Differential activation of NK cells by influenza A

pseudotype H5N1 and 1918 and 2009 pandemic H1N1 viruses

Running title: NK cell activation by influenza pps

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Abstract

Natural killer (NK) cells are the effectors of innate immunity and are recruited into the pulmonary 48 h after influenza virus infection. Functional NK cell activation can be triggered by the interaction between viral hemagglutinin (HA) and natural cytotoxicity receptors, NKp46 and NKp44, on cell surface. Recently, novel subtypes of influenza viruses such as H5N1 and 2009 pandemic H1N1 transmitted directly to the human population, with unusual mortality and mobility rates. Here, the human NK cell responses to these viruses were studied. Differential activation of heterogeneous NK cells (upregulation of CD69 and CD107a, and IFN-γ production as well as downregulation of NKp46) was observed following interactions with H5N1, 1918 H1N1, and 2009 H1N1 pseudotyped particles (pps), respectively, and the responses of CD56dim subset predominated. Much stronger NK activation was triggered by H5N1 and 1918 H1N1 pps as compared with 2009 H1N1 pps. The interaction of pps with NK cells and subsequent internalization were mediated by NKp46 partially. The NK cell activation by pps showed a dosage-dependent manner while an increasing viral HA titer attenuated NK activation phenotypes, cytotoxicity, and IFN-γ production. The various host innate immune responses to different influenza subtypes or HA titers may be associated with disease severity.
**Introduction**

Influenza is a contagious, acute respiratory disease caused by influenza viruses and has caused substantial human morbidity and mortality over the past century (24, 27). The 1918–1919 pandemic caused by influenza virus type A H1N1 was responsible for an estimated 50 million deaths (21). In recent years, novel subtype influenza viruses such as H5N1 and the 2009 pandemic H1N1 have been directly transmitted from animals to the human population. These infections were characterized by unusually high rates of severe respiratory disease and mortality among young patients (8, 18). Various genetic shifts have occurred in these viruses, allowing them to evade the host protective effects of specific anti-hemagglutinin (HA) or anti-neuraminidase (NA) antibodies (27). Therefore, host innate immunity in the early phase of infection, which includes a variety of pattern-recognition molecules, inflammatory cytokines, and immune cells such as macrophages and natural killer (NK) cells, plays a critical role in host defense.

NK cells are bone marrow-derived, large, granular lymphocytes and are key effector cells in innate immunity for host defense against invading infectious pathogens and malignant transformation through cytolytic activity and production of cytokines such as interferon (IFN)-γ (10, 28, 42, 51). In humans, NK cells account for approximately 10% of all blood lymphocytes and are identified by their expression of the CD56 surface antigen and their lack of CD3. Two distinct subsets of human NK cells have been defined according to cell
surface density of CD56 expression (10). The majority (~90% in blood) of human NK cells are CD56\textsuperscript{dim}, and a minor population (~10% in blood) is CD56\textsuperscript{bright}. These NK subsets are functionally distinct, with the immunoregulatory CD56\textsuperscript{bright} cells producing abundant cytokines and the cytotoxic CD56\textsuperscript{dim} cells probably functioning as efficient effectors of natural and antibody-dependent target cell lysis (11).

Many lines of evidence suggest that NK cells can be functionally activated by the interaction between natural cytotoxicity receptors (NCRs) on the cell surface and influenza HA protein or stress-induced proteins from infected cells (2, 13, 32, 43, 45). On the other hand, influenza virus is able to evade host immunity by infecting NK cells and triggering cell apoptosis or by attenuating NK cell lysis of H3N2-infected cells owing to alterations in HA binding properties (34, 38). The infiltration of macrophages and lymphocytes into the lung and strong inflammatory responses were detected in H5N1 and the 1918 and 2009 pandemic H1N1 infections. Nevertheless, little is known about the precise roles of NK cells in these infections.

In this study, the responses of NK cells to 1918 H1N1, 2009 H1N1, and H5N1 influenza A viruses were evaluated using three strains of influenza A virus pseudotyped particles (pps). Our findings may aid in understanding the pathogenicity of influenza viruses and their correlations with clinical severity.
Materials and methods

Isolation of primary human NK cells. Blood samples were obtained from healthy adult volunteers, with written informed consent. Ethical approval was given by Ethics Committee of Chinese CDC. Peripheral blood mononuclear cells (PBMCs) were isolated by separation on a Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient. CD56⁺CD3⁻ NK cells were enriched from PBMCs by magnetic bead separation using a NK cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The cells were counted and tested for viability by trypan blue exclusion. Purity was confirmed by flow cytometry. The resulting preparations routinely contained >95% CD56⁺CD3⁻ NK cells.

Preparation of influenza pseudotyped particles and coculture with NK cells. Influenza pps were generated as described previously (31, 58, 59). Briefly, influenza pps were produced using a retrovirus-based pseudotype system via the expression of influenza HA, NA, Gag-pol, and the GFP reporter gene in a 293T cell expression system.

We took the following measures on the quality control on pps preparations. Firstly, the HA, NA expression plasmids; Gag-pol encoding plasmids; and reporter plasmid-GFP were extracted by using Endofree plasmid maxi kit (Qiagen, USA) for transfection. To avoid the differences among different preparations, 293T cell was transfected by the same
amount of plasmids and all pps were prepared simultaneously. Secondly, after 24 h 
post-transfection, the supernatants were removed and replaced with serum-free DMEM 
medium. At another 48 h post-transfection, the pps were collected from the supernatants 
by filtrating through a 0.45µm Durapore PVDF membrane filter (Millipore, Ireland).
Thirdly, all pps were quantified by real-time PCR with primers and probe targeting the 
report gene GFP mRNA packaged in pps, p24 quantization ELISA assay, western blotting 
assay on HA/NA incorporated into pps, as well as hemagglutination assay. In this study, 
we normalized all pps with HA unit since less than 1 percentage of NK cells were found 
to be infected at MOI of 2 using wild-type virus 2009 H1N1, seasonal H1N1 or pps (data 
not shown). Therefore, the interaction of NK cells and pps was studied here and we used 
the same HAU rather than infectious viral particles.
Freshly purified NK cells at 1 × 10^6/ml were treated by A/California/7/2009 (H1N1), 
A/Brevig Mission/1/1918 (H1N1), and A/Anhui/1/2005(H5N1) pps (abbreviated as 
H1N1-2009, H1N1-1918, and H5N1pps) at different dosages respectively, overnight in a 
humidified atmosphere of 5% CO_2 at 37°C. NK cells cultured in growth medium (RPMI 
1640 (Invitrogen, Camarillo, CA) medium with 10% FBS (Sigma-Aldrich, Saint Louis, 
MO)) or treated by pps with HA and NA of a H3N2 virus strain (A/Brisbane/10/2007) or 
the supernatants from Gag-pol/GFP-transfected 293T cell were tested in parallel as 
controls.
**Immunophenotyping and intracellular staining of NK cells.** For surface marker staining, the cells were incubated for 15 min at 4°C with Fc-blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) containing human IgG, to block nonspecific Fc receptor binding. After washing with fluorescence-activated cell sorting (FACS) buffer consisting of 1× PBS, 0.1% sodium azide (Sigma-Aldrich, Saint Louis, MO), and 1% FBS, the cells were incubated in the dark at 4°C with fluorochrome-conjugated antibodies (Abs) to cell surface markers, at concentrations previously determined by titration. Next, the cells were washed twice in FACS buffer and fixed for 15 min at room temperature (RT) in fixation buffer consisting of 1× PBS and 1% paraformaldehyde (Sigma-Aldrich, Saint Louis, MO).

With regard to CD107a detection, NK cells were stimulated with pps in the presence of CD107a Abs (H4A3, BD Biosciences, San Diego, CA), monesin, and brefeldin A (Sigma-Aldrich, Saint Louis, MO). After 6 h incubation, the cells were treated with 20 mM EDTA, pH 7.2–7.4 for 15 min at RT. Surface marker staining was performed as described above. The cells were then permeabilized with Cytofix/Cytoperm buffer (BD Biosciences, San Diego, CA) for 20 min and washed in washing solution. Isotype control Ab was used for each staining combination.

**FACS analysis.** The following fluorescently labeled mAbs, purchased from BD Biosciences (San Diego, CA) or Biolegend (San Diego, CA), were used for FACS
analysis: anti-CD56 PE-cy5 (MEM-188), anti-CD3 PE-cy7 (SK7), anti-NKp46 PE (9E2), anti-NKp44 PE (P44-8), anti-NKp30 APC (P30-15), anti-CD16 FITC (3G8), anti-CD69 APC (FN50), anti-CD107a PE (H4A3), anti-NKG2D APC (1D11), anti-IL10R PE (3F9), and anti-2B4 FITC (CD244, C1.7). FACS data were collected using a FACS Aria I flow cytometer and analyzed using Diva analysis software (BD Biosciences, San Diego, CA).

NKp46 blocking test. Blocking experiments were performed by pre-incubating freshly purified NK cells with saturating amounts of NKp46 blocking antibodies (10\( \mu \)g/ml, 9E2) for 1 h. Then the cells were cocultured overnight with H1N1-2009pps, H1N1-1918pps, or H5N1pps at a dose of 200 HAU/ml. Corresponding isotype antibody was added as control.

Cytotoxicity assays. The assay of NK cell cytotoxicity was performed with a LIVE/DEAD Viability/Cytotoxicity kit for mammalian cells (Invitrogen, Camarillo, CA). Briefly, K562 target cells were stained with calcein AM and then cocultured for 3 h with NK cells at specific effector cell/target cell (E/T) ratios. After incubation, the cells were stained with ethidium homodimer-1. Cytotoxicity was analyzed by flow cytometry and calculated as the percentage of cells positive for calcein AM plus ethidium homodimer-1 among the total number of cells positive for calcein AM. Assays were performed at the indicated E/T ratios.
Cytometric bead array assay. Cytokines were determined in cell-free supernatants using human IFN-γ, macrophage inflammatory protein (MIP)-1α, tumor necrosis factor (TNF)-α, interleukin (IL)-17a, and IL-10 Flex Set reagents (BD Biosciences, San Diego, CA) according to the manufacturer’s protocol. The results are presented as the means of assays performed in duplicate wells. Data were analyzed by using BD Cytometric Bead Array 1.4 software.

Statistical analysis. Data are expressed as means ± SEM. Statistical significance was determined by paired or nonparametric tests using Graph Prism 5 software (GraphPad Software), and $P < 0.05$ was considered significant.
Results

Universal upregulation of CD69 surface expression in NK cells stimulated by influenza virus pps

To evaluate the NK cell response to pps, we first examined the expression of the early activation marker CD69 after coculture of NK cells with different pps. The C-type lectin-like glycoprotein CD69 is a sensitive and very early marker of leukocyte activation (26, 29, 50), is encoded within the NK gene complex (6), and is rapidly expressed on the cell surface from preformed intracellular stores in an RNA- and peptide synthesis-independent manner (41). Cross-linking of CD69 by Abs not only triggers NK cytotoxicity but also induces NK cell proliferation, CD25 and intracellular adhesion molecule-1 expression, TNF-α production, and Ca^{2+} mobilization (36).

Freshly purified NK cells were stimulated overnight with different influenza virus pps at dosages ranging from 50 to 1000 HAU/ml. The expression of CD69 and NKp46 on NK cells cultured in growth medium, supernatants from Gag-pol/GFP-transfected-293T cells, or H3N2pps (A/Brisbane/10/2007), respectively, show no significant difference (supplementary Fig. 1 and 2). Therefore, NK cells cultured in growth medium were presented as mock control in this study.

The changes in CD69 expression on NK cells are shown in Fig. 1. After incubation of NK
cells overnight in growth medium alone, less than 20% of the NK cells expressed CD69.

Universal upregulation of CD69 was observed in NK cells treated by H5N1, 1918 H1N1, and 2009 H1N1 pps (Fig. 1). Interestingly, differential regulation of CD69 expression was detected in heterogeneous NK cells (Fig. 1A, B and C). CD69 expression in CD56\textsuperscript{dim} NK cells appeared to be higher significantly than CD56\textsuperscript{bright} cells, both in terms of the percentage of CD69\textsuperscript{+} NK cells and the fold increase in median fluorescence intensity (MFI).

CD69 expression on NK cells showed dose-dependent upregulation with stimulation by pps over the concentration range of 50 to 1000 HAU/ml. CD56\textsuperscript{dim} cells showed greater activation and a higher percentage of CD69\textsuperscript{+} cells in response to H1N1-1918pps and H5N1pps than in response to H1N1-2009pps at each dose point (n=18, P< 0.01). After stimulation with H1N1-1918pps or H5N1pps overnight, about 60% (50 HAU/ml) to almost 100% (1000 HAU/ml) of CD56\textsuperscript{dim} cells stained positively for CD69. In contrast, CD69 expression on NK cells stimulated by H1N1-2009pps increased only slightly, in terms of both the percentage of CD69\textsuperscript{+} CD56\textsuperscript{dim} cells and the MFI (Fig. 1).

The differential activation of cells, especially the CD56\textsuperscript{dim} population, from all donors in response to different subtype viral pps suggests that influenza pps can directly trigger NK cell activation and may heterogeneously influence NK cell function.
Upregulation of CD107a and elevated cytotoxic activity in NK cells stimulated by pps

Two major antiviral effects of NK cells, lysis of virus-infected cells and IFN-γ production, were confirmed at the early stages of infection (16, 37, 49). After confirmed the NK cell activation by influenza pps, we then detected these two main functions of NK cells.

A more recently described marker of cytotoxic activity is the lysosome-associated membrane protein-1 (LAMP-1 or CD107a), which is expressed on the NK cell surface following cytotoxic granule exocytosis induced by exposure to MHC-deficient target cells (1, 26). It has been reported that CD69 is linked to the cytotoxic potential of NK cells (9, 33), and were shown here to be heterogeneously expressed. Therefore, we examined whether CD107a expression was also differentially stimulated by pps.

CD107a expression in response to pps was upregulated preferentially in the CD56dim subset of NK cells (Fig. 2A and 2C). Whereas incubation with pps stimulated a little increase of CD107a expression on the CD56 brightly cells, as compared with mock NK cells, there were no significant differences, regardless of the virus HAU dose (Fig. 2B). Compared with H1N1-2009pps, the H1N1-1918pps and H5N1pps triggered much greater NK activation, with significantly higher percentages of CD107a+ CD56dim cells and higher MFI values (Fig. 2C) at each dosage (n=18, P < 0.05). Although the level of CD107a expression decreased gradually with increasing HAU dose, it remained higher
than that in mock NK cells, even when stimulated with pps at 1000 HAU/ml.

The cytotoxic capability of NK cells was further investigated based on the above cellular markers of NK cell activation and function. After overnight interaction with pps, the NK cells were collected and tested for the ability to lyse K562 cells. All of the pps examined enhanced NK cell cytotoxicity. The H5N1pps and H1N1-1918pps produced stronger stimulatory effects, and these pp-treated NK cells showed significantly higher levels of cytotoxicity than that treated by H1N1-2009pp-stimulated NK cells at an E/T ratio of 20:1 (n=6, \( P < 0.01 \)). The NK cells showed gradual attenuation of lytic activity with increasing HA titers of pps, which is correlated significantly with CD107a expression (Fig. 2D). After coculture with pps at 1000 HAU/ml, H1N1-1918pp- and H5N1pp-stimulated NK cells still showed higher cytotoxic activity, with more than 40% cytotoxicity against K562 cells. In contrast, NK cells stimulated with increasing H1N1-2009pp titers showed mild lysis capabilities and even nearly to the level of mock NK cells at 1000 HAU/ml. At a dosage of 200 HAU/ml, H1N1-1918pp- and H5N1pp-stimulated NK cells showed significantly higher cytotoxic activity (n=6, \( P < 0.01 \)) than those stimulated by H1N1-2009pps at different ratios of E/T (Fig. 2E).

**IFN-\( \gamma \) induced by different influenza virus pps**

NK cells are an important source of innate immunoregulatory cytokines such as IFN-\( \gamma \), TNF-\( \alpha \), and MIP-1\( \alpha \), which coordinate the early immune response and contribute to the
subsequent T cell response following infection (10, 14). Therefore, we examined cytokine production in the supernatants of NK cells stimulated by pps.

IFN-γ, MIP-1α, TNF-α, IL-17a, and IL-10 were assayed after overnight incubation with each of the pps and with no stimulation. The cytokine baselines were variable after overnight incubation in the absence of any influenza pps: the concentrations of MIP-1α, TNF-α, and IL-17a were less than 20 pg/mL, the IL-10 level was much lower, and IFN-γ was not detected (data not shown).

All pps upregulated IFN-γ production by NK cells (Fig. 3). Consistent with the above NK activation findings, H1N1-1918pps and H5N1pps, as compared with H1N1-2009pps, exhibited much stronger stimulation of NK cells, with significantly enhanced IFN-γ production (n=18, P < 0.01), and IFN-γ production was reduced gradually with increases in the HA titer of pps. However, IFN-γ was modestly upregulated in response to H1N1-2009pps, and at the highest dosage, the IFN-γ level was decreased to almost the same level as in mock NK cells. No change was detected in the levels of the other cytokines after NK cell stimulation with any of the influenza pps at any dosages (data not shown).

**Internalization of NKp46 on CD56^dim NK cells stimulated by influenza virus pps**

NK cell function is controlled by the integration of signals from various stimulatory and
inhibitory cell surface receptors. The major activating receptors are NKG2D, 2B4, and the three NCRs (NKp46, NKp44, and NKp30) (35, 39, 53). Several recent studies have indicated that NCRs can recognize HA protein directly and do not require the presence of accessory or mediating molecules (2). Our findings that the differential expression of CD69 and CD107a and IFN-γ production from NK cells stimulated by different influenza virus pps have implications for the diverse activating extent of NK cells. In order to identify the putative receptor(s) responsible for the recognition of influenza pps by human NK cells and potentially involved in the activation of NK cells, we further investigated the surface expression of three NCRs and other activating receptors on NK cells stimulated by pps.

As reported previously (15, 54), almost all peripheral blood NK cells expressed NKp46 and NKp30, whereas NKp44 was absent. No changes were detected in NKp44 or NKp30 on NK cells after overnight stimulation by pps (data not shown), whereas NKp46 expression was downregulated in a dose-dependent manner by the three influenza pps. We compared NKp46 expression between CD56bright and CD56dim NK cells in individual donors, and the percentage of NKp46+ cells and the decrease percentage in MFI are shown in Fig. 4. As compared with mock, differed and significant downregulation of NKp46 expression was restricted to the CD56dim population responses to different pps (Fig. 4A and 4C), with no significant changes in the CD56bright subset (Fig. 4B). Marked reductions in NKp46 expression were detected with increases of pps dosage and no
significant difference in expression among the three pps at each dosage (Fig. 4C, n=18, $P > 0.05$). To determine whether NKp46 downregulation occurs via receptor internalization, NK cells were permeabilized and stained for the presence of intracytoplasmic NCRs. No significant alteration in total NKp46 (surface and intracellular) was observed, and there was no change with any of the influenza pps or any of the HA titers, compared with levels in mock-treated CD56$^{dim}$ cells (data not shown).

To identify molecules that may contribute to NK cell activation, multiplex analysis of NK cell phenotypic markers, including CD16, NKG2D, 2B4, and IL-10R, was performed. These markers on NK cells were not significantly altered by stimulation with influenza pps, compared with markers levels on mock-treated NK cells in the absence of pps (data not shown).

**NK cell activation by influenza virus pps partially mediated by NKp46**

The activation of NK cells in response to influenza pps was observed as changes in the expression of both CD69 and NKp46. Previously, it was reported that NKp46 can recognize influenza HA antigen and transfer activating signals to induce CD69 expression (5, 44). Thus, the induction of these two markers of NK activation may be linked.

To identify the role of NKp46 receptor in NK cells response to pps, our ongoing experiment was to compare NK cell functions with or without anti-NKp46 blocking Ab.
After the NKp46 receptor was blocked by the specific anti-NKp46 mAb, the expression of CD69 was remarkably inhibited (Fig. 5) in both CD56\textsuperscript{bright} subset (Fig. 5B) and CD56\textsuperscript{dim} subset (Fig. 5C), in terms of both the percentage of CD69\textsuperscript{+} NK cells and the MFI (n=6, P<0.05).

CD107a expression and cytokine production were also examined after being blocked by anti-NKp46. Interestingly, although these markers were all displayed downregulation in different extent, there were no significant differences as compared with those of NKp46 unblocked NK cells (supplementary Fig. 3 and 4).
A new type of influenza A H1N1 virus of swine origin emerged in March 2009, producing significant morbidity and mortality especially in young patients; this caused a great deal of public concern, based on previous experiences with the 1918 pandemic and H5N1 infections. Recent clinical and experimental studies suggested that enhanced inflammation and cell death in cases of severe influenza infection might have contributed to the severe immunopathology (21, 22). Innate immune responses, including the effects of NK cells, are important in minimizing influenza virus infection (17). NK cells may encounter influenza virions during viral infection. Several studies have suggested that influenza virus directly targets NK cells as part of an immunoevasion strategy; this is supported by the findings that influenza virus can bind to NCRs on NK cell surface and directly infect NK cells and trigger cell apoptosis (4, 7). To explore the interactions of human primary NK cells with the H5N1 and 1918 and 2009 pandemic H1N1 viruses as representative influenza A viruses, we used an in vitro model with pseudotyped virus particles expressing only HA and NA in a retrovirus-based system to stimulate resting human NK cells.

Retrovirus-based influenza pps containing only influenza virus HA and NA proteins are good tools for studying the antigenicity of influenza virus toward target cells, because the HA-specific activities of pps were shown to be similar to those of egg-grown influenza
viruses (59). We chose influenza pps instead of wild influenza viruses for the following reasons: 1) The use of pps avoids biosafety hazards, whereas the highly pathogenic H5N1 and 2009 pandemic H1N1 influenza viruses must be studied individually in a BSL-3 laboratory facility; 2) The surface HA and NA antigens of 1918 H1N1 influenza virus can be recreated using pps; and 3) The pps containing only HA and NA proteins simplified the study by eliminating the influence of internal gene segments.

The population of human NK cells is phenotypically and functionally heterogeneous. The immunoregulatory CD56$^{\text{bright}}$ cells (~10% in blood) are superior to CD56$^{\text{dim}}$ cells in their ability to produce abundant amounts of cytokines and chemokines. It has therefore been suggested that the major effector function of CD56$^{\text{dim}}$ cells is cytotoxicity (20, 56). The data presented here firmly establish that these three different influenza pps directly activate human NK cells and heterogeneously influence their functions in different subsets of NK cells. The expression of the early activation marker CD69 was universally upregulated on both CD56$^{\text{bright}}$ and CD56$^{\text{dim}}$ cells (Fig. 1), although the CD69 expression level was much higher on CD56$^{\text{dim}}$ cells than on CD56$^{\text{bright}}$ cells (Fig. 1B and 1C). However, the change of NKp46 was only detected on CD56$^{\text{dim}}$ subset cells (Fig. 4).

Additionally, functional activation is initiated by overnight pps stimulation (Fig. 2 and 3) including upregulation of CD107a preferentially in the CD56$^{\text{dim}}$ population (Fig. 2A-C), enhanced cytotoxic activity toward K562 cells (Fig. 2D and E) and IFN-γ production (Fig. 3). These observations suggest that different NK subset cells may be differentially...
concerned with NK cell activation and function in peripheral blood in response to influenza virus stimulation and the distinct intracellular signaling pathways that lead to cytokine secretion and cytotoxicity in NK cells may be complex (55, 60). Besides CD56<sup>bright</sup> NK cell response to those pps, CD56<sup>dim</sup> population acts as the primary effectors as reported elsewhere (26, 57).

NKp46 was considered to be a vital receptor potentially involved in the direct recognition to influenza virus without the need for accessory or mediating molecules (5, 44). On stimulation with each of the three influenza pps, downregulated surface expression of functional NKp46 was restricted to the CD56<sup>dim</sup> subset of NK cells (Fig. 4). Since total NKp46 expression (surface and intercellular) did not change, it may be that once binding to viral surface glycoproteins, NKp46 is being internalized and initiates the activation signaling.

NK activations by the pps did be inhibited by a blocking Ab against NKp46 receptor (Fig. 5). The expression of activation marker CD69 on pps-treated NK cells was suppressed significantly by anti-NKp46 although the blocking effect was incomplete. Oppositely, NK cell activation by pps and CD107a expression and IFN-γ production, displayed no significant difference with or without anti-NKp46 blocking antibody. It was recently demonstrated that besides NCRs, Toll-like receptor (TLR) 2 on NK cells could also directly recognize lipophosphoglycan of pathogens and some viral replication products.
can also activate NK cells indirectly by interacting with appropriate TLRs (3, 30). Our data obtained, together with these recent findings, suggest that NKp46 is not likely to be the only receptor involved in influenza pps-induced NK cell activation but rather may play a coreceptor role in the complex network of functional activities of NK cells, which has yet to be determined.

Fatal human respiratory disease associated with the 1918 pandemic influenza virus and the potentially pandemic H5N1 virus is characterized by severe lung pathology, including pulmonary edema and extensive inflammatory infiltration (22, 23, 52). Recent studies using animal models to investigate the mechanisms of severe influenza virulence have suggested a role of the innate immune system in complicating lung tissue recovery (21, 25, 47). Although the morbidity and mortality induced by the 2009 pandemic H1N1 virus are similar to those associated with seasonal H1N1 virus, 2009 pandemic H1N1 can also cause fatal respiratory disease in immunocompetent adults. Recent studies with challenge models in ferrets and mice have suggested that 2009 pandemic H1N1 causes more serious lung pathology than seasonal H1N1 (48). As compared with the H1N1-2009pps, the H1N1-1918pps and H5N1pps produced much greater stimulations, as evidenced by significant upregulation of CD69 and CD107a expression, stronger cytotoxic activity toward K562 cells, and higher levels of IFN-γ. The differential NK activation maybe partially related to receptor-binding affinity or preference of those viruses which was already proved in a lower level of NK cell lysis activated by human influenza H3N2 virus.
isolates due to their reduced affinity to both α-2,3- and α-2,6-linked receptors (38). Our findings implied that there was a stronger NK response to H5N1 and 1918 H1N1 virus infection in which excess innate immune responses: proinflammatory cytokines, macrophages and neutrophils were found in mice model or fatal cases (40). Although the levels of CD69 expression on H1N1-1918pp-stimulated cells were higher than those on H5N1pp-stimulated cells, H5N1pp showed a slightly stronger effect on NK cell function with regard to lytic capability and IFN-γ production. These observations also support a potentially critical difference between the pathogenesis of H5N1 and 1918 H1N1 infections. In contrast, H1N1-2009pp demonstrated a lower level of NK cell stimulation with modest changes in activation phenotype and cytotoxic activity, consistent with a milder pathogenesis of 2009 pandemic H1N1 than that of H5N1 and 1918 H1N1. Notably, NKp46 on NK cells, recognizing pps partially, showed no difference among these pps stimulations at the same HAU dosage. It is likely the effects exhibited by the interaction of NKp46 and pps not only determined by amount or density of receptors but also pathogen glycoproteins themselves certainly involved in the pathogenicity of influenza viruses (12, 19, 46). Moreover, the diversity of functional activation of NK cells might also imply the enormous network of NK cells in response to influenza virus infection. And a dosage-dependent manner was found in NK cell activation by pps. Yet, an increasing viral HA titer attenuated its activation. These observations suggest a dual role for NK cells in the early stages of influenza virus infection.
Although our results were obtained under *in vitro* experimental conditions, they nevertheless contribute to our understanding of the potential mechanisms of NK responses to influenza infection. Further studies on the underlying intracellular signaling pathway or other related viral components are required.
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Figure legends

Figure 1. Expression of the early activation marker CD69 on NK cells in response to influenza pps.

Freshly purified human NK cells from 18 different donors were cocultured overnight with H1N1-2009pps, H1N1-1918pps, or H5N1pps at doses ranging from 50 to 1000 HAU/ml.

(A) CD69 expression on mock-treated NK cells and on NK cells stimulated with pps at a dosage of 1000 HAU/ml from one representative donor. FACS plots are gated on CD56^+CD3^- lymphocytes. Percentages indicate the proportions of CD56^{bright} or CD56^{dim} cells that were positive for CD69. Data are based on the collection of 20,000 total events.

(B) CD69 expression on CD56^{bright} NK cells. The percentage of CD69^+ cells (left panel) and the fold increase in CD69 median fluorescence intensity (MFI) (right panel) are shown. (C) CD69 expression on CD56^{dim} NK cells. The percentage of CD69^+ cells (left panel) and the fold increase in CD69 MFI (right panel) are shown. *, P<0.05; **, P<0.01.

Figure 2. Expression of CD107a on NK cells in response to influenza pps and the cytotoxicity of NK cells toward K562 cells after stimulation by pps.

Freshly purified human NK cells from 18 different donors were cocultured overnight with H1N1-2009pps, H1N1-1918pps, or H5N1pps at doses ranging from 50 to 1000 HAU/ml. CD107a expression was assessed by intracellular staining, followed by flow cytometric analysis. For the cytotoxicity assay, K562 cells were prestained with calcein AM,
coclutured with pp-stimulated NK cells or mock-treated NK cells for 3 h, and then
stained with ethidium homodimer-1. Cytotoxicity was analyzed by flow cytometry and
calculated as the percentage of cells stained with calcein AM plus ethidium homodimer-1
cells among the total cells stained with calcein AM. (A) CD107a expression on
mock-treated NK cells and on NK cells stimulated with pps at a dose of 1000 HAU/ml
from one representative donor. FACS plots are gated on CD56^CD3^- lymphocytes.
Percentages indicate the proportions of CD56^{bright} or CD56^{dim} cells that were positive for
CD107a. Data are based on the collection of 50,000 total events. (B) CD107a expression
on CD56^{bright} NK cells. The percentage of CD107a^+ cells (left panel) and the fold increase
in CD107a MFI (right panel) are shown. (C) CD107a expression on CD56^{dim} NK cells.
The percentage of CD107a^+ cells (left panel) and the fold increase in CD107a MFI (right
panel) are shown. (D) Cytotoxic activity of mock-treated and pp-stimulated NK cells
from 6 individuals toward K562 cells at an E/T ratio of 20:1. (E) Cytotoxic activity of
mock-treated NK cells and NK cells from 6 individuals stimulated by pps at a dose of 200
HAU/ml toward K562 cells at different E/T ratio. E/T ratio, ratio of effector to target cells,
*, P<0.05; **, P<0.01.

Figure 3. Influenza pp-induced IFN-γ in NK cells.
Freshly purified human NK cells from 18 different donors were cocultured overnight with
H1N1-2009pps, H1N1-1918pps, or H5N1pps at doses ranging from 50 to 1000 HAU/ml.
IFN-γ was determined in cell-free supernatants using human IFN-γ Flex Set reagents (BD
Biosciences) according to the manufacturer’s protocol. Concentrations are shown in pg/mL. **, $P<0.01$.

Figure 4. Expression of NKp46 on NK cells in response to influenza pps.

Freshly purified human NK cells from 18 different donors were cocultured overnight with H1N1-2009pps, H1N1-1918pps, or H5N1pps at doses ranging from 50 to 1000 HAU/ml.

NKp46 expression was analyzed by surface staining and flow cytometric analysis. (A) NKp46 expression on mock-treated NK cells and on NK cells stimulated with pps at a dose of 1000 HAU/ml from one representative donor. FACS plots are gated on CD56$^+$CD3$^-$ lymphocytes. Percentages indicate the proportions of CD56$^{\text{bright}}$ or CD56$^{\text{dim}}$ cells that were positive for NKp46. Data are based on the collection of 20,000 total events.

(B) NKp46 expression on CD56$^{\text{bright}}$ NK cells. The percentage of NKp46$^+$ cells (left panel) and the decrease percentage in NKp46 MFI (right panel) are shown. (C) NKp46 expression on CD56$^{\text{dim}}$ NK cells. The percentage of NKp46$^+$ cells (left panel) and the decrease percentage in NKp46 MFI (right panel) are shown.

Figure 5. Enhanced NK cell activation partially induced by NKp46 downregulation.

Freshly purified human NK cells from 6 different donors were first incubated for 1 h with or without unlabeled anti-NKp46 mAb at a concentration of 10µg/ml, and then were cocultured overnight with H1N1-2009pps, H1N1-1918pps, or H5N1pps at a dose of 200 HAU/ml. Corresponding isotype antibody was added as control. (A) CD69 expression on
mock-treated NK cells and on NK cells stimulated with pps from one representative donor. FACS plots are gated on CD56^−CD3^− lymphocytes. Percentages indicate the proportions of CD56^{bright} or CD56^{dim} cells that were positive for CD69. Data are based on the collection of 50,000 total events. (B) CD69 expression on CD56^{bright} NK cells. The percentage of CD69^+ cells (left panel) and the fold increase in CD69 MFI (right panel) are shown. (C) CD69 expression on CD56^{dim} NK cells. The percentage of CD69^+ cells (left panel) and the fold increase in CD69 MFI (right panel) are shown. *, P<0.05; **, P<0.01.
References


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