, along with poor trogocytosis, were consistent with a lower activation state.

VACV-induced NK cells exhibit a delayed maturation profile

Various stages of murine NK cell maturation have been identified according to CD11b and CD27 expression (Chiossone et al., 2009). Maturity follows a progression beginning with immature $CD11b^{-}CD27^{+}$ NK cells that acquire CD11b to become positive for both markers. The double positive NK cells then shed CD27 to become part of the $CD11b^{+}CD27^{-}$ subset. The CD11b-$CD27^{+}$ and $CD11b^{+}CD27^{+}$ subsets are associated with cytokine production while the $CD11b^{+}CD27^{-}$ subset is considered the most cytolytically active. CD11b and CD27 expression have also been shown to define functionally distinct human NK cell populations (Fu et al., 2011).

Because NK^{VACV} cells exhibited lower cytotoxicity and appeared less activated than their LCMV-induced counterparts, we tested the hypothesis that a larger percentage of NK^{VACV} cells would be distributed among less cytolytically mature subsets. Indeed, there were significant differences between NK^{LCMV} and NK^{vaCv} cells in their distribution among the various maturity subsets (Fig. 4A). The most significant differences in subset frequency were for the $CD11b^{-}CD27^{+}$ and $CD11b^{+}CD27^{+}$ subsets (Fig. 4B). As hypothesized, a larger percentage of NK^{VACV} cells possessed the early immature $CD11b^{-}CD27^{+}$ phenotype than NK cells induced by LCMV. The relatively small percentages of $CD11b^{+}CD27^{-}$ NK^{LCMV} and NK^{VACV} cells might be explained by activity-dependent losses among this maturity subset following the destruction of multiple targets (Jewett and Bonavida, 1996; 1995), although the severity of these losses has been shown to vary according to the cytokine environment (Bhat and Watzl, 2007). The majority of NK^{LCMV} and NK^{VACV} cells are notably of the late immature $CD11b^{+}CD27^{+}$ phenotype that immediately precedes the cytolytically mature $CD11b^{+}CD27^{-}$ phenotype. The greater percentage of $CD11b^{+}CD27^{+}$ NK cells during LCMV infection, compared to VACV infection, suggests a larger reservoir of CD11 $b^{+}CD27^{+}$ NK^{LCMV} cells poised for progression to full cytolytic maturity.

NK cells from LCMV- and VACV-infected mice possess distinct NKR expression profiles

The cytolytic action of NK cells is controlled by a balance of signaling inputs through stochastically-expressed activating and inhibitory receptors (Lee and Biron, 2010; Taylor et al., 2000). Activating NK cell receptors (NKRs) are a diverse group of proteins whose members recognize various ligand types, including stress ligands (Bauer et al., 1999), cellular adhesion molecules (Bottino et al., 2003), and other non-MHC surface proteins (Bottino et al., 2003; Latchman et al., 1998). Activating receptors mediate signaling through kinases that promote NK cell functions (McVicar and Burshtyn, 2001). Inhibitory receptors generally recognize MHC ligands (Kärre et al., 1986), with some exceptions (Rahim et al., 2015; Stanietsky et al., 2009), and prevent NK cells from lysing potential target cells by signaling through intracellular phosphatases (Burshtyn et al., 1996) that remove phosphate groups from activated protein substrates (Campbell, 2016). When NK cells engage a cell with few ligands to bind inhibitory receptors, any activating signals present in the NK cell will predominate and lead to cytolysis.

The prevalence of various NKRs within the populations of LCMV- and VACV-induced NK cells, and the intensity of each NKR's expression on the NK cells staining positively for that particular NKR (i.e. expression level), were examined at day 3 post-infection. Five activating receptors (Ly49D, Ly49H, NKG2D, NKp46, and DNAM-1) and six inhibitory receptors (Ly49A, Ly49C/I, Ly49F, Ly49G2, NKG2A, and PD-1) were evaluated. Expression of the SLAM family receptor 2B4, for which both stimulatory (Cannons et al., 2011) and inhibitory activity (Waggoner et al., 2010) have been reported, was also examined. Comparative analyses revealed differential expression of several NKRs between LCMV- and VACV-infected mice. Both the prevalence of a receptor among the NK cell population and its expression level on positively staining NK cells were considered. Cohorts of LCMV- and VACV-infected mice, consisting of 4-5 animals, were harvested on day 3 post-infection, and splenic NK cells were analyzed for percentage of NK cells expressing a given NKR (i.e. prevalence) and the MFI of the NKR(+) cells (i.e. expression level). The LCMV- and VACV-infected cohorts were compared using a parametric, unpaired t-test using Welch's correction, which does not assume equal standard deviations. The inhibitory receptors Ly49C/I and 2B4 were more prevalent among NK^{LCMV} cells while NKG2A (another inhibitory receptor) was more prevalent among NK^{VACV} cells (Supp. Table 1). With regard to differences in expression level (of receptor-positive cells) between NK^{LCMV} and NK^{VACV} cells, five receptors (Ly49D, Ly49F, Ly49H, NKG2A, and DNAM-1) were higher in NK^{VACV} cells relative to NK^{LCMV} cells, and five (Ly49C/I, Ly49G2, NKG2D, 2B4, and PD-1) were lower (Supp. Table 2). Each receptor was surveyed in a minimum of four experiments, and the reported data are from representative experiments.

The NKR expression analysis assumed that the NK cells present in the spleen were representative of circulating NK cells; however, the potential differences in receptor prevalence and expression levels between compartments were considered. The peritoneal cavity was the primary site of inoculation for both the LCMV- and VACV-infected mice. For all receptors examined, except NKp46 and PD-1, the prevalence and expression levels on NK cells from the spleen and peritoneal exudate were compared. For each NKR, ratios were

computed for the percentage of NKR(+) splenic NK cells to the percentage of NKR(+) peritoneal exudate NK cells in individual mice (Supp. Table 3).

Most NKRs showed either no differences in prevalence between the spleen and peritoneal cavity, indicated by an average ratio of 1, or had similar relative differences between these two compartments for both LCMV- and VACV-infected mice. Ly49A(+) NK cells were more common in the spleens of VACV-infected mice, while there was no difference in the percentages of Ly49A(+) NK cells between the spleens and peritoneal cavities of LCMV-infected mice. This observation suggests that Ly49A is present on more NK cells in the spleen than in the peritoneal cavity during VACV infection. Ly49D(+), Ly49G2(+), and Ly49H(+) NK cells were less common in the spleens of VACV-infected mice than in the peritoneal cavity, but present at similar percentages in both compartments of LCMV-infected mice, suggesting a slight migration of these cells to the peritoneum during VACV infection.

DNAM-1 appeared to be present on fewer NK cells in the spleens of LCMV-infected mice than in the peritoneal cavity whereas there was little difference between compartments in VACV-infected mice. The lower percentage of DNAM-1(+) NK cells in the spleens of LCMV-infected mice compared to the percentage during VACV infection indicates either a mobilization of splenic DNAM-1(+) NK cells to the primary site of infection in the peritoneum or the proliferation of DNAM-1(+) NK cells already present in the peritoneum. This result is particularly interesting as it offers an explanation for the unexpected observations that expression of the activating receptor DNAM-1 exhibits a strong inverse correlation to GzmB expression (data not shown) and expression of DNAM-1 is higher in NK^{VACV} cells than in NK^{LCMV} cells (Supp. Tables 1, 2).

Ratios of expression levels on NKR(+) splenic NK cells to expression levels on NKR(+) peritoneal exudate NK cells were also computed (Supp. Table 4) for individual mice. For most NKRs, the average ratio of expression level on NKR(+) NK cells in the spleen to the expression level on NKR(+) peritoneal exudate NK cells was close to 1 for both LCMV- and VACV-infected mice, or exhibited similar differences between the two compartments for both LCMV- and VACV-infected mice. DNAM-1 expression level appeared similar on DNAM-1(+) NK cells between compartments for VACV-infected mice (average ratio of 0.96); however, in LCMV-infected mice, DNAM-1 expression appeared lower in splenic NK cells compared to peritoneal exudate NK cells (average ratio of 0.59). Ly49G2 expression in NK^{VACV} cells was similar between the spleens and peritoneal cavities of VACV-infected mice, yet slightly lower in the splenic NK cells of LCMV-infected mice than in the NK cells within their peritoneal cavities.

Expression of the inhibitory NK cell receptor NKG2A is higher in VACV-induced NK cells than in LCMV-induced NK cells

The differential expression of NKG2A between VACV- and LCMV-induced NK cells, as well as the strength of this receptor's statistically significant inverse correlation with GzmB expression, made it the best potential marker of poor cytolysis against $CD4^{act}$ cells to emerge from the screen. The low p-value and high R-squared value for the correlation between NKG2A and GzmB indicate that there is a highly significant and strong inverse correlation between NKG2A and GzmB expression levels (Supp. Table 2). Strong

correlations between NKG2A and GzmB in this system of virus-induced NK cell-mediated killing of $CD4^{act}$ cells were also notable because of previous reports linking NKG2A to NK cell-mediated killing of specific $CD4^{act}$ subsets during autoimmune pathologies (Leavenworth et al., 2011).

NKG2A displayed both increased prevalence and expression level in NK^{VACV} cells when compared to NK cells from LCMV-infected mice (Fig. 5A-C). Both the prevalence and expression level of NKG2A exhibited a strong inverse correlation to the cytotoxic effector GzmB (Fig. 5D, E). In trogocytosis assays with SpDiI18C-labeled YAC-1 target cells, the expression level (Fig. 5G), but not prevalence (Fig. 5F), of NKG2A inversely correlated with membrane exchange between NK cells and YAC-1 targets. The prevalence and expression levels of NKG2A correlated inversely with CD69 prevalence and expression levels, respectively (Fig. 5H-I). These data are consistent with observations that CD94, the co-receptor of NKG2A, has been shown to mitigate the activity of CD69 in NK cells (Borrego et al., 1999) and decreased NKG2A expression is associated with the upregulation of CD69 expression in γδ T cells (Wang et al., 2012).

The disparities between the prevalence of NKG2A(+) NK^{LCMV} and NK^{VACV} cells, and expression levels between these NK cells, appeared greatest in the less mature subsets. NKG2A was most prevalent among NK cells with the less mature $CD11b^{-}CD27^{+}$ phenotype (Figs. 5J-L). The greatest percentage of NKG2A expression was observed in $CD11b^{-}CD27^{+}$ NK^{VACV} cells, of which an average of 88% express NKG2A (Fig. 5L) compared to 62% of NK^{LCMV} cells (Fig. 5K). For NK^{LCMV} and naïve NK cells, NKG2A prevalence in the intermediately mature $CD11b^{+}CD27^{+}$ NK cell population was slightly lower than the prevalence observed in the total NK cell populations (Fig. 5J, K), yet similar for NK^{VACV} cells (Fig. 5L). Expression levels for NKG2A were also higher in the $CD11b^{-}CD27^{+}$ subsets, with NK^{VACV} cells having the highest expression levels of NKG2A (Fig. 5M-O). These results suggest that that NKG2A expression is also a hallmark of immaturity among NK cells, especially among poorly activated populations.

VACV-induced NK cells express low levels of the IRF4 transcription factor

Type 1 IFN is induced at high levels in many virus infections and has been shown to be a major activator of NK cells (Gidlund et al., 1978; Oehler et al., 1978; Santoli et al., 1978a; 1978b; Trinchieri et al., 1978; Welsh, 1978). The WR strain of VACV used here is a weak type 1 IFN (IFN-I) inducer, like most orthopoxviruses. Furthermore, although IFN-I may be produced during infection with VACV, the effective IFN-I titer is suppressed by an arsenal of viral accessory proteins (Perdiguero and Esteban, 2009). Average VACV-induced serum IFN-I titers at day 3 post-infection are lower than 3 [$\log_2$] units per 50 μL of serum, in contrast to more than 10 [$\log_2$] units for LCMV-infected mice and 6 [$\log_2$] units for mice examined after treatment with the potent IFN-I inducer poly I:C (Fig 6A). NK^{LCMV} cells had lower apparent expression of the interferon α/β receptor alpha chain (IFNAR1) than NK^{VACV} cells (Fig. 6B), perhaps due to either a possible ligand-mediated blocking of the staining antibody or a genuine downregulation of IFN-I receptors following activation (Branca and Baglioni, 1982).

Expression of IRF4 among NK^{VACV} cells is generally lower than among NK^{LCMV} cells (Fig. 6C) although variability among the LCMV-infected mice precludes statistical significance of the difference in IRF4 prevalence between the LCMV- and VACV-infected cohorts (Fig. 6D). IRF4(+) NK cells had lower expression levels of IRF4 (Fig. 6E) in NK^{VACV} cells than in NK^{lCmv} cells. IRF4 is a lymphoid cell-specific transcription factor that belongs to the IRF family, whose members are defined by the ability to bind the interferon-stimulated response element (ISRE). IRF4 is induced in human NK and T cells by IFN-I (Lehtonen et al., 2003) and mitogens, often acting in concert with other transcription factors such as PU.1, phosphorylated STAT6, and Bcl6 to promote cytokine production in T cells (Ahyi et al., 2009; Hu et al., 2002). IRF4 is also required for the proper function of mature B and T cells (Mittrücker et al., 1997). There were strong positive correlations between IRF4 and GzmB with respect to both prevalence and expression levels when NK^{VACV} and NK^{LCMV} cells were compared (Fig. 6F, G). Similarly, strong positive correlations were observed between IRF4 and CD69 (Fig. 6H, I). CD69 expression is known to be subject to induction by IFN-I (Gerosa et al., 1991) and might be expected to coincide with IRF4 if the expression of IRF4 is IFN-driven. There was a significant inverse correlation between the prevalence of NKG2A and IRF4 (Fig. 6J) while no correlation was observed between the expression levels of NKG2A and IRF4 (Fig. 6K). It is noteworthy that, despite the variability in IRF4 prevalence among NK cells from LCMV-infected mice, these cells maintain the correlations between IRF4 prevalence and the prevalence of GzmB, CD69, and NKG2A (Fig. 6F, H, J).

NK cell-dependent suppression of VACV-specific CD4 T cell responses late in VACV infection

The poor cytolytic capacity of the NK^{VACV} cells motivated us to question whether NK cells had as much effect on the T cell response to VACV as they have on the T cell response to LCMV and other viruses. First we questioned whether the depletion of NK cells had an effect on VACV titers. An increase in VACV titers after NK cell depletion might suggest that NK cells were directly controlling the virus. Alternatively, a decrease in viral titers would be consistent with NK cells controlling the T cells that regulate viral titer. VACV titers were measured at days 4, 6, 8, and 10 post-infection in the liver and fat pads, and found to be similar in NK cell-depleted mice and undepleted controls (Supplementary Fig. 1). Virus was cleared in the liver by day 8 and lingered longer in the fat pads, but titers were similar in both groups of mice. These data indicate that any increase in the antiviral CD4 T cell population in the absence of NK cells would not be due to marked differences in antigen load.

The failure of NK^{VACV} cells to kill $CD4^{act}$ cells effectively early in infection, relative to NK cells induced by other virus infections, was likely due to the poor cytolytic capacity of the NK^{VACV} cells during this discrete time period. However, it was unclear if adaptive immunity was completely unaffected by NK cells in the VACV system, or whether effects would be noticed after a longer time period of infection. Thus, the total number and percentages of CD4 and CD8 T cells were examined over a time course spanning day 4 to day 14 post-infection. The number and percentage of total CD4 T cells present within the spleen lymphocyte population of NK cell-depleted mice were comparable to the values observed in

non-depleted control mice until day 14, when both the number and frequency of CD4 T cells appeared slightly lower in the absence of NK cells, perhaps due to resolution of the response (Fig.7A, C). The CD8 T cell numbers were not statistically different at any time point, although the percentage may have been slightly higher in the absence of NK cells (Fig. 7B, D). Because $CD4^{act}$ cells, identified by the expression of CD43 and high expression of CD44 ($CD43^{+}CD44^{hi}$), are the target of NK cell-mediated killing during viral infections, it was important to determine if these $CD4^{act}$ cells were culled by NK cells during days 4 to 14 post-infection. On most days during this time course, the presence of NK cells had no significant effect on the frequencies of $CD4^{act}$ cells except for on days 6 and 10 p.i. when $CD4^{act}$ cells were slightly more abundant in the absence of NK cells (Fig. 7E). There were no NK cell-dependent differences in the frequencies of $CD8^{act}$ cells on any of the days examined (Fig 7F).

As another indicator of the mobilization of the T cell response into he infection, spleen leukocytes were stimulated with antibody to CD3 and tested for IFNγ production in intracellular cytokine assays (Fig. 7G, H, solid lines). Background stimulation without anti-CD3 antibody is shown in the dotted lines. There were no significant differences in the responses of either CD4 or CD8 T cells to NK cell deletion, except perhaps for the day 14 CD4 T cells, for which the NK cell-depleted samples appeared to have higher percentages. For better statistical reliability, T cells from mice from four day 14 experiments were stimulated with anti-CD3 and tested for IFNγ production. Fig. 7I and J show modest but statistically higher frequencies of IFNγ producing both CD4 and CD8 cells, with the CD4 cells being of particularly high significance ($p = 0.0002$). To test for specific T cell responses, the T cell responses to 14 VACV-encoded CD4 T cell epitopes and 5 VACV-encoded CD8 T cell epitopes were evaluated during the course of infection. Responses to the CD4 epitopes were very weak and not easily distinguished from background responses during the earlier stages of infection. However, with background subtracted, weak but elevated responses to some epitopes, such as B5R, could be detected at Day 14 and were slightly higher in NK-depleted mice (Fig. 7K). The percentages of T cells responsive to the CD8 epitopes were more easy to detect but did not seem very different in the NK-depleted vs. control group, as shown with the immunodominant CD8 epitope, B8R (Fig. 7L). These data suggest that, despite their poor cytolytic activity, NK^{VACV} cells might still have a modest effect on the virus-specific CD4 T cell response over time.

Discussion

This report addresses the question of whether the NK cell-mediated control of adaptive immunity could be a universal phenomenon in viral infections. We demonstrate that NK^{VACV} cells, in contrast to NK cells induced by the other virus infections previously tested (Waggoner et al., 2011), including LCMV, exhibited poor capacity to lyse activated CD4 T cells *in vivo*. NK^{VACV} cells were also poorly cytotoxic against YAC-1 lymphoma cells *ex vivo* and against allogeneic leukocytes *in vivo*. In comparison to NK^{LCMV} cells, this poor cytotoxicity of NK^{VACV} cells was associated with lower levels of mRNA encoding the cytolytic effector molecule GzmB and also lower levels of GzmB protein. The NK^{Vacv} cells skewed more dramatically towards an immature differentiation state than NK^{LCMV} cells and expressed intermediate levels of the activation molecule CD69 and high levels of the

inhibitory NKR NKG2A. This reduced differentiation and cytolytic activity correlated with a poor type 1 IFN response and relatively low expression of the IFN-associated transcription factor, IRF4. Expression of IRF4 correlated with the expression of granzyme B and other activation and maturation markers.

Despite the poor cytolytic activity at the peak of the NK cell response, NK^{VACV} cells maintained some level of activity over and above naïve NK cells and clearly were responding to infection. This was indicated by the moderate expression of activation markers and effector molecules. Indeed, extensive analyses of five experiments at day 14 post-infection showed an elevated CD4 T cell response to the class II MHC-presented VACV-encoded epitope B5R in the absence of NK cells. There also was a significantly elevated response to CD4 T cells stimulated with anti-CD3 at this time. This is consistent with the concept that these cytolytically weak NK cells could still have a modest though weak impact on the CD4 T cell response over a sustained period of time. In contrast to the effect on CD4 T cells, there was little effect of NK cell depletion on the epitope-specific CD8 T cell epitopes, which in the LCMV system have been reported to be poor targets for activated NK cells due to elevated expression of CD48, which drives negative signals to NK cells by virtue of the receptor 2B4 (Waggoner et al., 2010).

The ability of NK cells to exert direct control over VACV in the mouse model has long been a subject of controversy and our data in this report (Supplementary Fig. 1) indicate that they do not directly control VACV in males of the C57BL/6 mouse strain. Previous work suggesting that NK cells control VACV might be accounted for by the possibility that gamma delta (γδ) T cells are the lymphocyte population behind this phenomenon. The γδ T cells express high levels of the NK cell marker asialo-GM1 and low levels of NK1.1. Our method of anti-NK1.1 mAb-mediated depletion has been carefully designed to use a dose of anti-NK1.1 sufficient for robust elimination of NK cells while leaving other known NK1.1-expressing populations, such as γδ T cells, unperturbed because they express levels of NK1.1 that are insufficient to make them vulnerable to opsonization and antibody-dependent cell cytotoxicity (ADCC). Nevertheless, our findings that VACV viral load in the liver and fat pads was unaffected by the lack of NK cells allowed us to analyze the influence of NK cells on the developing T cell response without having to worry that any differences in the absence of NK cells were being caused by differences in viral load rather than the NK cells themselves. Indeed, as mentioned above, our data indicate that, as expected, there was only a modest and barely detectable increase in the CD4 T cell response when NK cells had been depleted.

Our data demonstrate that NK^{VACV} cells are poorly activated and ineffective at killing $CD4^{act}$ cells. IFN-I is a strong driver of the NK cell response (Welsh, 1978) and low levels of IFN-I or other cytokines found during VACV infection seem sufficient for the weak activation of some crucial NK cell functions. Previous work from the Yang laboratory has supported the requirement of IFN-I signaling (Martinez et al., 2008), and the STAT1 transcription factor on which it depends (Fortin et al., 2013), in the VACV system. Genetic studies with knockout mice for toll-like receptor 2 (TLR2) and myeloid differentiating factor 88 (MyD88) have suggested that signaling through the TLR2-MyD88 pathway is necessary for the activation of NK cells during VACV infection (Martinez et al., 2010). These studies

suggest that VACV-induced NK cells still depend on the relatively weak IFN-I signaling present during VACV infection, yet these cells also might require other activating signals to augment their cytotoxicity.

The finding that expression of the inhibitory receptor NKG2A was higher in VACV-induced NK cells than in LCMV-induced NK cells was intriguing because of reports that NKG2A might play a role in inhibiting the NK cell-mediated control of autoreactive $CD4^{act}$ cells that otherwise lead to autoimmune disorders (Leavenworth et al., 2011; 2010; Lu et al., 2007). The data presented here reveal that NKG2A expression was not only higher in NK^{VACV} cells relative to NK^{LCMV} cells, but it also had strong inverse correlations with the activation marker CD69 and the cytotoxic effector GzmB. Our data suggest that NKG2A is a marker of a poorly activated NK cell; however, further studies are required before functional importance can be assigned to NKG2A in modulating the ability of NK cells to kill virus-induced $CD4^{act}$ cells. Interestingly, in human NK cells, the transcription factor Gata-3 increases transcription from the NKG2A promoter (Marusina et al., 2005), and there are possible binding sites for Gata-3 in the mouse NKG2A promoter. A hallmark of VACV infection is a weak type I interferon response, and IFN-I has been shown to suppress Gata-3 transcription from an alternate first exon in human Th_2 cells (Huber et al., 2014).

In conclusion, NK^{VACV} cells are phenotypically distinct underperformers among NK cells from viral infections with regard to their abilities to kill $CD4^{act}$ cells and other targets. This report reveals that the lower overall cytotoxicity of NK^{VACV} cells is associated with lower activation and a shift of the NK cell population towards lower maturity states. A poor type 1 IFN response is likely at least partially responsible for this NK^{VACV} cell phenotype, which is also associated with low expression of GzmB and IRF4. NKG2A was more highly expressed among NK^{VACV} cells compared to their LCMV-induced counterparts, but we have no evidence that this NKR is functionally important other than being a marker of poorly activated cells. Overall these data show why $CD4^{act}$ cells are poorly killed by NK^{VACV} cells during cytotoxicity assays *in vivo*. This might suggest that NK cells are poor regulators of adaptive immunity during VACV infection. However, when the long-term effects of NK cell depletion were examined, there were substantially higher percentages of CD4 T cells specific for one VACV-encoded epitope late in infection. This would argue that the role of NK cells in regulating adaptive immunity may indeed be a universal phenomenon, even with viruses like VACV that are poor inducers of NK cell activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- NK^{VACV} cells are atypically poor killers of CD4 T cells early in infection.
- NK^{VACV} cells are less cytotoxic and mature than CD4 T cell-killing NK^{LCMV} cells.
- The inhibitory receptor NKG2A is enriched in NK^{VACV} cells.
- Poor cytotoxicity of NK^{VACV} cells corresponds to low IFN-I and IRF-4 expression.
- Nevertheless, NK^{VACV} cells still partially reduce the CD4 response by day 14 of infection.

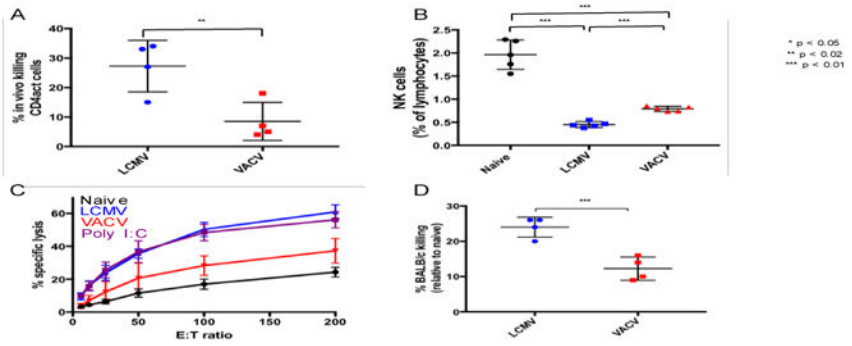

Figure 1.

Comparison of cytolytic potential between LCMV-and VACV-induced NK cells. **A.** Percentage of labeled donor splenic $CD4^{act}$ cells from day 4 LCMV-infected mice killed in day 3 LCMV-infected and VACV-infected hosts after delivery of 6×10^7 labeled LCMV-infected (day 4) splenocytes, from NK cell-depleted donors, by tail vein injection and recovery in splenocyte preparation following 5 hour incubation. **B.** Splenic NK cell frequencies among naïve, LCMV- and VACV-infected mice at day 3 post-infection. **C.** Cytotoxicity of naïve, virally-induced, and poly I:C- activated splenic NK cells against ^{51}Cr-labeled YAC-1 cells. **D.** Killing of BALB/c donor splenocytes in LCMV-and VACV-infected C57BL/6J hosts after delivery of 6×10^7 labeled naïve BALB/c splenocytes by tail vein injection and recovery in splenocyte preparations following 2-hour incubation. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$. NK cell gating scheme: Singlets (FSC)>Lymphocytes(SSC)>NK 1.1(+) CD3(-).

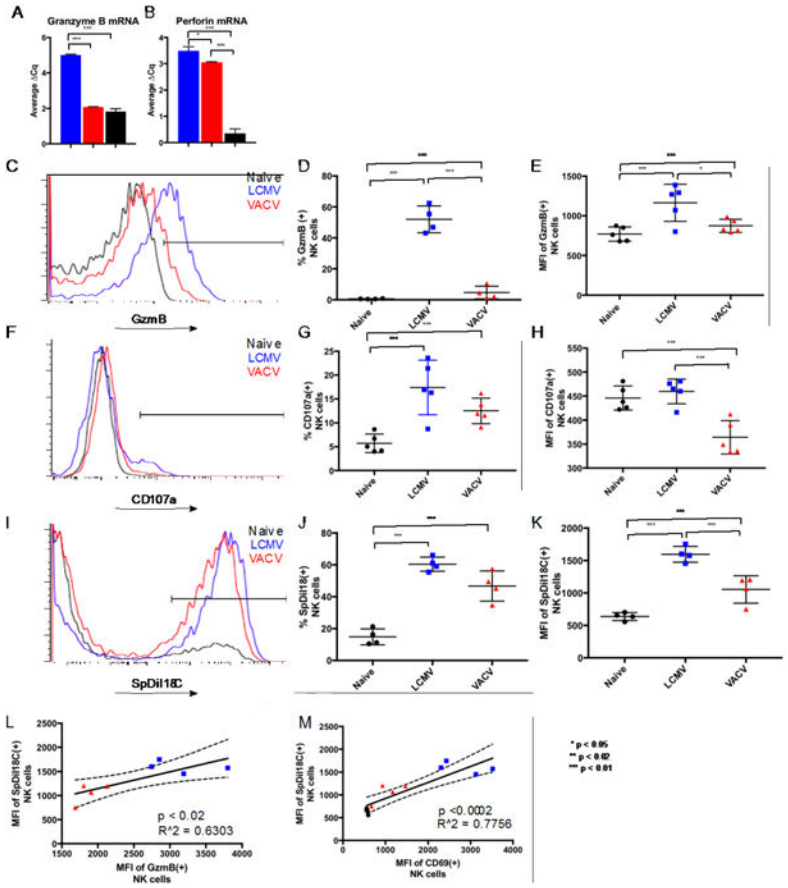

Figure 2.

Comparison of cytolytic indicators between LCMV-and VACV-induced NK cells. **A, B.** GzmB and Perforin mRNA levels in day 3 purified splenic NKp46(+) NK 1.1(+) CD3(-) NK^{LCMV} (blue bars) and NK^{VACV} (red bars) cells compared to bulk LCMV-infected splenocytes (black bars). **C.** Overlaid flow cytometry profiles showing GzmB protein expression in NK cells from representative naive and infected mice (each histogram represents NK cells from a single mouse). **D.** Prevalence of GzmB(+) NK cells among total splenic NK cells. **E.** Expression level of GzmB in GzmB(+) splenic NK cells. **F.** Overlaid flow cytometry profiles showing steady state exposure of CD107a in the plasma membrane of splenic NK cells, after one hour *ex vivo* incubation with no additional stimulus, from representative naive and infected mice (each histogram represents NK cells from a single mouse) **G.** Percentages of splenic NK cells with detectable surface CD107a. **H.** Amounts of CD107a trafficked to the surface of CD107a(+) splenic NK cells. **I.** Comparison of trogocytosis among splenic NK cells from representative individual naïve, LCMV-, and VACV-infected mice in overlaid flow cytometry profiles. **J.** Frequency of membrane transfer to splenic NK cells. **K.** Amount of labeled YAC-1 target cell membrane absorbed by naïve and infected splenic NK cells through trogocytosis. **L, M**. Association of intensity of transferred SpDiI18C-labeled target membrane with GzmB and CD69 expression levels on splenic NK cells. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$. NK cell gating scheme: Singlets (FSC)>Lymphocytes(SSC)>NK 1.1(+) CD3(-).

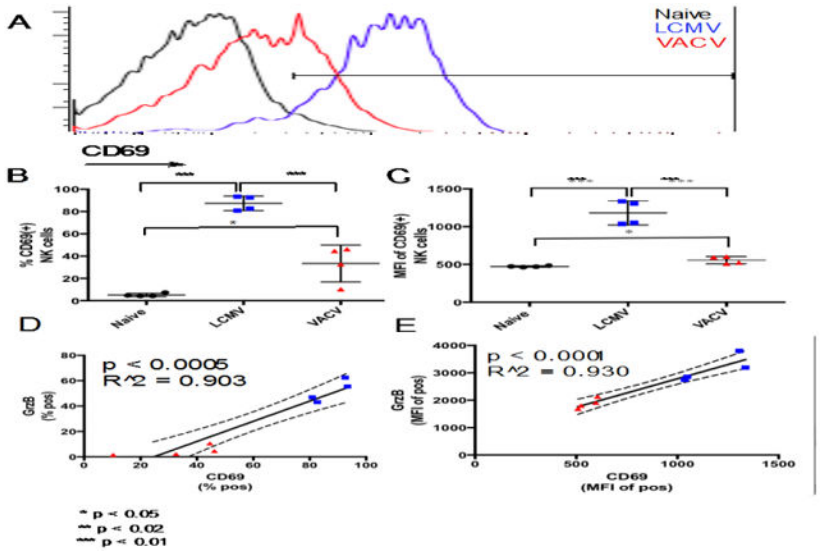

Figure 3.

Comparison of NK cell activation between day 3 LCMV- and VACV-induced splenic NK cells. **A.** Overlaid flow cytometry profiles showing CD69 protein expression in NK cells from representative naive and infected mice (each histogram represents NK cells from a single mouse). **B, C.** CD69 prevalence and expression level in NK cells. **D, E.** Correlation of CD69 prevalence and expression level to GzmB. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$. NK cell gating scheme: Singlets (FSC)>Lymphocytes(SSC)>NK 1.1(+) CD3(-).

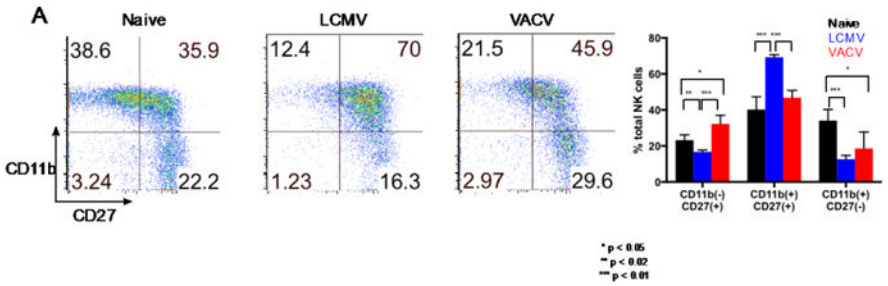

Figure 4.

Differences in maturation between day 3 post-infection LCMV- and VACV-induced splenic NK cells. **A.** Expression of the maturity markers CD11b and CD27. **B.** Distribution of NK cells among CD11 b/CD27 subsets. Bars represent distribution of NK cells across CD11b/CD27 subsets for cohorts of naïve (black bars, n=4), LCMV-infected (blue bars, n=4), and VACV-infected (red bars, n=4) mice. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$. NK cell gating scheme: Singlets (FSC)>Lymphocytes(SSC)>NK 1.1(+) CD3(-).

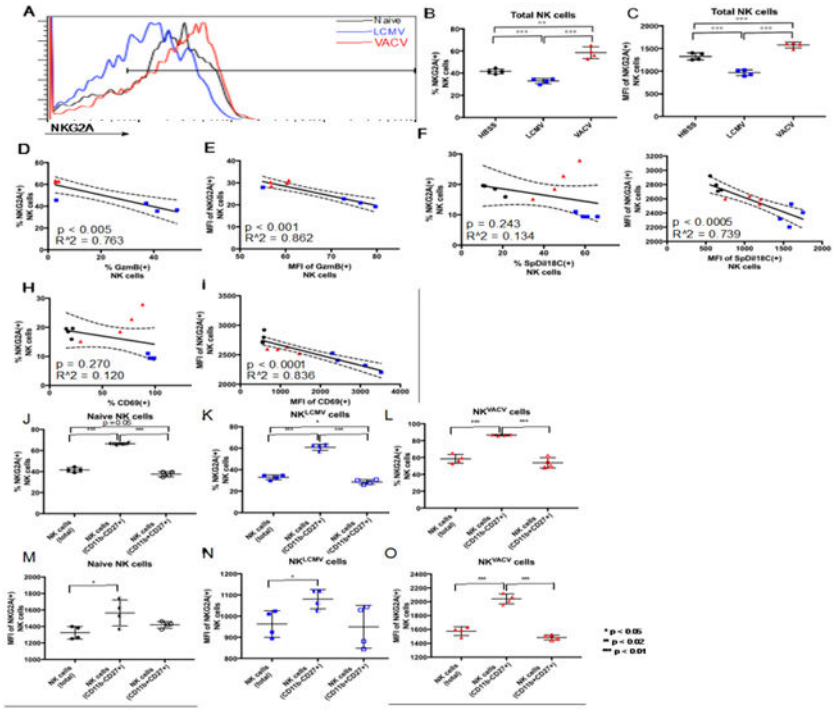

Figure 5.

Differential expression of NKG2A between LCMV- and VACV-induced splenic NK cells. **A.** Overlaid flow cytometry profiles showing NKG2A protein expression in NK cells from representative naive and infected mice (each histogram represents NK cells from a single mouse). **B, C.** NKG2A prevalence and expression level in NK cells. **D, E.** Correlation of NKG2A prevalence and expression level to GzmB. **F.** Correlation of percent NKG2A(+) NK cells to the percentage of NK cells engaged in membrane exchange. **G.** Correlation of NKG2A expression level in NKG2A(+) NK cells to the degree of trogocytosis in SpDiI-18C(+) NK cells. **H, I.** Correlation of NKG2A prevalence and expression level to CD69. **L-O.** Prevalence and expression levels of NKG2A for CD11 b-$CD27^+$ and CD11b $^+CD27^+$ NK cell maturity profiles compared to total NK cells for naïve NK cells (**J, M**), NK^{LCMV} cells (**K, N**), and NK^{VACV} cells (**L, O**). * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$. NK cell gating scheme: Singlets (FSC)>Lymphocytes(SSC)>NK 1.1(+) CD3(-).

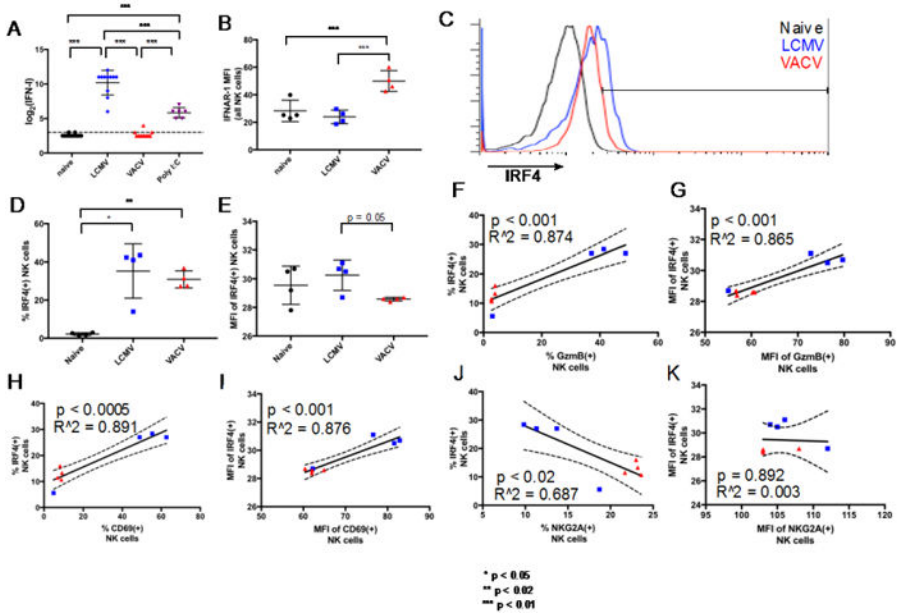

Figure 6.

Comparison of IFN-I response and IRF4 expression between LCMV- and VACV-induced splenic NK cells. **A.** Serum IFN-I titers. **B.** Average IFNAR1 expression on all NK cells present in naïve, LCMV-, and VACV-infected mice. **C.** Overlaid flow cytometry profiles showing IRF4 protein expression in NK cells from representative naive and infected mice (each histogram represents NK cells from a single mouse). **D, E.** IRF4 prevalence and expression levels in NK cells. **F-I.** Correlation of IRF4 prevalence and expression levels to GzmB and CD69. **J, K.** Correlation of IRF4 prevalence and expression level to NKG2A. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$. NK cell gating scheme: Singlets (FSC)>Lymphocytes(SSC)>NK 1.1(+) CD3(-).

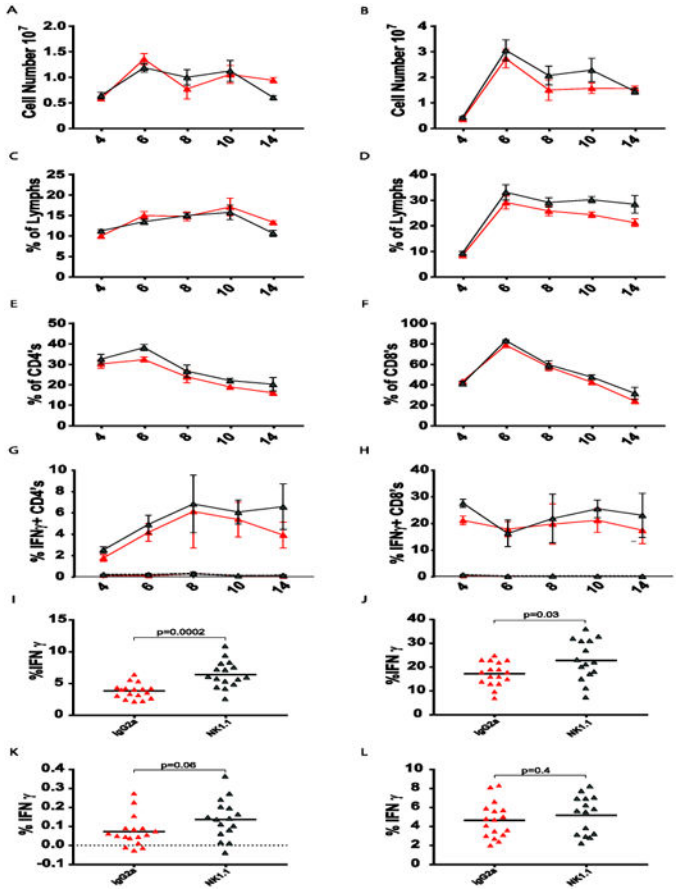

Figure 7.

Effects of VACV-induced NK cells on T cell populations. VACV-infected mice were treated with either monoclonal antibody against NK1.1 (red) or the non-depleting isotype control antibody (grey) prior to infection. Data in panels A-H are pooled from three identical experiments, with n = 8-10 per group, except for day 14, which was one experiment in a complete time course with n = 5 per group. Day 14 data from panels I-L are pooled data from four identical experiments. **A, B.** Comparison of CD4 and CD8 T cell population size in the presence and absence of NK cells. **C, D.** CD4 and CD8 T cell percentages in the presence and absence of NK cells. **E, F.** $CD4^{act}$ and $CD8^{act}$ cell percentages in the presence and absence of NK cells, as indicated by co-expression of CD43 and CD44. **G, H.** Percentages of IFNγ(+) CD4 and CD8 T cells responsive to activation by antibody to CD3ε (solid lines) or control background (dotted lines), in the presence and absence of NK cells. **I, J.** Percentages of anti-CD3-activated IFNγ(+) CD4 and CD8 T cells in the presence and absence of NK cells at day 14 post-infection. **K.** B5R-specific antiviral IFNγ (+) CD4 T cell response at day 14 p.i. with the background IFNγ (+) percentage subtracted. **L.** The immunodominant B8R-specific antiviral IFNγ (+) CD8 T cell subpopulation at day 14 with the background IFNγ (+) percentages subtracted. T cell pre-gating scheme: Singlets (FSC)>Lymphocytes(SSC).