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The Functional Impairment of Natural Killer cells during Influenza Virus Infection

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Abstract

Natural Killer (NK) cells play a critical role in clearing influenza virus, which primarily infect lung epithelial cells. However, the ability of influenza virus to infect and manipulate NK cells has not been studied. In this context, we hypothesized that influenza virus can target NK cells leading to functional impairment in their ability to mediate cytotoxicity and cytokine/chemokine generations. Here, we show influenza virus, PR8, can enter inside the NK cells. This infection did not alter the expression levels of activating, inhibitory or developmental receptors of NK cells. However, infection of NK cells by PR8 reduced the cytotoxicity to tumor cells that represent ‘*induced-self*’ and ‘*missing-self*’. PR8-infection also significantly down regulated the NCR1, NKG2D, Nkpr1c, Ly49D and CD244 receptors-mediated generation of pro-inflammatory cytokines and chemokines. Mutations in the nonstructural protein 1 (NS1) of influenza virus further augmented the functional impairment of NK cells. Our observations demonstrate the presence of novel but yet to be explored mechanism by which the influenza virus can evade immune detection.

Influenza virus is the etiological agent that causes acute respiratory disease with a high rate of mortality, especially among the elderly and children¹. A large number of influenza infection-induced deaths are reported every year all over the world. Infection with influenza virus generates both innate and adaptive immune responses. B and T cells play important roles in influenza virus clearance². Involvement of NK cells in influenza infection has been recognized³ and confirmed by *in vivo* deletion studies using anti-asialo GM1 or NK1.1 antibodies⁴. NK cells through their natural cytotoxicity and cytokine/chemokine production control influenza at the early stages of infection^{5–7}. Recent studies highlighted the direct involvement of one of the NK cell activation receptor, NCR1 in recognizing influenza-derived HA protein^{8–10}. Mice with NCR1-dead mutation were more susceptible to influenza infections compared to wild type (WT)¹⁰. Thus, NK cells play a critical role in the clearance of influenza infection.

Influenza virus primarily targets the lung epithelial cells for infection and replication. Recent studies indicate that macrophages and dendritic cells can also be infected by influenza virus^{11–13}. However, the ability of this virus to infect and manipulate effector lymphocytes

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has not been explored. Since NK cells are one of the major lymphocyte population in lungs¹⁴, we hypothesized that they can be targeted by influenza virus. Does influenza virus infect NK cells? If so, does this infection affect their effector functions? To answer these questions, we performed both *in vitro* and *in vivo* experiments. Our *in vitro* results demonstrate that mouse-adopted human influenza virus, PR8, can non-productively infect NK cells. Furthermore, NK cells from infected lung contained PR8-derived matrix protein (M2) confirming the ability of this virus to enter inside the NK cells. *In vitro* infection of NK cells with PR8 did not lead to infectious levels of viral titers and failed to alter the expression levels of NK activating or inhibitory receptors. However, PR8 infection led to a reduction in the natural cytotoxicity of NK cells. In addition, PR8-infection significantly down regulated the ability of NK cells to generate pro-inflammatory cytokines and chemokines. Infecting NK cells with a PR8 virus that contained mutations in the non-structural protein 1 (NS1) further augmented these functional impairments. Our present observations provide novel insights into an unknown molecular mechanism used by the influenza virus to manipulate the innate immune system.

RESULTS

NK cells express sialic acid side chains specific for influenza viral entry

Influenza viruses bind to the terminal sialic acid of membrane glycoproteins and glycolipids of susceptible cells. They enter into host cells through the interaction of their hemagglutinin (HA) protein with α -2,3 and/or α -2,6-linked sialic acids (SA). In addition to lung epithelial cells, multiple cell types including macrophages¹⁵ and DCs^{16,17} have been shown to express these specific SA side chains. However, the presence of these SAs in immune effectors such as NK cells has not been explored. Towards this, we first analyzed the expression of α -2,3 and α -2,6 SA in NK cells. Results presented in Figure 1a demonstrate the presence of significant levels of α -2,3 and α -2,6 SA in spleen-derived NK cells. Influenza virus targets the upper respiratory system; therefore, we also analyzed the expression of these SA in lung-resident NK cells. As shown in Figure 1b, lung NK cells expressed ample and moderate levels of α -2,3 and α -2,6 SA, respectively. IL2-activated NK cells also expressed comparable levels of α -2,3 and α -2,6 SA (Figure 1c). This suggests both primary and IL2-activated NK cells can be targeted by influenza virus.

Detection of PR8 infection in NK cells

To assess the successful entry of influenza virus into the NK cells, we analyzed the infected cells for the presence of PR8-derived M2 protein through confocal microscopy. IL2-activated NK cells were cultured in chamber slides, exposed to PR8 virus at a multiplicity of infection (MOI) 1 for 1 h, washed, cultured for 12 h and stained with anti-NCR1, anti-LAMP1, and anti-M2 antibodies. Since it is known to be uniquely expressed in NK cells, NCR1 staining was used throughout this study. Figure 2 shows the expected peripheral membrane staining of NCR1 receptor. After attaching to the SA-containing glycoproteins, influenza viruses use clathrin-mediated endocytosis as a major mode of cellular entry¹⁸. These clathrin-coated vesicles differ from lysosomes, endosomes and cytotoxic secretory vesicles that are positive for Lysosomal-associated membrane protein-1 (LAMP1)¹⁹. Figure 1d shows that virus infected NK cells possess small unique punctated vesicular structures that are M2 positive. This did not overlap with LAMP1 staining patterns. These distinct structures are similar to those described earlier for intracellular influenza viruses that are localized inside clathrin-coated endocytic vesicles¹⁸. Our results strongly suggest that PR8 virus can successfully enter inside the NK cells.

To characterize the *in vivo* infection of NK cells, we intra nasally inoculated C57BL/6 mice with 5000 PFU of PR8. Lungs along with trachea were removed at different days of post

infection (DPI), embedded in OCT, sectioned and stained for NCR1 and anti-M2 antibodies. Our results presented in Figure 2 demonstrate, localization and persistence of M2 protein inside the NK cells up to 10th day of post infection. NK cells were identified with their unique NCR1 staining pattern in the lung sections. NK cells in the lung sections of mock-infected C57BL/6 mice showed no evidence of viral M2 protein staining. In contrast, NK cells in the lung sections of infected mice consistently showed a strong staining M2 protein. Maximal amount of viral entry inside the NK cells occurred on day 4 of post infection (Figure 2). PR8 viral titer also peaks on day 4 of post infection (data not shown); thus, the level of viral infection in NK cells proportionately correlated with the viral titer. On day 4 of post infection, M2 staining was also evident in epithelial and other cell types in the lung (Figure 2). Interestingly, on day 7 and 10 of post infections, while the PR8 is cleared in most part of the lung tissues, our data shows persistence of M2 protein in NK cells.

To further confirm the *in vivo* infection of NK cells, we isolated lung-resident lymphocytes from mock-infected and day 4 post-infected mice. NK cells were marked by anti-NCR1 antibody and additionally stained for LAMP1 and viral M2 proteins (Figure 3a). Staining of trachea from day 4 post infection mice with anti-M2 antibody revealed the presence of considerable amount of M2 protein in epithelial cells indicating the successful infection of these mice (Figure 3b). NCR1⁺ NK cells from mock-infected lung tissues contained LAMP1 positive cytotoxic granules; but, were negative for viral M2 protein. However, NK cells derived from the day 4 post infection were positive for M2 protein confirming our *in vitro* observations (Figure 3a). These observations demonstrate for the first time that influenza virus can successfully enter into NK cells.

PR8 infection does not affect the expression of activating or inhibitory receptors

NK cell functions are regulated by activating and inhibitory receptors²⁰. To determine whether PR8 infection affect the surface expression of NK cell receptors, we examined CD122, CD11b, CD43, CD49b, CD51, CD69 (developmental and functional markers); NCR1, NKG2D, NKR1c, CD244, Ly49D (activation receptors); and NKG2A, Ly49A, Ly49C/I, Ly49G2 (inhibitory receptors) on gated CD3⁻NK1.1⁺ cells. Flow cytometric analysis showed that expressions of all these molecules were comparable between PR8-infected and mock-infected NK cells (Figure 4a, 4b & 4c). This result indicated that PR8 infection does not affect surface expression of these NK receptors.

PR8 infection impairs the cytotoxic potential of NK cells

To determine the effects of PR8 infection on NK cell functions, we first tested the cytolytic potential of mock- and PR8-infected NK cells against EL4^{H60}, YAC-1 and RMA/S target cells. EL4^{H60} is a cell line stably expressing H60, which is a ligand for NKG2D. This cell line was established through transfection of EL4 cells that lack any known NKG2D ligands with a H60-encoding cDNA²¹. As seen in Figure 5a, PR8 infected NK cells showed reduced cytotoxicity against EL4^{H60} compared to that of mock-infected. YAC-1 is a tumor cell line that naturally expresses H60. The killing of this target by infected NK cells was also down regulated (Figure 5b). Cells that lack or have reduced expression of self MHC Class I molecules are also susceptible to NK-mediated cytotoxicity. PR8-infected NK cells showed reduced cytotoxicity against RMA/S cells, which express lower levels of MHC class I (Figure 5c). Taken together, these data show that PR8 infection of NK cells could negatively modulate their cytotoxic potentials.

PR8 infection of NK cells impairs anti-viral cytokine and chemokine generation

Lung resident NK cells generate multiple anti-viral cytokines and pro-inflammatory chemokines during influenza infection. To determine the effect of PR8 on NK cells, we next analyzed the generation of IFN- γ , GM-CSF, MIP-1 α , MIP-1 β and RANTES following

receptor-mediated activations. We stimulated PR8- and mock-infected NK cells with mitogenic anti-NCR1, -NKG2D, -Ly49D, -NKPR1c, and -CD244 antibodies. Secreted cytokines and chemokines were measured by multiplex assays. As seen in Figure 6, mock-infected NK cells produced a large amount of IFN- γ and GM-CSF when stimulated with plate-bound antibodies. However, PR8-infected NK cells were severely impaired to produce these cytokines. Further, generation of chemokines MIP-1 α , MIP-1 β and RANTES from infected NK cells were also significantly reduced by PR8-infection (Figure 6). Although significant reductions were seen, the effect on the generation of GM-CSF and RANTES were less compared to others, indicating a possible differential regulation by the PR8 virus. Substantial reduction in cytokine and chemokines could be due to the inability of the PR8-infected NK cells to produce cytokines or due to a virus-induced defect in the ability of the infected NK cells to secrete. To distinguish between these two possibilities, we next analyzed the levels of intra cellular IFN- γ in response to anti-NKG2D antibody. Consistent with above findings, percentages of IFN- γ -positive NK cells were reduced after PR8 infection (Figure 7). Collectively, these results demonstrate a direct infection of NK cells by PR8 can impair their ability to generate cytokines and chemokines.

Mutations in PR8-NS1 augment the functional impairment of NK cells

PR8 infection did not alter the expression levels of activating receptors of NK cells. Hence, it is possible that the PR8 virus may target signaling pathways in NK cells. Our earlier work in NK cells demonstrated that phosphatidylinositol 3-kinase subunit p85 (PI3K-p85) plays a critical role in their cytokine/chemokine generation and cytotoxicity²². Further, we and others have shown that the PR8-derived NS1 has the ability to directly interact with PI3K-p85 subunit^{23–25}. Since PR8 can enter inside the NK cells, it could allow the viral NS1 to bind and alter the functions of PI3K-p85 with a direct impact on the effector functions. Based on these observations, we hypothesized that abolishing the binding ability of NS1 to PI3K-p85 could result in loss of PR8's ability to manipulate NK cell functions. Towards this, we generated a NS1-mutated PR8 virus (PR8-NS1-SH2/SH3-mt). NS1 contains two polypyrrole motifs (164–167: PSLP and 212–216: PPLTP) that are required to bind to the p85 subunit of the PI3K. The NS1 protein also contains a consensus SH2 binding site that is similar to the previously described YXXM motif (89–93: YLTDM). The five prolines and the tyrosine were swapped to alanines or phenylalanine in order to generate a mutated NS1 that failed to interact with PI3K-p85 (Figure 8a)^{24,25}.

Using this PR8-NS1-SH2/SH3-mt and the wild type PR8 viruses, we infected the IL2-activated NK cells. NK cells were infected with 1 MOI of PR8 or PR8-NS1-SH2/SH3-mt for 1 h, washed and 24 h later expression patterns of activating (NCR1, NKG2D, CD244 and Ly49D), inhibitory (NKG2A, Ly49A, Ly49C/I and Ly49G2), and developmental markers (CD122, CD11b, CD43, CD49b, CD51 and CD69) were analyzed. None of these receptors were modulated as a result of PR8-NS1-SH2/SH3-mt infection (**data not shown**). Next, we quantified the levels of functional impairment of the PR8-NS1-SH2/SH3-mt-infected NK cells. Ability of NK cells to mediate cytotoxicity against tumor cells was quantified. Contrary to our expectations, infection of NK cells with NS1 mutant PR8 virus further reduced NK cytotoxicity compared to those infected with PR8 (Figure 8d). Similar to cytotoxicity, generation of IFN- γ in response to anti-NCR1 or anti-NKG2D were also further reduced (Figure 8e).

One possible explanation for these observations could be infection-induced cell death. Earlier studies have shown that the binding of NS1 to PI3K-p85 redirected signaling events to initiate pro-survival pathways and thereby delayed host cell apoptosis^{24,25}. Thus, the mutated NS1 that is unable to bind to PI3K-p85 could have failed to initiate these pro-survival pathways resulting in increased apoptosis. Therefore, 24 h after infection, we tested NK cells with Annexin V (early phase of apoptosis) and 7AAD (late phase of apoptosis). Our results show

that infection with PR8 caused moderate early and late phase of NK cell death (Figure 8f) and infection with PR8-NS1-SH2/SH3-mt virus only slightly augmented this cell death. We conclude that the functional impairment caused by the PR8 in NK cells may not depend on the ability of NS1 to manipulate the functions of PI3K-mediated signaling cascades. Thus, our results suggest the existence of additional novel mechanisms by which influenza virus can negatively regulate the effector functions of lymphocytes.

DISCUSSION

Influenza A virus is a negative-strand RNA virus that can cause severe disease in humans and animals. Influenza virus primarily infects and replicates in the lung epithelial cells. However, their ability to infect and modulate the functions of effector lymphocytes has not been analyzed. Here, our results show that the PR8 virus can enter inside and modulate the functions of NK cells.

Influenza enters the host cells by specifically binding to glycoproteins that possess α -2,3 and α -2,6-linked sialic acids. Our current results show that NK cells express abundant quantities of these glycosyl side chains facilitating the binding and entry of PR8 virus. *In vitro* exposure to the virus followed by staining for viral M2 protein showed the presence of PR8 virus inside the NK cells. Further, NK cells that are present in the lung sections or isolated from infected mice confirmed the presence of viral proteins. Since viral hemagglutinin (HA) functions as ligand that facilitates the entry into the susceptible cells and as an activating ligand for NCR1 receptor, it is possible that PR8 indeed uses NCR1 to enter inside the NK cells. It is also important to note that NCR1 is uniquely expressed on NK cells and not on T, B or NKT cells. Therefore, if NCR1 is used as a conduit for viral entry, NK cells could be the only target for viral manipulation. PR8 entry into NK cells were analyzed using anti-M2 and not anti-HA antibodies. This argues against the possibility that NK cells preferentially internalized NCR1/HA complexes and not the whole PR8 virus. Since PR8 inside the NK cells underwent only a limited replication, it appears that influenza virus does not use the NK cells as a typical host cell similar to that of lung epithelial cells. Limited replication of influenza virus on NK cells is not surprising as its infection in macrophage and DC is usually non-productive^{11,12}. Limited infection of influenza virus in human NK cells was also observed¹³. If not for replication, does influenza infection affects NK cell functions? Our results show PR8 can affect both cytotoxicity and cytokine/chemokine generation of NK cells.

One possible mechanism by which PR8 can manipulate effector functions is to alter the expression of activating receptors in NK cells. NK cells express multiple activation receptors. NCR family contains three human (NKp46, NKp44 and NKp30) and one murine (NKp46) members²⁶⁻²⁸. Recognition of HA and hemagglutinin-neuraminidase (HN) of Sendai virus⁹ on the host cells by NCR constitutes a critical step for the activation of NK cells during influenza infection. NKG2D plays an important role in the recognition of virus infected cells through the recognition of 'induced-self' ligands. NKG2D is expressed on all human and murine NK cells and it recognizes MIC-A/B29, ULBP-1/2/3³⁰ (in human) and H6031/32, Rae-1 $\alpha/\beta/\gamma/\delta/\epsilon$ 5, Mult-133 (in mouse). Ly49D associates with both DAP10 and DAP12^{34,35} and recognizes H2-D^d as its ligand³⁶. Nkrp1c is a subunit of NK1.1 complex and it is a unique cell marker expressed on NK and NKT cells that associates with FcR γ to mediate its signal³⁷. CD244 is expressed both human and murine NK cells and recruits SH2 domain containing SAP to propagates its signals. A direct infection of NK cells by PR8 did not alter the expression levels of any of these activation receptors. Further, our results also indicate that PR8 did not have any effect on inhibitory, developmental or functional status-related receptors on NK cells.

NK cells control influenza infection by destroying the infected cells in order to release the viral particles that can be neutralized by specific antibodies³⁸. Mice that lack NK cells succumbed to influenza, demonstrating their critical role in controlling influenza infection^{3,4,39}. For that reason, PR8 could have evolved evasion strategies that target the effector functions of NK cells. Our results show that infection of NK cells with PR8 lead to a significant reduction of their cytotoxicity against tumor cells that represent '*induced-self*' (EL4^{H60} and YAC-1) and '*missing-self*' (RMA/S). This indicates that a direct infection by influenza virus can interfere with ability of NK cells to effectively recognize and kill the host cells that harbor this virus.

NK cells are also a critical source of multiple pro-inflammatory cytokines and chemokines. Our present findings indicate that a direct infection of NK cells by PR8 reduces the generation of IFN- γ , GM-CSF, MIP-1 α , MIP-1 β and RANTES when stimulated through NCR1, NKG2D, Ly49D, Nkpr1c or CD244. These findings have significant implications on both innate and adaptive immune responses. NK cells can control infection by secreting IFN- γ that directly inhibit influenza replication resulting in protection⁴⁰. Therefore, a reduction in the level of IFN- γ can increase the severity of the disease. NK-derived cytokines and chemokines also regulate B and T cells functions^{41,42}. For instance, INF- γ secreted by NK cells is also one of the key regulator of antibody isotype switching and secretion, controlling the quality of B cell responses⁴³. During influenza infection, NK cells are required for the generation of influenza virus-specific CTL *in vitro* and *in vivo*⁴⁴. NK cells also participate in adaptive immune responses by clearing influenza-infected cells through antigen-specific antibodies by ADCC⁴⁵. Thus, this ability of PR8 to manipulate NK cell functions suggests a novel mechanism employed by influenza virus with implications to both the innate and adaptive immune responses.

Infection of NK cells with PR8 did not alter the expression levels of any of the activating or inhibitory receptors. Further, the viral titers from the culture supernatant indicate that this infection is moderate and the NK cells do not support active replication of PR8. Irrespective of these, critical effector functions mediated by the infected NK cells were significantly compromised. Thus, it is possible that the PR8 virus may target signaling pathways that regulate the cytotoxicity and cytokine/chemokine generation. We and others have shown that the PR8-derived NS1 has the ability to directly interact with and modulate the function of PI3K-p85 subunit whose phosphorylation regulates NK-mediated cytokine generation and cytotoxicity^{23-25,22}. Towards this, we used a PR8 virus that expressed a mutated NS1 protein (PR8-NS1-SH2/SH3-mt) that failed to interact with PI3K-p85^{24,25}. In contrary to our expectations, mutation of NS1 resulted in the additional reduction of NK cell-mediated cytotoxicity and cytokine generation. Based on these results, we conclude that PR8 employs novel mechanisms that does not involve SH2/SH3 domain of NS1 to target signaling pathways in NK cells.

Viral infections can lead to cell death. However, to achieve high level of replication, during early phase of infection, influenza virus uses its NS1 protein to delay host cell apoptosis via the activation of PI3K/Akt-dependent pro-survival pathways²⁴. NS1 protein with mutations in the SH2/SH3-binding motifs failed to interact with SH domains of PI3K-p85 β and did not activate the PI3K/Akt pathway²⁵. Thus, disruption of the NS1 binding to p85 could have induced apoptosis. In our study, infection of NK cells with PR8 or NS1-mutated PR8 caused moderate levels of cell death (23–29%) compared to that of mock-infected (8–14%). However, the moderate level of cell death alone does not explain the severe reductions in the effector functions of NK cells. Taken together, we conclude that influenza virus can down modulate the effector functions of NK cells; however, the molecular mechanism is yet to be understood.

METHODS

Mice, virus and cell lines

The C57BL/6 mice were maintained in pathogen-free conditions at the Biological Resource Center (BRC) of the Medical College of Wisconsin (MCW), Milwaukee, WI. All the animal protocols used were approved by the IACUC, BRC, MCW, Milwaukee, WI. Target cells, EL4^{H60}, RMA/S and YAC-1 and their culture conditions were described⁴⁶. Madin-Darby Canine Kidney (MDCK) were purchased from ATCC (Rockville, MD) and cultured in RPMI 1640 medium with 10% FBS. Mouse adapted human influenza virus A/PR/8/34 (PR8) was used as described²³. NS1 mutant PR8 virus was described previously^{24,25}. Viral titers were determined by plaque-forming assays.

PR8 infections

***In vivo*:** C57BL/6 mice were intra nasally challenged with of 5000 PFU of PR8 virus in sterile PBS in a total volume of 30 μ l through one nostril. Mock infections were performed using only sterile PBS without the virus. Following day 4, 7, 10 post infection, lungs were collected and used for lymphocytes isolation or tissue mounting. For lymphocyte preparations, lung was minced finely into pieces, washed, 500ul 10 mg/ml collagenase (C5138-100mg, Sigma, St Louis, MO) and 500ul 1mg/ml DNase I, bovine pancreas (D4527-20KU, Sigma, St Louis, MO) were added for digestion 1h at 37 with gentle vortex (400 rpm). Remaining tissue pellets were transferred into cell strainer and smashed gently with 5ml syringe plunger, washed. Cell suspensions were incubated with 5ml RBC lysis buffer for 5 min, then washed. For generating cryosections, whole lungs were mounted with OCT and used in immunofluorescence analyses. ***In vitro*:** IL-2 activated NK cells were incubated with PR8 virus at a multiplicity of infection 1 (MOI). After one hour at 37°C, cells were washed three times and cultured in RPMI 1640 medium with 10% FBS and 1000U/ml of IL2 for additional 12 or 24 h before used in different analyses.

Confocal microscopy

Lymphocytes from digested lung tissues at days 4 of post infection were purified using Ficoll density gradient. Purified lymphocytes were cultured in poly-L-lysine coated 8-wells chamber slides. Cell preparations or tissue sections were fixed with 2% paraformaldehyde. Single cell preparations cultured in chambers slides were additionally permeabilized before staining. Slides were blocked for 1 h with 10% FBS and 1% BSA in PBS followed by goat anti-mouse NCR1 (BD Biosciences, San Jose, CA), anti-M2 and anti-mouse LAMP1-PE antibodies (e-Bioscience, San Diego, CA) for overnight. After 3 washing with PBS, cells were incubated with rabbit anti-goat Alexa Flour 488 (Invitrogen, Carlsbad, CA) and goat anti-mouse Alexa Flour 633 for additional 1 h and slides were mounted with VECTASHIELD. Images were obtained using Olympus FluoView FV1000 MPE microscope that is equipped with multiphoton capabilities (MaiTai DSBB-OL: 710nm–990 nm MaiTai DSHP-OL: 690nm–1040nm).

NK cell preparation and analysis of α -2,3 and α -2,6-linked sialic acid

NK cells were purified as described⁴⁶. Single cell suspension of spleens were made and used. IL2-activated splenic NK cells were generated using following protocol. Briefly, single cell suspensions from spleen were passed through nylon wool columns to deplete adherent populations consisting of B cells and macrophages. Nylon wool-non-adherent cells were cultured with 1000U/ml of IL2 (NCI-BRB-Preclinical Repository, Maryland, MD). Purity of the NK cultures was checked by flow cytometry and preparations with more than 90% of NK1.1⁺ cells were used. The distribution of α -2,3 and α -2,6-linked sialic acids on prepared NK cells were detected by flow cytometry. Primary or IL2-activated NK cells (1×10^6) were

blocked with anti-mouse CD16/32 (1 μ g/10⁶cells) followed by staining with anti-CD3 PB (145-2C11, e-Bioscience, San Diego, CA), NK1.1-APC (PK136, e-Bioscience, San Diego, CA) and 100 μ l of 1:400 dilution of SNA-FITC or 1:800 MAA-FITC (Vector Laboratories, Burlingame, CA) for 30 min at 4°C. Cells were washed, resuspended in FACS washing buffer and analyzed by flow cytometry in LSR-II using FACSDiva software (Becton Dickinson, Franklin Lake, NJ).

Flow cytometric analysis of NK markers after virus infection

Cell preparations were stained with fluorescent-labeled mAbs as described⁴⁶. Antibodies for NK1.1 (Nkpr1c) (PK136), CD3 ϵ (145-2C11), NKG2D (A10), NKG2A (16a11), CD11b (M1/70), CD43 (1B11), CD49b (DX5), CD51 (RMV-7), CD69 (H1.2F3), CD122 (5H4) and Ly49I (YLI-90) were obtained from e-Bioscience (San Diego, CA). Antibodies for CD244 (2B4), Ly49A (A1), Ly49D (4E5), Ly49C/I (5E6) and Ly49G2 (4D11) were obtained from BD Biosciences (San Jose, CA). Antibody for NCR1 (259018) was purchased from R&D system (Minneapolis, MN). Standard flow cytometry analysis was carried out in LSR-II using FACSDiva software (Becton Dickinson, Franklin Lakes, NJ).

NK cell cytotoxicity assay

NK-mediated cytotoxicity was quantified using ⁵¹Chromium (⁵¹Cr)-labeled target cells, including, EL4^{H60}, YAC-1 and RMA/S. 24 h after infection, infected or mock-infected NK cells were mixed with target cells at varied E:T Ratio and incubated at 37°C for 4 h. Plates were spun down, and supernatants were harvested and the release of ⁵¹Cr into culture supernatants were measured using gamma counter. Percent specific lysis was calculated using amounts of absolute, spontaneous and experimental ⁵¹Cr-release from target cells⁴⁶.

Quantification of cytokines and chemokines

PR8-, PR8-NS1-SH2/SH3-mt- or mock-infected NK cells were activated with titrated concentrations of plate-bound anti-NCR1 (259018), anti-NKG2D (A10), anti-Ly49D (4E5), anti-Nkpr1c (PK136) and anti-CD244 (2B4) mAbs for 18 h. The secreted IFN- γ , GM-CSF, MIP-1 α , MIP-1 β and RANTES in the supernatants were quantified by conventional ELISA or multiplex assays (Bio-Rad, Richmond, CA). Intracellular staining of IFN- γ in mock or PR8 infected NK cells was performed using plate bound anti-NKG2D (5 μ g/ml) activation as described⁴⁷.

Apoptosis assay

PR8 or NS1 mutant PR8 virus infected NK cells were stained with Annexin V and 7-ADD using Annexin V-PE apoptosis detection kit (BD Pharmingen, Franklin Lake, NJ). Flow cytometry was performed in LSR-II using FACSDiva software (Becton Dickinson, Franklin Lake, NJ).

Statistics

Statistical analysis was performed by two-tail, unpaired, Student's t-test using Microsoft Excel 2003 software to compare the differences in results obtained using PR8 infected and mock infected NK cells. p values of ≤ 0.05 were considered significant.

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Figure 1.

NK cells can be infected by PR8. α -2,3 and α -2,6 SA are expressed on primary splenic NK cells (a), primary lung NK cells (b) or IL2-activated splenic NK cells (c). Total splenocytes, lung-derived lymphocytes or IL2-activated NK cells were stained with anti-CD3, anti-NK1.1 antibodies and lectins SNA-FITC or MAA-FITC that are specific for sialic acid side chains. Gated NK cells ($CD3^{-}NK1.1^{+}$) were analyzed for the expression of α -2,3 and α -2,6 SA and presented in a, b and c. Percent SA positive $CD3^{-}NK1.1^{+}$ and the Mean Fluorescence Index (MFI) are shown. Negative controls that were used to calculate the percent SA positivity are shown only with the MFI. (d) Intracellular entry of PR8 virus in NK cells. IL2-activated splenic NK cells were mock- or infected with 1 MOI PR8 for 1 h, washed and 24 h later were stained

with anti-NCR1, anti-LAMP1 and anti-M2 antibodies. Individual or merged images of all three or shown. Data presented in a–d are representatives of 3–5 experiments each. Representative images of more than 50 individually analyzed cells are shown in d.

Figure 2.

PR8 can infect NK cells *in vivo*. Lung sections from the PR8 infected mice were stained for NK cells (anti-NCR1) and virus (anti-M2). Lung sections from days 4, 7 and 10 of post infection along with mock-infected controls are shown. Data presented are representative of more than 20 individual image panels obtained. A group of three mice were analyzed for each time point.

Figure 3.

Primary NK cells from infected mice contain PR8 virus. (a) Lymphocytes from the lungs of mock or PR8 infected mice were isolated and stained for NK cells (anti-NCR1), lysosomal/endosomal vesicles (LAMP1) and PR8 virus (anti-M2). (b) Tissue sections containing trachea of infected mice (4 DPI) were used as positive controls for anti-M2 antibody staining. Data presented are representative of more than 50 individual cells analyzed. A total of three mice were mock or PR8 infected and their NK cells were purified and analyzed on day 4 of post infection.

Figure 4.

Expression of NK cell receptors are unaffected after PR8 infection. IL2-activated NK cells were mock or PR8 infected and stained for activating (a), inhibitory (b) and developmental/functional (c) NK cell markers. Percent positive cells and the respective MFI are shown in parenthesis. Data presented is a representative of three independent experiments.

Figure 5.

Natural cytotoxicity of NK cells are reduced after PR8 infection. Ability of NK cell to mediate cytotoxicity against EL4^{H60} (a, 'induced-self'), YAC-1 (b, 'induced-self and allo') and RMA/S (c, 'missing-self') was analyzed. IL2-activated NK cells were mock or PR8 infected (1 MOI) for 1 h, washed and 24 h later harvested and used in ⁵¹Cr-release assay at indicated E:T Ratio. Data presented are average mean values of three independent experiments with standard deviations.

Figure 6.

Cytokine and chemokine generation from major activation receptors of NK cells are significantly reduced after PR8 infection. IL2-activated NK cells were mock or PR8 infected (1 MOI) for 1 h, washed and 24 h later harvested and activated by immobilized-plate-bound anti-NCR1, anti-NKG2D, anti-Ly49D, anti-Nkrp1c and anti-CD244 antibodies. Culture supernatants were tested for indicated cytokines and chemokines in a multiplex assay. Data presented are average mean values of three independent experiments with standard deviations.

Figure 7.

Level of intracellular IFN- γ severely reduced in PR8 infected NK cells. IL2-activated NK cells were mock-infected (middle panel) or with 1 MOI of PR8 (right panel) for 1 h and 24 h later harvested and activated by immobilized-plate-bound anti-NKG2D for 2 h followed by adding Brefeldin-A and incubating for additional 8 h. Mock-infected, non-activated NK cells were used to determine the background levels of intracellular IFN- γ (left panel). Activated NK cells were harvested and stained for intracellular IFN- γ . Cells shown are gated for CD3⁻NK1.1⁺ NK population. Percentages of CD3⁻NK1.1⁺ NK cells were shown for intracellular IFN- γ positivity. Data presented are representatives of 3 independent experiments.

Figure 8.

Infection with PR8-NS1-SH2/SH3-mt virus augments the functional impairment of NK cells. (a) NS1-mutated PR8 virus (PR8-NS1-SH2/SH3-mt) was generated by reverse genetics. Three putative motifs in NS1 protein that have the potential to interact with PI3K-p85 are altered in the PR8-NS1-SH2/SH3-mt virus. A tyrosine $\rightarrow$ phenylalanine in a consensus SH2 binding motif (89–93: YLTDM); prolines $\rightarrow$ alanines in two polyproline motifs (164–167: **PSLP** and 212–216: **PPLTP**) were made. (b) Ability of NK cell to mediate cytotoxicity against EL4^{H60} after PR8-NS1-SH2/SH3-mt virus infection. IL2-activated NK cells were mock-, 1 MOI of PR8 or PR8-NS1-SH2/SH3-mt virus infected for 24 h, harvested and used in ⁵¹Cr-release assay at indicated E:T Ratio. (c) Impairment in the NCR1 and NKG2D-mediated IFN-

γ generation after PR8 or PR8-NS1-SH2/SH3-mt virus infection. **(d)** Infection-induced cell death after PR8 or PR8-NS1-SH2/SH3-mt virus infection. IL2-activated splenic NK cells were mock, PR8 or PR8- NS1-SH2/SH3-mt virus infected for 1h, washed and 24 h later tested with Annexin V and 7AAD. Data presented in b–d are average mean values or representatives of 3–5 independent experiments.