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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.

1. 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 h 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, 2010; Waggoner and Kumar, 2012; 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 $CD4^{act}$ 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

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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.

2. Materials and methods

2.1. 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.

2.2. 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.

2.3. 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).

2.4. 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): $A3L_{270–277}$ (KSYNYMLL); $A11R_{198–206}$ (AIVNYANL); $A18R_{49–63}$ (PKGFYASPSVKTSLV); $A20R_{233–247}$ (DNIFIPSVITKSGKK); $A47L_{138–146}$ (AAFEFINSL); $B2R_{46–60}$ (VKDKYMWCYSQVNKR); $B5R_{46–60}$ (FTCDQGYHSSDPNAV); $B8R_{20–27}$ (TSYKFESV); $D13L_{486–500}$ (PKIFFRPTTITANVS); $E9L_{179–193}$ (PSVFINPISHTSYCY); $F15L_{55–69}$ (TPRYIPSTSISSSNI); $I1L_{7–21}$ (QLVFNSISARALKAY); and $K3L_{6–15}$ (YSLPNAGDVI).

2.5. 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 260 × *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.

2.6. 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 min 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 h 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.

2.7. 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.

2.8. In vivo cytotoxicity assay

Targets were incubated with labeling dye in HBSS for 15 min 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 h. 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.

2.9. Chromium release assay

YAC-1 target cells were labeled with $Na_2{}^{51}CrO_4$ (Perkin Elmer) for 1 h, and then combined with lymphocytes from individual mice. Targets and effectors were incubated at 37 °C and 5% CO_2 for 4 h before centrifugation at 260 × *g* for 5 min. 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.

2.10. 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.