

Influenza Virus Directly Infects Human Natural Killer Cells and Induces Cell Apoptosis[∇]

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Influenza is an acute respiratory viral disease that is transmitted in the first few days of infection. Evasion of host innate immune defenses, including natural killer (NK) cells, is important for the virus's success as a pathogen of humans and other animals. NK cells encounter influenza viruses within the microenvironment of infected cells and are important for host innate immunity during influenza virus infection. It is therefore important to investigate the direct effects of influenza virus on NK cells. In this study, we demonstrated for the first time that influenza virus directly infects and replicates in primary human NK cells. Viral entry into NK cells was mediated by both clathrin- and caveolin-dependent endocytosis rather than through macropinocytosis and was dependent on the sialic acids on cell surfaces. In addition, influenza virus infection induced a marked apoptosis of NK cells. Our findings suggest that influenza virus can directly target and kill NK cells, a potential novel strategy of influenza virus to evade the NK cell innate immune defense that is likely to facilitate viral transmission and may also contribute to virus pathogenesis.

Influenza is an acute respiratory virus infection that continues to pose endemic, zoonotic, and pandemic threats to human health, with significant morbidity and mortality (17). At the early phase of viral infection, innate immunity plays important roles in host defense by limiting viral replication and helping to initiate an adaptive immune response. Natural killer (NK) cells are key effector cells in innate immunity and play a critical role in the first line of host defense against acute viral infections by directly destroying infected cells without the need for prior antigen stimulation (7, 20). As influenza illness and virus transmission usually occur in the first few days of infection, the virus has to devise strategies to evade host innate immune responses, including NK cell immunity (15, 21).

NK cells can recognize and kill influenza virus-infected cells (2, 10, 23); to counteract this killing, however, influenza virus has developed an escape strategy that inhibits NK cell cytotoxicity by increasing the binding of two inhibitory receptors to the infected cells after infection (1). The individuals with complete NK cell deficiency developed life-threatening varicella zoster virus and cytomegalovirus infection, but no severe influenza virus infection occurred (30, 40). Indeed, the interaction between human NK cells and influenza virus remains poorly understood. After influenza virus infection, respiratory epithelial cells release inflamma-

tory chemokines that recruit NK cells to the site of infection (12). As a lytic virus, numerous influenza virus particles are released from the infected epithelia and macrophages (5, 9, 33). In the infected microenvironment, NK cells undoubtedly encounter these infective virus particles. It is therefore important to investigate the direct interaction of NK cells with influenza virus. Patients with severe influenza virus infection were shown to have diminished NK cells in peripheral blood and an almost complete absence of pulmonary NK cells, together with marked apoptosis (13, 42). During influenza virus infection in mice, a transient increase of NK cytotoxicity is followed by a marked decrease in NK cell activity, with a virus dose-dependent effect (8, 28). These data suggest that influenza virus may directly target NK cells as part of its immunoevasion strategies. However, no reports of the direct effects of influenza virus on human NK cells have so far been available.

In this study, we demonstrated that influenza virus infects and replicates in primary human NK cells. Viral infection was dependent on sialic acids on the cells. The entry was mediated by both clathrin- and caveolin-dependent endocytosis rather than macropinocytosis. Influenza virus infection induced a marked apoptosis of NK cells, which contributed to reduced NK cell cytotoxicity. This, to the best of our knowledge, is the first paper to demonstrate that influenza virus can directly infect NK cells and induce cell apoptosis. These findings suggest that influenza virus may have developed a novel strategy to evade NK cell innate immune defenses, which is likely to facilitate viral transmission and may also contribute to virus pathogenesis.

MATERIALS AND METHODS

Isolation of primary human NK cells. Peripheral blood mononuclear cells were isolated from whole-blood samples obtained from the Hong Kong Red Cross by Ficoll-Hypaque (Pharmacia) gradient centrifugation (44). NK cells were magnetically separated from peripheral blood mononuclear cells with NK

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cell isolation kit II (Miltenyi Biotec). The purity of isolated CD56⁺ CD3⁻ NK cells was consistently >97%, as determined by flow cytometry. NK cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% autologous serum. The research protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster.

Influenza virus preparation and infection of NK cells. As described in our previous study (44), influenza virus A/Hong Kong/54/98 (H1N1) was cultured in Madin-Darby canine kidney (MDCK) cells and was purified by adsorption to and elution from turkey red blood cells. The virus titer was determined by titration in MDCK cells, with daily observation of cytopathogenic effect, and confirmed by hemagglutination assay. MDCK cells were routinely maintained in Dulbecco's modified Eagle's medium (Invitrogen).

NK cells or MDCK cells were incubated with influenza virus H1N1 at a multiplicity of infection (MOI) of 2 in the absence of human serum. After 1 h of viral adsorption, unadsorbed viruses were washed away by excess phosphate-buffered saline. Mock-infected cells were treated in parallel except that the virus was not added. In some experiments, NK cells were activated by stimulation with 500 U/ml recombinant human interleukin 2 (IL-2) (Invitrogen) prior to influenza virus infection. For the inhibition of viral endocytosis, NK cells were preincubated with chlorpromazine, nystatin, and cytochalasin D (Sigma-Aldrich) at the concentrations noted in Fig. 4 for 30 min and then infected by influenza virus. Every agent was present during the infection process. To determine whether virus infection was dependent on sialic acid, NK cells were first treated with *Arthrobacter ureafaciens* sialidase (Sigma) for 30 min and then extensively washed prior to infection. In some experiments, NK cells were pretreated with 20 μ M Z-Asp-Glu-Val-Asp-fluoromethylketone (Z-DEVD-FMK; BD Biosciences) for 30 min prior to infection, and this agent was present during the infection process.

Quantitative reverse transcriptase-PCR (RT-PCR). Total cellular RNA was extracted by using TRIzol reagent (Invitrogen) at the time points indicated in Fig. 2. The cDNA was transcribed from DNase-treated RNA with Superscript II RT (Invitrogen) and quantified by a quantitative PCR assay with an ABI Prism 7700 sequence detection system (Applied Biosystems) as described in our previous reports (5, 44). The sequences of specific primers and probes for the influenza matrix (M) gene and β -actin were described in our previous study (44). The expression of M gene mRNA was normalized to 10^4 copies of β -actin.

Immunofluorescent staining. Virus- or mock-infected cells were fixed with methanol-acetone (1:1) and then analyzed by immunofluorescent staining using an antibody reagent (Dako Cytomation) containing monoclonal antibodies for influenza viral M and nucleoprotein (NP) antigens to determine influenza virus infection. For the colocalization assay, 1 hour after infection, NK cells were stained with anti-influenza NP-fluorescein isothiocyanate (A1; Chemicon) and anti-early endosome antigen 1 (EEA1) (N-19) or anti-clathrin (H-300; both from Santa Cruz). EEA1 and clathrin were examined with Alexa Fluor 647 donkey anti-goat antibody and goat anti-rabbit antibody (both from Invitrogen), respectively. Images were obtained with a Leica DMLB microscope (Leica Microsystems AG) or a Zeiss 510-Meta confocal microscope (Carl Zeiss).

Electron microscopy. NK cells were exposed to influenza virus on ice for 90 min to allow viral binding and then shifted to 37°C for viral internalization. Twenty minutes later, cells were washed and fixed in 2.5% glutaraldehyde, followed by processing for electron microscopy as described in our previous study (19). The ultrathin section was viewed on a Philips EM208S transmission electron microscope (Philips Electron Optics).

Flow cytometry. The following monoclonal antibodies were used in this study: anti-CD56 (NCAM16.2), anti-CD3 (UCHT1), anti-NKp46 (9E2), anti-perforin (G9), anti-granzyme B (GB11), anti-caspase 3 (C92-605; all from BD Biosciences), and anti-influenza NP (H16-L10-4R5; Chemicon). For surface staining, NK cells were stained with specific antibodies. For intracellular staining, cells were fixed, permeabilized, and then labeled with the antibodies indicated above. To avoid nonspecific staining, cells were incubated with FcR blocking reagent (Miltenyi Biotec) prior to the specific staining. NK cell apoptosis was determined with the Annexin V-fluorescein isothiocyanate kit according to the manufacturer's instructions (Immunotech). All data were acquired on a BD FACS Aria with FACS Diva (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Cytotoxicity assay. The cytotoxicity assay of influenza virus-infected NK cells was performed with a Live/Dead cell-mediated cytotoxicity kit (Invitrogen) as described in our previous study (41). Briefly, K562 target cells were stained with 3,3'-diiodoacetylcarboxyanine perchlorate (DiOC₁₈) and then cocultured with NK cells at specific effector cell/target cell (E/T) ratios in the presence of propidium iodide (PI) for 2 h. After incubation, cytotoxicity was analyzed by flow cytometry and calculated as the percentage of DiO⁺ PI⁺ cells out of the total number of DiO⁺ cells.

Statistical analysis. Data are expressed as means \pm standard errors of the means (SEM). Statistical analysis was performed by Student's paired *t* test or

one-way analysis of variance (ANOVA) with a multiple-comparison test using Prism 5 (GraphPad Software). A *P* of <0.05 was considered significant.

RESULTS

Influenza virus directly infects primary human NK cells.

We first determined whether influenza virus could directly infect NK cells. Freshly isolated primary human NK cells were infected with H1N1 virus for 1 h, extensively washed to remove unadsorbed virus, and then cultured for 6 h. Using RT-PCR, we detected the M gene in influenza virus-exposed NK cells but not in mock-treated NK cells (data not shown). In addition, the expression of influenza viral antigens was also detected in the virally exposed NK cells by immunofluorescent staining. In parallel, purified resting primary human T cells, which cannot be directly infected by influenza virus (24), were included as a negative control. After treatment with influenza virus, no positive staining was found in these cells (Fig. 1A). The confocal microscopy further showed the expression of viral antigens in the infected NK cells (Fig. 1B). The attachment and entry of virus into NK cells were confirmed by electron microscopy, which showed that virus particles bound to the cell membrane and within vesicles inside NK cells (Fig. 1C). Using flow cytometry to detect intracellular viral antigen, we found that about 50% of NK cells were infected by H1N1 virus at an MOI of 2 at 6 h postinfection. As a positive control, MDCK cells were similarly treated with influenza virus at an MOI of 2. At 6 h postinfection, all the MDCK cells were infected by the virus (Fig. 1D).

We further examined whether the activated NK cells could be infected by influenza virus. NK cells were activated by recombinant human IL-2 and then treated with H1N1 virus. As shown in Fig. 1E, confocal microscopy with intracellular staining of viral antigens demonstrated that influenza virus also successfully infected IL-2-activated NK cells.

Influenza virus replicates in primary NK cells. After infection, influenza virus could replicate within NK cells. The expression of viral M gene mRNA and NP protein was detected at a series of time points postinfection by quantitative RT-PCR and flow cytometry. The expression of the viral M gene increased from 1 h onwards to peak at 6 h after infection and then decreased (Fig. 2A). In contrast, the expression of viral protein by flow cytometry analysis peaked at 12 h after infection (Fig. 2B). At the same time points, the supernatants from infected NK cells were collected and inoculated onto MDCK cells to determine whether infectious progeny were produced. However, influenza virus infection did not lead to increasing titers of infectious virus in the NK cell supernatants and therefore is abortive in nature (data not shown). We further investigated whether inactivated influenza virus could infect NK cells. The cells were exposed for 1 h to virus inactivated by UV or by heat at 100°C. Upon inactivation by UV, which did not alter viral proteins and fusion activity (3), influenza virus could still bind to NK cells (Fig. 2C). In contrast, the heat treatment at 100°C denatured the hemagglutinin and impaired viral binding to cell surfaces; therefore, the heat (100°C)-inactivated influenza virus did not bind NK cells (Fig. 2C).

Influenza virus infection is dependent on the sialic acids on NK cells. The sialic acid on target cells is now commonly recognized as the receptor for influenza virus infection. Upon

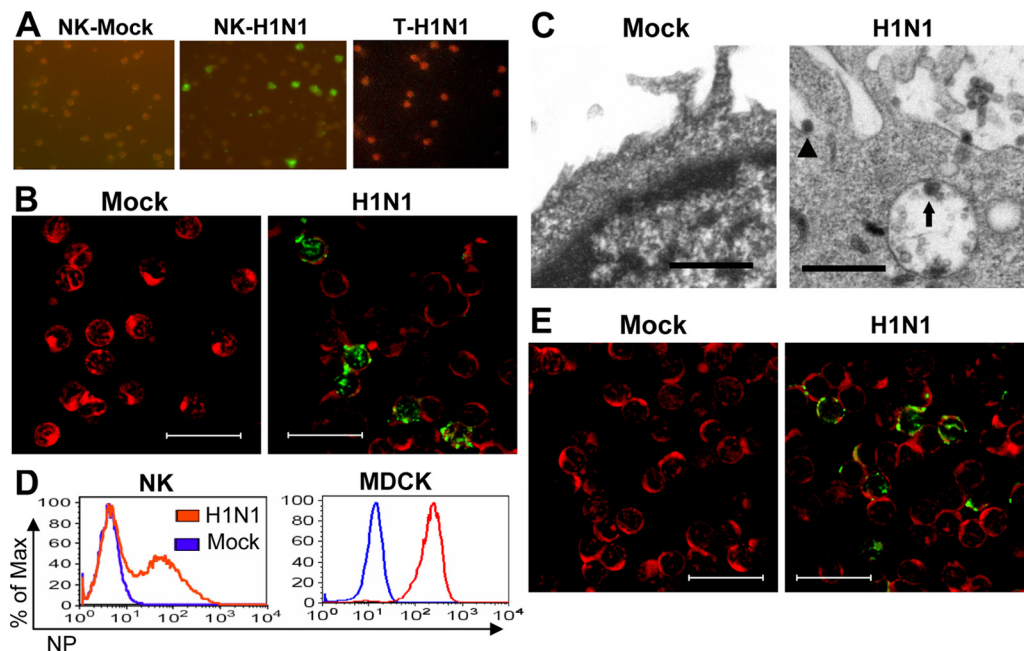


FIG. 1. Influenza virus directly infects primary human NK cells. (A) Fresh NK cells were treated with (NK-H1N1) or without (NK-Mock) influenza H1N1 virus for 1 h, extensively washed, and then cultured for 6 h. Cells were fixed and permeabilized for intracellular staining of viral antigens (green) with Evan's blue (red) as a counterstain. Fresh purified primary human gamma delta T cells treated with H1N1 virus (T-H1N1) were included as a negative control. (B) The NK cells that were infected or not infected with influenza virus as described for panel A were examined by confocal microscopy for intracellular staining of viral antigens. Scale bars, 20 μ m. (C) A negative-contrast, thin-section transmission electron microscope was used to view mock (left)- or H1N1 (right)-infected NK cells at 20 min after viral infection. Viruses bound to (triangle) and were internalized by (arrow) NK cells. Scale bars, 0.5 μ m. (D) Fresh NK cells were infected with (red line) or without (blue line) H1N1 virus for 6 h as described for panel A. The expression of intracellular NP protein was examined by flow cytometry. MDCK cells infected with H1N1 at the same MOI during the same time as NK cells were included as a positive control. (E) IL-2-activated NK cells were treated (right) or not treated (left) with influenza H1N1 virus for 6 h as described for panel A. Cells were fixed and permeabilized for intracellular staining of viral antigens (green), with Evan's blue (red) as a counterstain. Scale bars, 20 μ m. Images are representatives of three independent experiments.

binding to sialic acids on the cell surface, the virus is internalized by receptor-mediated endocytosis. We next determined the role of sialic acid in the influenza virus infection of NK cells by treating cells with serial concentrations of sialidase prior to viral infection, aiming to cleave the sialic acids on NK cells. Six hours after infection, intracellular viral antigen was detected by flow cytometry. As shown in Fig. 3A, the blockade of influenza virus infection by sialidase in NK cells was dose dependent. The frequency of virally infected NK cells decreased by around half at the concentration of 100 mU of sialidase/ml, and almost total blockade of the infection was found when 500 mU of sialidase/ml was applied. Cell viability was not affected by the sialidase treatment, as evidenced by no changes in annexin V binding and PI uptake of the treated NK cells (Fig. 3B). In addition, the treated cells also showed major histocompatibility complex (MHC) class I staining similar to that of mock-treated cells (Fig. 3C). These data suggested that influenza virus infection of NK cells is mediated by sialic acids. In addition, double staining against viral protein and anti-EEA1 was performed at 1 h after influenza virus infection. Confocal microscopy analysis showed that these two proteins colocalized (Fig. 3D), indicating that influenza virus entered NK cells via endocytosis.

Influenza virus infection of NK cells is mediated by both clathrin- and caveolin-dependent endocytosis but not macropinocytosis. Recent research has demonstrated that numerous viruses employ the following endocytic pathways to infect different cells:

clathrin-mediated endocytosis, caveolin-mediated endocytosis, macropinocytosis, and clathrin- and caveolin-independent pathways (6, 26). We thus attempted to identify the endocytic pathway(s) exploited by influenza virus for infecting NK cells. We first examined whether clathrin-mediated endocytosis was involved in infection, because it is the most established route of entry. Double staining of viral antigen and clathrin showed that these two proteins colocalized (Fig. 4A). Flow cytometry analysis further demonstrated that both the frequency of infected cells and the geometric mean fluorescent intensity of viral antigen were significantly decreased by clathrin inhibitor chlorpromazine treatment in a dose-dependent pattern (Fig. 4B). These data indicated that clathrin-dependent endocytosis was exploited by influenza virus to infect NK cells.

We next defined the role of caveolin-mediated endocytosis in an influenza virus infection of NK cells. Nystatin was applied to specifically block this endocytic pathway (18). Similarly to chlorpromazine, nystatin significantly inhibited the viral infection in a dose-dependent way. Decreased frequencies of infected cells and decreased intensity of viral antigen were observed at 6 h postinfection (Fig. 4C), suggesting that caveolin-mediated endocytosis was also involved in the viral infection.

Finally, we examined whether macropinocytosis contributed to influenza virus infection of NK cells. We used cytochalasin D, the most commonly used inhibitor (36), to inhibit this path-

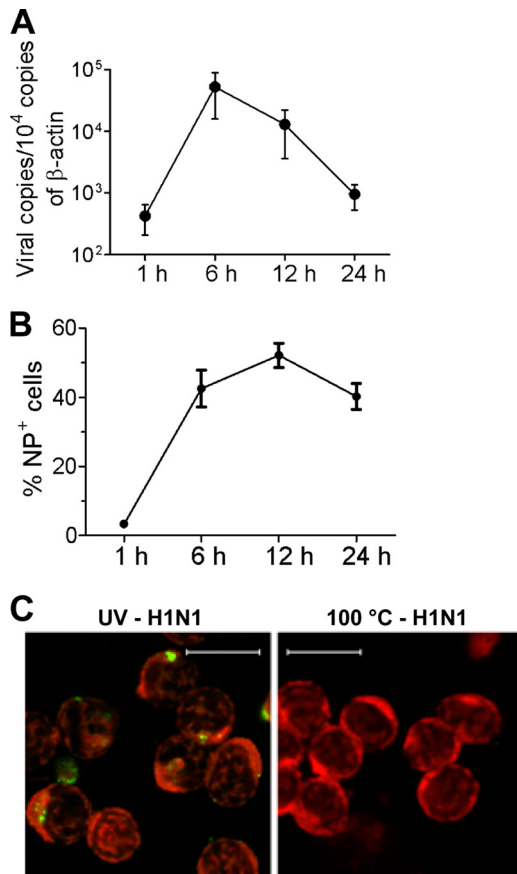


FIG. 2. Influenza virus replicates in primary human NK cells. (A and B) NK cells were infected with H1N1 virus for the indicated times. M gene mRNA expression levels (A) and percentages of cells that were infected (B) were determined by quantitative RT-PCR and flow cytometry, respectively. The data shown are means \pm SEM of results for four different donors. (C) NK cells were treated with UV- or heat (100°C)-inactivated H1N1 virus as described in the legend of Fig. 1. After 6 h, samples were stained and examined by confocal microscopy for the expression of viral antigen (green), with Evan's blue (red) as a counterstain. Scale bars, 10 μ m. Images are representative of samples from three independent experiments.

way during influenza virus infection of NK cells. The expression of viral antigen was determined by flow cytometry at 6 h postinfection. Neither the percentage of infected cells nor the intensity of viral antigen was decreased (Fig. 4D), indicating that macropinocytosis was not required for influenza virus entry into NK cells.

Direct influenza virus infection kills NK cells. In order to determine the effect of influenza virus infection on NK cell viability, the cells were stained with annexin V and PI at 24 h after infection; annexin V and PI were selected as the markers of early apoptosis and late apoptosis, respectively. As shown in Fig. 5A, the frequencies of both annexin V⁺ PI⁻ and annexin V⁺ PI⁺ cells were remarkably increased after viral infection. The percentage of annexin V⁺ cells in the total number of virally infected NK cells was significantly higher than that in mock-infected cells (Fig. 5B). The activation of caspase 3, another biological marker for induction of apoptosis, was also examined in NK cells. Similarly, the percentage of ac-

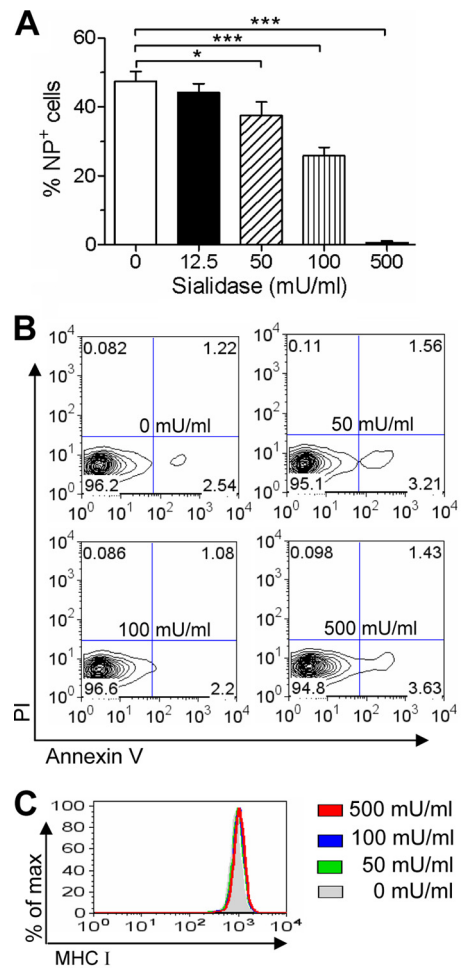


FIG. 3. Influenza virus infection of NK cells is dependent on sialic acids. (A) NK cells were pretreated with serial concentrations of *Arthrobacter ureafaciens* sialidase for 30 min at 37°C, extensively washed, and then infected with H1N1 virus as described in the legend of Fig. 1. After 6 h, samples were examined by flow cytometry for frequencies of infected cells. The data shown are means \pm SEM of results for four different donors. Results of one-way ANOVA with Tukey multiple-comparison test analysis are shown (*, $P < 0.05$; ***, $P < 0.001$). (B and C) After being treated with *Arthrobacter ureafaciens* sialidase at the indicated concentrations for 30 min, NK cells were examined for apoptosis by determining annexin V binding and PI uptake (B) and for surface MHC class I expression (C). (D) One hour after influenza virus infection of NK cells, the colocalization of intracellular viral antigen (green) and EEA1 (red) was determined by confocal microscopy. Scale bars, 10 μ m. Images are representative of three independent experiments.

tive caspase 3-positive cells was about fourfold higher in virally infected NK cells than that in mock-infected cells (Fig. 5C). In addition, by costaining active caspase 3 and viral NP protein, we demonstrated that almost all of the apoptotic NK cells were influenza virus-infected cells (Fig. 5D).

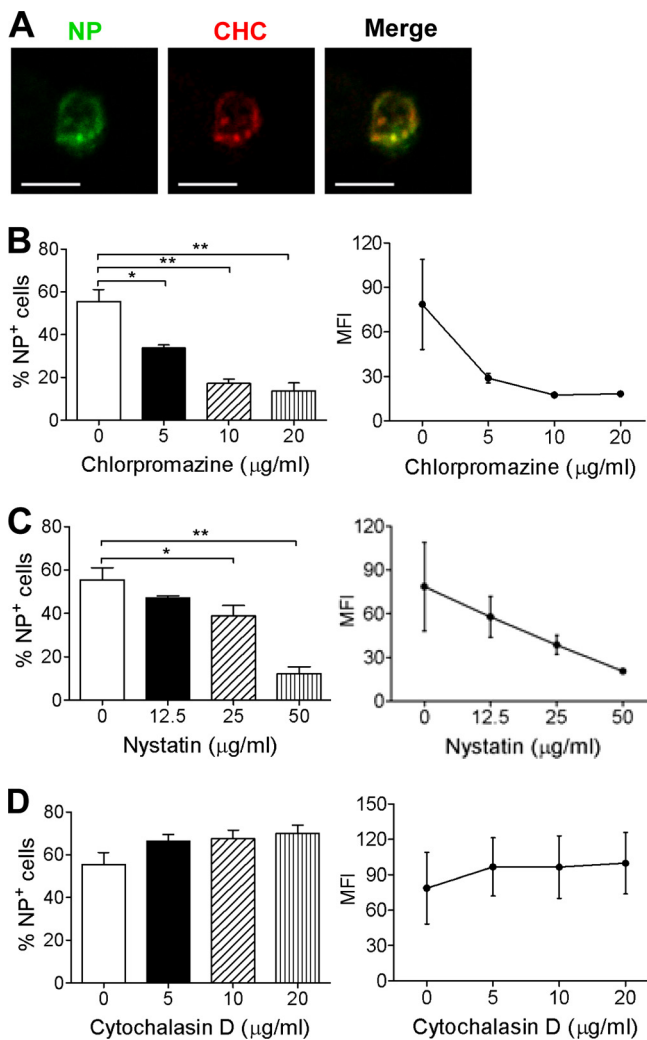


FIG. 4. Influenza virus infection of NK cells is mediated by both clathrin- and caveolin-dependent endocytosis but not macropinocytosis. (A) One hour after influenza virus infection of NK cells, the colocalization of intracellular viral antigen (green) and clathrin (red) was determined by confocal microscopy. Scale bars, 10 μm. CHC, clathrin heavy chain. Images are representatives of three independent experiments. (B to D) NK cells were preincubated with chlorpromazine (B), nystatin (C), and cytochalasin D (D) at the indicated concentrations for 30 min and then infected by H1N1 virus. The frequency of infected cells (left) and the geometric mean fluorescence index of viral antigen (right) at 6 h postinfection were analyzed by flow cytometry. The data shown are means ± SEM of results for four different donors. The results of one-way ANOVA with Tukey multiple comparison test analysis are shown (*, $P < 0.05$; **, $P < 0.01$).

We next examined whether UV-inactivated or heat (100°C)-inactivated influenza virus could also kill NK cells. As shown in Fig. 5E, unlike with live virus, the UV-inactivated or heat (100°C)-inactivated virus did not increase NK cell apoptosis compared to that in mock-infected cells, which suggested that only live H1N1 virus can induce the apoptosis of NK cells. We further determined whether caspase 3 inhibitor could preserve NK cell survival. As shown in Fig. 5F, Z-DEVD-FMK significantly reduced NK cell apoptosis induced by influenza virus.

Following the observation that influenza virus induced NK

cell apoptosis, we continued to determine NK cytotoxicity. At 24 h postinfection, total NK cells were collected and examined for their killing of K562 cells. As shown in Fig. 6A, influenza virus-infected NK cells showed significantly lower cytotoxicity than mock-infected cells at all E/T ratios. NKp46 is a major lysis receptor for fresh NK cells and contributes to cell cytotoxicity (38, 39). We then examined NKp46 and granule expression in virally infected NK cells. The expression of both surface and intracellular NKp46 and of perforin was remarkably decreased in virally infected NK cells, compared to that in mock-infected cells (Fig. 6B and C). With PI staining to exclude the dead cells, we further examined NKp46 expression in the gated PI-negative cells and also found that NKp46 was significantly decreased in these cells, although the change was minor (Fig. 6D and E). As a control, the expression of MHC class I was not decreased in the PI-negative cells with influenza virus infection (Fig. 6F). The decreased expression of NKp46 was not due to masking by viral hemagglutinin, as the hemagglutinin-binding site on NKp46 was different from that for specific antibody binding, indicated by the fact that both the UV-inactivated virus- and hemagglutinin protein-bound NK cells exhibited staining of NKp46 by the antibody that was similar to that of untreated cells (data not shown). The minor change in NKp46 expression of infected NK cells might be related to the cells that were undergoing apoptosis but still had complete cell membranes and could not take up PI. Taken together, these results suggest that direct influenza virus infection induced NK cell apoptosis, which would contribute to the reduced NK cell cytotoxicity.

DISCUSSION

Viruses and NK cells are in a constant battle. In response to NK cells, many viruses have developed a variety of strategies to evade the activity of these cells, aiming for viral survival and transmission (15, 21, 31, 35). In this study, we demonstrated for the first time that influenza virus could directly infect primary human NK cells via two distinct pathways. Viral infection induced marked NK cell apoptosis. These findings suggest that influenza viruses may have developed a novel strategy to evade the innate immune defense of NK cells, which is likely to facilitate viral transmission and may also contribute to virus pathogenesis.

It is interesting that influenza virus could directly infect and replicate in primary human NK cells, because NK cells are generally regarded as effector cells of innate immunity that kill virally infected cells during acute infection, although direct viral infection of NK cells has been shown for persistent virus infections, such as with herpes simplex virus (43), Epstein-Barr virus (14), human immunodeficiency virus (4), and ectromelia virus (32). Indeed, with innate immune cells, only macrophages were demonstrated to be susceptible to influenza virus infection and to support viral replication, according to our previous data and other studies (5, 33, 44). Here, using different techniques, including quantitative RT-PCR, flow cytometry, and confocal and electron microscopy, we demonstrated that NK cells are susceptible to influenza virus, which causes acute infection. The comparison of virus infections between NK and MDCK cells infected at the same MOI and for the same times

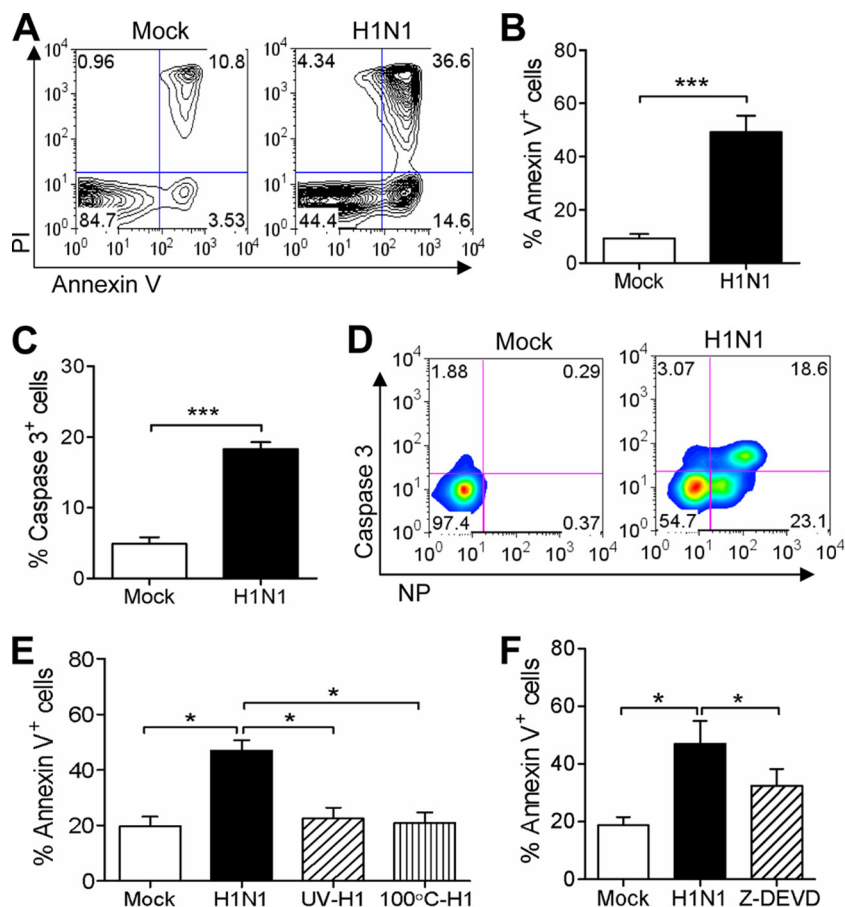


FIG. 5. Influenza virus infection induces marked NK cell apoptosis. NK cells were infected with H1N1 virus as described in the legend of Fig. 1. After 24 h, cell apoptosis was examined for annexin V binding and PI uptake (A and B) or for the intracellular expression of active caspase 3 (C). (A) Representative plots for mock (left)- or H1N1 virus (right)-infected NK cells. The frequency of annexin V (B)- or active caspase 3 (C)-positive cells was analyzed by flow cytometry. The results of Student's paired *t* test analysis are shown (***, $P < 0.001$). (D) At 24 h postinfection, NK cells were examined for intracellular expression of both active caspase 3 and viral NP protein by flow cytometry. (E) NK cells were not treated or treated with live, UV-inactivated, or heat (100°C)-inactivated H1N1 virus. After 24 h, frequencies of annexin V-positive cells were determined by flow cytometry. (F) NK cells were pretreated with 20 μ M Z-DEVD-FMK for 30 min and then infected by influenza virus. After 24 h, frequencies of annexin V-positive cells were determined by flow cytometry. The data shown are means \pm SEM of results for four different donors. Results of one-way ANOVA with Tukey multiple-comparison test analysis are shown (*, $P < 0.05$).

indicated that the efficiency of influenza virus infection of NK cells is lower than that of MDCK cells.

Following virus infection, virus replication can be either productive (i.e., with fully infectious virus being produced and released from the cell) or abortive (with no new infectious virus being produced). In the case of NK cells, we found that influenza viruses undergo abortive replication within NK cells, as evidenced by the increased expression of viral mRNA and internal protein after infection. Such a block in the complete replication of the virus may occur at one of several steps in the cycle of replication, including during viral ribonucleoprotein (vRNP) sorting, transport of subviral components to the cell membrane, assembly, packaging, budding, and final virus release. In addition, apart from the viral components, the host components are also involved in these processes (25). Therefore, any incompatibility between the requirements of the virus and what is available in the host cell might lead to an abortive infection. In fact, as with NK cells, the lymphocytes in general could not support viral replication, although they express viral

protein with the help of macrophages (24, 27). In mammals, replication-competent influenza A viruses are generally recovered from the superficial epithelium of the respiratory tract (9), where the viruses replicate and spread. In addition, the enhanced apoptosis of infected cells might also be partly responsible for the abortive infection of NK cells by influenza virus.

Studies have shown that influenza virus infection of target cells is a multiple-step process. Upon binding to sialic acids on the cell surface, influenza virus is internalized into the endosome by receptor-mediated endocytosis. The fusion between viral and endosomal membranes leads to the release of vRNPs into the cytoplasm. vRNPs are then imported into the nucleus for viral replication (18, 36). In this study, the dose-dependent inhibition of viral infection by sialidase indicated that influenza virus infection of NK cells is dependent on the sialic acids. For the infection of different cells, such as MDCK, BS-C-1, and HeLa cells, influenza virus has been reported to exploit clathrin-mediated endocytosis, caveolin-mediated endocytosis, or

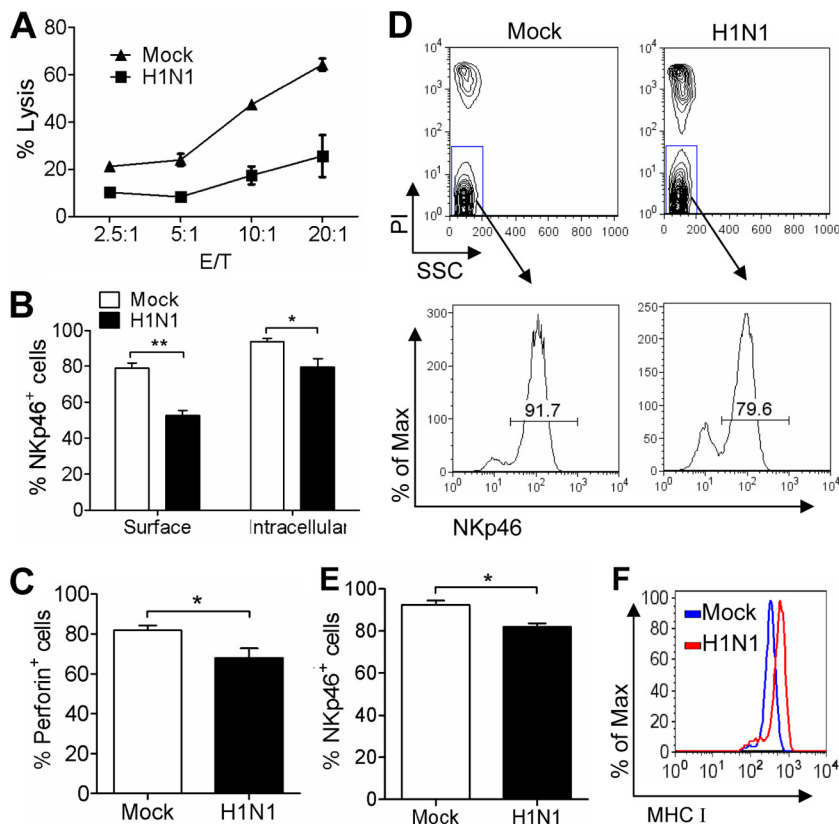


FIG. 6. Influenza virus infection reduces NK cell cytotoxicity. (A) Twenty-four hours after H1N1 infection, total NK cells were collected and cocultured with target K562 cells at the indicated *E/T* ratios for 2 h. Samples were examined by flow cytometry for percentages of specific lysis. (B and C) Twenty-four hours after H1N1 virus infection, total NK cells were examined by flow cytometry for the frequency of surface or intracellular NKp46-positive cells (B) or intracellular perforin-positive cells (C). (D to F) At 24 h after viral infection, NK cells were examined for PI uptake (D) and their surface expression of NKp46 (E) and MHC class I (F) on gated PI-negative cells was determined. The data shown are means \pm SEM of results for four different donors. Results of Student's paired *t* test analysis are shown (*, $P < 0.05$; **, $P < 0.01$).

the clathrin- and caveolin-independent pathways, depending on the type of target cells (29, 34, 37). Here, as a new target cell, we determined the endocytic pathways for influenza virus to infect NK cells. With colocalization assay and specific inhibition, we demonstrated that influenza virus infected NK cells by both clathrin- and caveolin-dependent endocytosis rather than by macropinocytosis.

In the host innate immunity, NK cells are key effector cells and can rapidly destroy virus-infected cells during the acute infection, limiting viral replication and transmission. However, in this study, we demonstrated that influenza virus directly infected and killed NK cells to restrain their activity. The reduced NK cell cytotoxicity herein reflects mainly increased cell death. It is beneficial for the better understanding of the interaction between influenza virus and NK cells to study whether other factors, such as viral components, can modulate NK cell activity, which is currently under investigation by us. Indeed, our findings are compatible with clinical observations. Patients with severe influenza virus infection were found to have diminished numbers of NK cells in peripheral blood and an almost complete absence of pulmonary NK cells together with marked apoptosis (13, 42). These findings support the hypothesis that influenza virus may directly infect NK cells, resulting in their apoptosis *in vivo*. Consistent with our finding,

other studies have also demonstrated that some viruses directly target NK cells to evade host natural antiviral immunity by direct infection and killing of NK cells, such as human immunodeficiency virus, herpes simplex virus, and ectromelia virus (4, 22, 31, 32).

At the early phase of viral infections, NK cells respond to eliminate the invading viruses; however, viruses have evolved a variety of strategies to evade NK cell activity, aiming to maintain a balance between NK cell responses and viral NK escape mechanisms, as each strives for survival (21, 31, 35). The major function of viral NK cell immunoevasion is to allow a high level of viral replication before the onset of the specific immune response (15), which is particularly beneficial for viruses to expand and spread (11, 16). In the case of influenza viral infection, NK cells recognize and kill virus-infected cells (23); in order for replication and subsequent spread, influenza virus needs to develop some mechanisms to combat the NK cell response. Previous study has shown that influenza virus infection increased the levels of binding of two inhibitory receptors to inhibit NK cell cytotoxicity (1). We therefore postulate that our findings here may represent a novel strategy of influenza virus to evade the NK cell innate immune defense by directly infecting NK cells and inducing cell apoptosis. Indeed, among their distinct NK cell immunoevasion mechanisms, many vi-

ruses use induction of NK cell apoptosis as a common strategy (21, 31, 35). This novel evasion strategy has obvious advantages for influenza virus, allowing it to replicate to the high titers necessary for successful transmission to new hosts before the onset of specific immune responses and to cause clinical illness (15). An intensive understanding of the interaction of influenza viruses and NK cells is important for better understanding influenza pathogenesis and for developing a more effective prophylaxis and treatment of this disease.

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