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NKp46 and NKG2D Recognition of Infected Dendritic Cells Is Necessary for NK Cell Activation in the Human Response to Influenza Infection

Monia Draghi,‡* Achal Pashine,‡† Bharati Sanjanwala,‡ Ketevan Gendzekhadze,‡ Claudia Cantoni,‡ David Cosman,‡ Alessandro Moretta,‡ Nicholas M. Valiante,‡ and Peter Parham‡*‡

At an early phase of viral infection, contact and cooperation between dendritic cells (DCs) and NK cells activates innate immunity, and also influences recruitment, when needed, of adaptive immunity. Influenza, an adaptable fast-evolving virus, annually causes acute, widespread infections that challenge the innate and adaptive immunity of humanity. In this study, we dissect and define the molecular mechanisms by which influenza-infected, human DCs activate resting, autologous NK cells. Three events in NK cell activation showed different requirements for soluble mediators made by infected DCs and for signals arising from contact with infected DCs. IFN-α was mainly responsible for enhanced NK cytolytic activity and also important for CD69 up-regulation, whereas IL-12 was necessary for enhancing IFN-γ production. Increased CD69 expression and IFN-γ production, but not increased cytolytic activity, required recognition of influenza-infected DCs by two NK cell receptors: NKG2D and NKp46. Abs specific for these receptors or their known ligands (UL16-binding proteins 1–3 class I-like molecules for NKG2D and influenza hemagglutinin for NKp46) inhibited CD69 expression and IFN-γ production. Activation of NK cells by influenza-infected DCs and polyinosinic:polycytidylic acid (poly(I:C))-treated DCs was distinguished. Poly(I:C)-treated DCs did not express the UL16-binding protein 3 ligand for NKG2D, and in the absence of the influenza hemagglutinin there was no involvement of NKp46.

Influenza virus (flu), a major human pathogen, causes ~36,000 deaths annually in the United States alone. During epidemic and pandemic outbreaks the morbidity and mortality can be much higher (1–3). Such statistics, plus growing perception that pandemic flu is imminent and fears that the virus will be used to further human conflict, heighten the need to understand how humans defend themselves against this common infection.

Whereas much is known about the effector mechanisms of adaptive immunity that terminate most influenza infections (4, 5), study of the innate immune response to influenza and its recruitment of Ag-specific cells is in its infancy. Dendritic cells (DCs) and NK cells are cells of innate immunity that cooperatively interact to determine the course of an innate response and its switch to adaptive immunity (6–12). NK cells are particularly implicated in defense against viral infections, which they provide through killing of infected cells and the production of cytokines, notably IFN-γ (13–15). Although recent studies have emphasized the role of DCs in promoting adaptive immune responses to influenza (16–18), less has been said of the part played by NK cells and NK-DC cooperation (19).

Sites of NK-DC interactions may be either lymphoid organs (20–24) or nonlymphoid peripheral tissues (25, 26). In tissues, immature DCs (iDCs) have efficient mechanisms for the detection and uptake of pathogens. Upon infection they mature, increasing Ag presentation, costimulation, and secretion of chemokines and proinflammatory cytokines (27, 28). The latter can recruit NK cells to the infected tissue where they can interact with DCs. Communicating through direct cell contact and secreted soluble factors, the DC and NK cells coordinate their response. DC-derived IL-12 and NK-derived IFN-γ create a positive feedback loop that drives cell-mediated (Th1) immunity (24, 29, 30).

NK cell function is controlled by the integration of signals from various stimulatory and inhibitory cell surface receptors. Inhibitory receptors of human NK cells including CD94/NKG2A, killer Ig-like receptors, and some leukocyte Ig-like receptors are specific for different HLA class I determinants (31–33). The major activating receptors are NKG2D, 2B4, and the natural cytotoxicity receptors Nkp30, NKp46, and NKp44 (34–41). Of the latter, NKp46 and Nkp30 are on most human peripheral blood NK cells.
whereas, NKP44 is only on activated NK cells (37, 42). Although endogenous natural cytotoxicity receptor ligands remain undetermined, NKP44 binds the influenza hemagglutinin (HA) protein as well as the HLA-neuraminidase of parainfluenzavirus (43, 44). NKG2D mediates NK cytology of tumors and CMV-infected cells (45, 46). The endogenous ligands for human NKG2D are MHC class I chain-related proteins (MIC)A and MICB and another family of class I-related molecules, the UL16-binding proteins (ULBP)1–3. Some NKG2D ligands are up-regulated in cells infected with CMV (47, 48), but the signals regulating the expression of MIC and ULBP in the context of other viral infections have yet to be defined.

The fact that influenza does not naturally infect mice, and that human and mouse NK cell receptors have some striking differences (49, 50), question the applicability of murine models for NK-DC cooperation in response to this human pathogen. Consequently, we established an autologous in vitro system to study the molecular mechanisms governing human NK cell activation by influenza-infected DCs. Our study shows that NK cell activation requires several cues mediated by cell surface and secreted factors, including the recognition by NKG2D and NKP46 of influenza-induced ligands on the infected DCs. Although previously identified as mediators of NK cell killing (35, 43, 51, 52), the NKG2D and NKP46 receptors are now shown to have a more constructive role as seen here in the activation of NK cells by influenza-infected DCs.

Materials and Methods

Cell isolation and generation of monocyte-derived DCs

PBMC were prepared from buffy coats (Stanford Blood Center, Stanford, CA) by separation on a Ficoll-Hypaque gradient. Samples were obtained with the informed consent of the subjects. All protocols for blood collection were approved by the Stanford University Review Board. iDCs were generated as described previously (53). Briefly, monocytes were obtained by positive selection, using anti-CD14 microbeads (Miltenyi Biotec), and generated as described previously (53). Briefly, monocytes were obtained by positive selection, using anti-CD14 microbeads (Miltenyi Biotec), and cultured for 6 days in RPMI 1640 medium supplemented with 10% FCS, IL-4 (20 ng/ml; Peprotech), and GM-CSF (50 ng/ml; Peprotech). The iDCs were checked for CD1a and CD80 expression and the absence of CD14.

DC/NK cocultures

NK cells were thawed, washed, and resuspended in RPMI 1640 with 10% FCS. iDCs were washed in RPMI 1640 medium to remove GM-CSF and IL-4. NKDC cocultures were performed in 96-well U-bottom plates. Cells were treated with the indicated doses of influenza virus strain Panama/2007/99 strain (H3N2) (allantoic fluid containing 2560 HA units (HAU)/mL), or polyinosinic:polycytidylic acid (poly(I:C)) (Sigma-Aldrich). In 2007/99 strain (H3N2) (allantoic fluid containing 2560 HA units (HAU)/mL) or poly(I:C) (Sigma-Aldrich). In experiments where blocking of NK cell activation and for neutralizing cytokines

The same mAbs, anti-NKG2D, anti-ULBP1, anti-ULBP2, anti-ULBP3, anti-2B4, anti-CD56, and anti-HA, used for staining were used unlabeled as purified Abs for blocking experiments. All Abs were used at a concentration of 10 µg/ml, except for anti-HA (30 µg/ml). Anti-NKp30 (2F52, IgM) and anti-NKp46 (KL247, IgM), which were from hybridoma culture supernatants, were used at 1/2 dilutions. All Ab concentrations for blocking studies were chosen based on published information (34, 36, 38). Flow cytometry was performed using a FACScan cytometer (BD Biosciences) and FlowJo software (Tree Star). In DC/NK cell cocultures, DC and NK cells were distinguished by side and forward scatter parameters. Staining specificity was confirmed by isotype-matched control Ab. Dead cells were excluded from the gate by staining with propidium iodide (PI) or dead cells discriminating receptor (Miltenyi Biotec).

Experimental protocols and Abs for blocking NK cell activation

For experiments requiring several cues mediated by cell surface and secreted factors, including the recognition by NKG2D and NKP46 of influenza-induced ligands on the infected DCs. Although previously identified as mediators of NK cell killing (35, 43, 51, 52), the NKG2D and NKP46 receptors are now shown to have a more constructive role as seen here in the activation of NK cells by influenza-infected DCs.

Flow cytometry

The following Abs were used: anti-CD86-FITC, anti-CD83-PE, anti-CD80-PE, anti-CD1a-FITC, anti-CD14-PE, anti-CD56-PE, anti-CD3-FITC, anti-CD19-FITC, DC54-PE, CD69-PE, anti-NKG2D-PE (clone 1D11), and anti-MICA/B-PE (clone 6D4) were purchased from BD Biosciences. Anti-NKp30-PE (clone Z25), anti-NKp46-PE (clone BARB281), anti-NKp44-PE (clone Z231), and anti-2B4-PE (CD244, clone C1.7.1) were purchased from Beckman Coulter; anti-NKG2C-PE was obtained from eBioscience. Abs specific for cytokines were as follows: anti-IL-12p40, IL-12p70, IL-1β, IL-6, IL-15, TNF-α, IFN-γ, IFN-α, sheep polyclonal anti-IFN-β, and mouse monoclonal anti-IFN-γ receptor mAb (PBL Biomedical Laboratories). The anti-IFN-α, anti-IFN-γ, and anti-IFN-β receptor Abs were used at concentrations of 1.5, 1.5, and 2.5%, respectively, of the commercial preparation as described previously (19). To block nonspecific binding of the Abs to Fc receptors, cells were first incubated for 10 min with Fc-blocking reagent (Miltenyi Biotec) that contains human IgG to block nonspecific Fc receptor binding. Staining for NKG2D was obtained from Biodesign International. The anti-HA-purified Ab was biotinylated using EZ-Link NHS-PEO solid Phase Biotinylation Kit (Pierce). Indirect immunofluorescence staining was performed using streptavidin-PE (BD Biosciences). For surface stainings, the cells were incubated for 15 min at 4°C with an Fc-blocking reagent (Miltenyi Biotec) that contains human IgG to block nonspecific Fc receptor binding. Staining for ULBPs was performed in two steps, using a goat anti-mouse IgG-FITC (BD Biosciences) as a secondary Ab. To detect viral NP, intracellular staining of DCs or NK was conducted. Cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 20 min at 4°C. TNF-α production was detected by adding 1 µl/m (BD Pharmingen) to DC, NK, or DC/NK cultures 4 h following infection with influenza for a further 12 h. Cells were fixed with anti-CD86-FITC or anti-CD56-FITC, then fixed and permeabilized with Cytofix/Cytoperm buffer, and anti-TNF-α-PE (BD Biosciences) was added for 30 min at 4°C. Analysis of all experiments was performed using a FACSscan cytometer (BD Biosciences) and FlowJo software. In experiments where blocking of NK cell activation and for neutralizing cytokines

The viability of DCs after infection with influenza virus was determined by staining with annexin V-FITC and PI (BD Biosciences). Analysis was performed on a FACSscan (BD Biosciences) using FlowJo software.

Detection of cytokines in the cell supernatants

Combined detection of cytokines IL-12p40, IL-12p70, IL-1β, IL-6, TNF-α, IFN-α, IFN-γ, GM-CSF, and the chemokine IL-8 was determined in cell-free supernatants using Beadlyte human flex-kit reagents (Upstate Cell Signaling Solutions) according to the manufacturer’s protocol. Results are the mean of assays performed in duplicate or triplicate wells. In some experiments, IFN-γ in the supernatants was determined using a commercially available ELISA kit (Quantikine Immunoassay; R&D Systems).

Cytotoxicity assays

Cytolytic activity of NK cells against autologous iDCs was measured in 51Cr release assays. Target cells were autologous DCs incubated with 10 µCi of Na251CrO4 for 1 h at 37°C and then washed three times and incubated for 4 h with NK cells (effectors) previously cultured with DC and influenza virus or in medium alone for 20–24 h. Supernatants were collected and the radioactivity determined with a gamma counter (Beckman Coulter). Assays were performed in triplicate at the indicated E:T ratios. The percentage of lysis was determined by normalizing the formula of specific lysis = 100 × (sample release–spontaneous release)/total release spontaneous release. In some experiments, Abs to block NK cell-activating receptors were added at the same amounts described above.
Sequencing of MICA alleles

Genomic DNA was obtained from PBMC using the QIAmp DNA Blood kit (Qiagen). Using two generic primers, a 2201-bp MICA gene fragment was amplified as described previously (54). The reaction was performed at the annealing temperature of 65°C. Exons 2, 3, and 4 were sequenced separately using primers described previously (55). For ambiguous allele combinations, 2201-bp MICA PCR product from four donors were cloned using TOPO TA cloning kit for sequencing (Invitrogen Life Technologies) according to the manufacturer’s recommendations. Sixteen clones, each corresponding to one of the two alleles from each donor, were analyzed using exon-specific primers to detect microsatellites present in exon 5.

Results

NK cell activation by influenza-infected DCs

Influenza-infected DCs were tested for their capacity to stimulate autologous NK cells. Culture of purified NK cells for 20–24 h with iDC (1:1 ratio) and 10 HAU/ml virus (Panama/2007/99-H3N2) induced a vigorous activation, as seen from increased CD69 expression (Fig. 1A) and IFN-γ production (Fig. 1B). When resting NK cells were cultured with iDCs and no virus, or with virus and no DCs, neither CD69 expression nor IFN-γ secretion increased (Fig. 1, A and B). Thus, NK cell activation required the presence of influenza-infected DCs. With less virus (0.1 and 1 HAU/ml) NK cells were not stimulated, and with more virus (>10 HAU/ml) the DCs were killed (data not shown) (17). A 10 HAU/ml viral dose combined DC viability, as assessed by PI staining, with productive infection as seen from cell surface expression of the viral HA and intracellular expression of the viral NP. During coculture, NK cells maintained >95% viability while very few became influenza infected (data not shown).

The enhanced CD69 expression of NK cells responding to infected DCs was less when the cells were separated by a semipermeable membrane in a Transwell system. This event in NK cell activation therefore depends upon cellular contact with infected DCs and soluble factors secreted by them (Fig. 1C). By contrast, enhanced IFN-γ production by NK cells was completely dependent upon contact with infected DCs (Fig. 1D). NK cells cultured with influenza-infected DCs also increased their cytolytic capacity to kill autologous, uninfected iDCs (Fig. 1E) and the Daudi cell line (data not shown). Unlike CD69 and IFN-γ induction, the enhancement of NK cell cytolysis by infected DCs was greater after culture in Transwells than when the DC and NK cells were cultured together. This result suggests that direct contact with infected DCs partially suppressed and/or exhausted the NK cells’ lytic potential (Fig. 1F). In summary, NK cell contact with infected DCs had differential effects on three events in NK cell activation; for enhanced IFN-γ production cell contact was essential, for up-regulation of CD69 it was beneficial, and for increasing cytolysis it was an impedance. These differences imply that distinct molecular mechanisms govern the three events in NK cell activation by flu-infected DCs.
DC activation by influenza infection

To identify molecules that contribute to NK cell activation by flu-infected DCs, a multiplex analysis of DC cytokines and chemokines was performed. Cultures of NK cells with DCs and influenza virus (10 HAU/ml) were compared with control cultures of DC alone, DCs with virus, or NK cells with virus. DC production of several cytokines and the chemokine, IL-8, was altered by flu infection. Produced in large quantities, IL-6 and IL-8 were detectable 5 h after infection and accumulated over 22 h of culture (Fig. 2A). IFN-α, IL-1β, TNF-α, IL-12 (both heterodimer, p70 and free H chain, p40), and IL-10 appeared at a later time (22 h) and lower concentration than IL-6 and IL-8 (Fig. 2B). IL-15 and GM-CSF were not detected in any culture, and no soluble factor examined was produced by cultures of NK cells with virus but no DCs (data not shown). Except for a small, consistent increase in IFN-α, the addition of resting NK cells did not significantly alter the cytokine and chemokine concentrations in the supernatants compared with cultures of flu-infected DCs in the absence of NK cells. Influenza infection also increased DC expression of the maturation markers CD83, CD86, and MHC class I, events that were also unaffected by the addition of NK cells (Fig. 2C).

Time-course experiments showed that the kinetic of IFN-γ production in cultures of NK cells with infected DC paralleled that of the DC factors produced late in culture: IFN-α, IL-1β, TNF-α, IL-12, and IL-10 (data not shown). Neutralization of IFN-γ with specific Abs had no effect on DC maturation or on their cytokine and chemokine response to flu infection, either in the presence or absence of NK cells. Addition of heat-killed virus to the DC cultures induced none of the cytokines stimulated by live virus, and the further addition of NK cells had no effect (data not shown). Intracellular staining showed that TNF-α was produced only by DC and not by the NK cells. Neither was the proportion of TNF-α-producing, flu-infected DC changed by the presence or absence of NK cells (data not shown). Thus, resting NK cells do not appreciably modulate DC responses to influenza virus in a 20-h culture, as assessed by TNF-α analysis. In contrast, activated NK cells have been shown to activate DC when cultured for longer times (48 h) in the absence of virus and presence of LPS (9, 11).

Molecular mechanisms governing NK cell activation by influenza-infected DCs

We examined the contribution of stimulatory NK cell receptors (NKG2D, NKp46, NKp44, NKp30, and 2B4) and soluble factors secreted by DCs (e.g., IL-12 and IFN-α) to the activation of NK cells by flu-infected DC (Fig. 1). As reported previously (40, 42), flow cytometric analysis showed that almost all peripheral blood NK cells expressed NKG2D, NKp46, NKp30, and 2B4, whereas NKG2C was restricted to a minor NK subset and NKp44 was absent (data not shown). Culturing NK cells for 20–24 h with flu-infected, or uninfected, DCs did not perturb this pattern of NK cell receptor expression (data not shown). Further analysis of the absent or low-expressing NKp44 and NKG2C receptors was not pursued.

NK cell up-regulation of CD69 involves NKG2D, NKp46, and type 1 IFNs

The effect of adding Abs specific for stimulatory NK cell receptors to the coculture of NK cells with flu-infected DCs was examined. Up-regulation of CD69 was inhibited by anti-NKG2D and anti-NKp46, but not by Abs specific for NKp30, 2B4, and CD56 (Fig. 3, A and B). These observations indicate that the NKG2D and NKp46 receptors are part of the mechanism that up-regulates CD69 and that binding of specific Ab blocks their function. When

![FIGURE 2.](http://www.jimmunol.org/)

Coculture with NK cells does not perturb maturation-marker expression and production of secreted factors by influenza-infected DCs. Supernatants from cultures of DCs alone, DCs with virus (10 HAU/ml), or DCs with virus and NK cells (1:1 ratio) were collected after 5, 10, and 22 h to assess cytokine content (A and B). For infected DCs (○), IL-6 and IL-8 were detected at all time points; addition of NK cells (■) did not alter the levels significantly (A). For uninfected DCs (□), low levels of IL-8 and IL-6 were detected; these too were unaffected by NK cell presence (data not shown). B, IL-12 (p70 and p70), IL-10, IFN-α, TNF-α, and IL-1β were detected only after 22 h of culture: DCs alone (◇), DCs with virus (◇), or with virus and NK cells (■). Data, mean ± SD (n = 3), are representative of three independent experiments. C, CD83, CD86, and MHC class I expression by DCs after 20–24 h of culture under the conditions described for A and B. Dead cells (PI+) were excluded from the gate. Data shown are from a representative experiment of three performed.
Signals from influenza-infected DC enhance NK cell expression of CD69. NK and iDCs were cocultured for 20–24 h with influenza virus (10 HAU/ml) in the presence or absence of the mAbs indicated that have been shown to be neutralizing in other studies (34, 36, 38). A, Cell surface CD69 was determined for NK cells in the cultures containing either anti-NKG2D (IgG1), anti-NKp46 (IgM), anti-NKp30 (IgM), anti-2B4 (IgG1), anti-CD56 (IgG1), or medium alone. Because of their observed negative effects, anti-CD56 acted as an isotype control for anti-NKG2D and anti-2B4, whereas anti-NKp30 acted as an isotype control for anti-NKp46. Data are reported as MFI. Similar results were obtained from four independent experiments, each involving a different donor. B, Fluorescence histograms of CD69 expression for NK cells cultured alone (dashed histogram; MFI, 14) or with flu-infected DC (10 HAU/ml virus) in the absence (open histogram; MFI, 90) or presence of anti-NKp46 (MFI, 57), anti-NKG2D (MFI, 36), and anti-type I IFNs (shaded gray histograms), or a mixture of anti-NKp46, anti-NKG2D, and anti-type I IFNs (shaded dark gray histogram; MFI, 25). The Ab concentrations are based on previous studies and are given in Materials and Methods.

Enhanced NK cell cytolytic activity. NK cells cultured with DCs and influenza virus (10 HAU/ml) in the presence or absence of neutralizing anti-IL-12 or anti-IFN-α Abs were tested for cytolytic activity of autologous, iDCs at the E:T ratios indicated. A, B, and C, NK cells cultured with flu-infected DCs in a Transwell system for 20–24 h were tested for their capacity to kill IDCs and flu-infected DCs in the presence or absence of the blocking mAbs indicated. The E:T ratio was 40:1 in B and 80:1 in C. When targets were flu-infected DCs, the frequencies of infected cells were measured by staining with anti-NP mAb. Excluding dead cells, the frequencies of NP⁺ DCs were 41% (B) and 76% and 90% for the DCs infected with 5 and 10 HAU/ml (C), respectively. The viability of flu-infected DCs used as targets was >80% in all experiments.

Enhanced NK cell cytolysis is induced by IFN-α

Signals from infected DCs that increase the cytolytic potential of NK cells after coculture were investigated. Because enhanced NK cytotoxic activity was contact-independent (Fig. 1F), we concentrated on IFN-α and IL-12, soluble factors known to augment NK killing (57, 58) and produced by DCs upon influenza infection (Fig. 2B). A neutralizing Ab against IFN-α completely inhibited enhanced NK cytolysis of autologous DCs, whereas anti-IL-12 had no effect (Fig. 4A). Thus, the enhancement of NK cytolysis by flu-infected DCs appears largely governed by IFN-α, contrasting with the combination of soluble and cell surface factors required for CD69 up-regulation. NK cells activated by flu-infected DCs in the Transwell system also lysed autologous, uninfected DCs, but did not kill flu-infected DCs. This pattern of lysis was unaffected by neutralizing Abs specific for activating NK cell receptors (Fig. 4, B and C). This insensitivity is likely due to the maturation of DCs as a consequence of flu infection, and is consistent with previous observations that NK cell-mediated cytolysis of mature DCs is less effective (10, 59). Moreover, additional mechanisms of viral escape from NK recognition may be responsible for the resistance to killing of flu-infected DCs by NK cells as previously suggested.
The means and SD are shown for three replicate cultures. IL-12 also reduced IFN-γ concentrations of 11–90 pg/ml (data not shown and Fig. 2A). The concentration of IL-12, IL-12p70, was produced when DCs were cultured with the virus, or with the virus and NK cells, and reached concentrations (pg/ml) of 10–90% of the virus infection, as occurs when influenza and anti-HA mAb are added together (data not shown).

An alternative interpretation we needed to consider was that the inhibitory effects of anti-NKG2D and anti-NKp46 were a consequence of redirected, NK cell-mediated lysis of the DCs. In that mechanism, the Ab’s Fab would be bound to the NK cell receptor and its Fc to the DC’s Fc receptors (61, 62). Decreases in NK cell activation observed would then be due to DC lysis and not to blocking of specific signals necessary for CD69 expression and NK cell IFN-γ production. This interpretation cannot apply to the monoclonal anti-NKG2D, which is an IgM and therefore unable to mediate redirected lysis. In contrast, the monoclonal anti-NKG2D is an IgG1 known to redirect NK cell lysis toward the P815 mastocytoma cell line (63). Consequently, we compared the viability of infected DCs when cultured with NK cells in the presence or absence of anti-NKG2D (Fig. 6, A–C). Anti-NKG2D Ab did not facilitate DC killing, but actually increased their viability. In a second test, the impact of the blocking Ab’s Fc portion was examined. Equivalent inhibition of NK cell IFN-γ production was obtained with F(ab’)2 and intact IgG (Fig. 6D), showing that the Ab’s Fc is not required for the inhibitory effect, which therefore cannot be due to redirected lysis. In summary, neither anti-NKG2D nor anti-NKp46 causes decreased NK cell activation by redirected killing of the influenza-infected DCs.

**FIGURE 5.** Molecular mechanisms governing IFN-γ production by NK cells responding to influenza-infected DCs. Culture conditions and mAb additions were as described in Fig. 3. A, IFN-γ concentrations were determined for supernatants from cocultures containing the blocking mAbs indicated or medium alone. B, IFN-γ concentrations (pg/ml) are shown for cocultures made in the presence of anti-IL-12, an isotype-control Ab, or medium alone. Shown are the mean and SD for three replicate cultures and are representative of three independent experiments. C, IFN-γ production in the presence of anti-IL-12 or anti-IFN-α-blocking Abs is compared with that observed in the absence of Ab. D, DCs were incubated overnight with influenza virus. Cells (frequency of HA positive was >90%) were washed and cultured for 24 h with NK cells in the absence or presence of anti-HA mAb, an isotype control (both at 30 µg/ml), or anti-NKp46. The concentration of IFN-γ in the cell supernatants was measured. NK cells cultured alone were used as a control. Addition of anti-HA, the isotype control, or anti-NKp46 did not alter the frequencies of infected DC as measured by intracellular staining using anti-NP mAb (data not shown).
Influenza virus and poly(I:C) induce DCs to express the ULBP ligand for NKG2D

Human NKG2D recognizes two families of ligands: ULBP1–3 and MICA/B (64 – 66). Their expression on uninfected and flu-infected DCs was compared using specific mAb and flow cytometry (Fig. 7, A and B). All NKG2D ligands were at low, or undetectable, levels on uninfected DCs. Influenza infection up-regulated ULBP1–3, but not MICA or MICB. The presence of NK cells in culture did not affect the expression of NKG2D ligands by uninfected or infected DCs. Recognition of ULBP1–3 could therefore explain the NKG2D-dependent NK cell activation by influenza-infected DCs. To test this hypothesis directly, blocking of ULBP with specific mAbs was performed. As predicted, blocking NKG2D-ULBP interactions with a mixture of anti-ULBP1–3 mAbs, an isotype control Ab, or no Ab. Data represent mean IFN-γ concentration (pg/ml) ± SD (n = 3).

MIC molecules are highly polymorphic and encoded by genes in the MHC. Certain MHC haplotypes have the nonfunctional MICA*010 allele or lack the MICA and B genes altogether (67). To rule out the possibility that the lack of MICA expression was due to such factors, we sequenced the MICA alleles for the five donors analyzed in our flow cytometry experiment. All donors were negative for MICA*010 and the MICA gene deletion (data not shown). Moreover, four of five donors possessed MICA*00801, an allele known to be recognized by the anti-MICA/B mAb 6D4 used in this study (68). Thus, it is unlikely that the observed lack of MICA/B expression was due to the inability of the 6D4 mAb to recognize all the MIC allotypes of our donor panel.

dsRNA, an agonist of TLR3, is a byproduct of influenza virus replication (17, 69, 70). On treatment with synthetic dsRNA poly(I:C), DCs up-regulated ULBP1 and -2, but to a lesser extent than after flu infection, and ULBP3 expression was unchanged (Fig. 8A). Poly(I:C) treatment of DCs had no effect on MICA or MICB expression, as also seen for flu infection. Differences in the up-regulation of ULBP by poly(I:C) and flu infection could reflect quantitative differences in the signals induced, or qualitative differences in which flu infection produces

FIGURE 7. NKG2D ligand expression by influenza-infected DCs. A, Expression of MICA and -B, and ULBP1, -2, and -3 was determined by flow cytometry of DCs cultured for 20–24 h in medium alone (■), with influenza virus (10 HAU/ml) (□), or with virus and NK cells (▲). Representative data from five independent experiments with different donors are shown. Dead cells (PI+) were excluded from the gate. B, Percentage of increase in ULBP1–3 expression by DCs from five different donors after infection is compared with uninfected DCs. Data represent the mean percentage of expression of ULBP1, -2, or -3 expression ± SD (n = 5). C, Shown is the IFN-γ concentration of supernatants from NK cells cultured with influenza-infected DCs in the presence of a mixture of anti-ULBP1–3 mAbs, an isotype control Ab, or no Ab. Data represent mean IFN-γ concentration (pg/ml) ± SD (n = 3).

FIGURE 8. Poly(I:C) induces a subset of the NK cell-activating signals induced by influenza infection of iDC. A, Expression of MICA/B, ULBP1, ULBP2, and ULBP3 was determined for DC cultured (20–24 h) in medium alone, with poly(I:C) (100 μg/ml), or with influenza virus (10 HAU/ml). NK and iDCs were cultured for 20–24 h with poly(I:C) in the presence or absence of the blocking mAbs indicated, and IFN-γ (B and C) or CD69 expression by NK cells (D) was determined. Results are representative of three independent experiments with different donors.
signals additional to those induced by poly(I:C). The increased expression of ULBP1 and -2 on poly(I:C)-treated DC enhanced their capacity to stimulate NK cells, as shown by the reductions of NK cell IFN-γ production and CD69 expression seen in the presence of Abs specific for ULBP1 and -2 (Fig. 8B) or their receptor, NKG2D (Fig. 8, C and D). Unlike virus-infected DCs, the NK cell-activating functions of poly(I:C)-treated DCs were not perturbed by anti-NKp46 (Fig. 8, C and D) or anti-HA (Fig. 8B). This result is consistent with HA interacting with NKp46 and providing a flu-specific signal for the activation of human NK cells.

Discussion

We studied an in vitro model for the early activation of human NK cells during the innate immune response to an influenza infection. The investigation has defined mechanisms used by influenza-infected, human DCs to activate resting, autologous NK cells. In the course of 1 day the virus infects and stimulates the maturation of DCs, which as mature, infected-DC then activate the NK cells. Without DCs, the virus had little effect on CD69 expression and IFN-γ production by NK cells. Likewise, during this 1-day time frame NK cells did not noticeably perturb the infected DCs, although preactivated NK cells have previously been shown to stimulate DCs after longer periods of coculture (9, 11).

NK cells were discovered and named for their cytolytic activity. Subsequent research shows NK cells to have a more extensive range of effector and regulatory functions in host defense, including interaction with DC (9–11, 19, 22, 24). Although DC lysis is one possible consequence of such encounters (9, 10, 71, 72), another is a cellular cooperation that influences the subsequent course of the innate and adaptive immune response. As for the NK cells, their NKG2D and NKp46 receptors were first defined in the context of cytotoxicity (35, 51, 52), but are shown in this study to be central to human NK-DC cooperation in the response to influenza infection. Consistent with this conclusion, a recent study in the mouse model reports that mice lacking NKp46 fail to control influenza infection (73). Our results argue that NK cells from these mice fail to interact optimally with DCs, thereby compromising multiple host defense mechanisms countering the infection.

The function of CD69 on activated NK cells is presently uncertain. A recent study shows that CD69 promotes T lymphocyte sequestration in lymphoid organs by inhibiting the chemotactic function of S1P1 (sphingosine 1-phosphate receptor-1) (74). Although there is no direct evidence that the S1P1 and CD69 complexes can

![FIGURE 9. Model for NK cell activation by autologous, influenza-infected DCs. Three events in NK cell activation—enhanced cytolyis, up-regulation of cell surface CD69, and the enhanced production of IFN-γ—depend differentially upon soluble factors secreted by the infected DC and the engagement of NK cell receptors by influenza-induced ligands on the DC surface. Enhanced NK cell cytolyis is dependent upon DC-derived IFN-α and does not require direct contact between the NK cell and the DCs (A). Up-regulation of cell surface CD69 requires type I IFNs secreted by the DCs and direct contact with the DCs, which provides for recognition of the influenza HA and ULBP on the DCs by their respective NK cell receptors, NKp46 and NKG2D (B). Enhanced production of IFN-γ requires cell-cell contact and dual recognition of HA and ULBP, but the important DC-derived cytokine for this event in NK cell activation is IL-12 (C). For both up-regulation of CD69 (B) and production of IFN-γ (C), additional endogenous ligand(s) for NKp46 may also be involved in activating NK cells.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.2695)
also alter the trafficking of activated NK cells, the new findings suggest that CD69 may play a role in retaining NK and T cells in lymph nodes, the sites of interaction between NK and DCs (20–24).

Of the many changes induced by influenza infection of DCs, we identified four to be important for human NK cell activation. These were the secretion of IL-12 and type I IFNs and the cell surface expression of ULBP's and HA. The cytokines secreted by the DC diffuse and bind to their cognate receptors on the NK cell surface. In direct intercellular contact between DC and NK cells, the ULBP's and HA on the DC presumably bind to their cognate receptors on the NK cell surface—NKGD2 and NKp46, respectively. Three features of NK cell activation—CD69 expression, enhanced IFN-γ production, and increased cytolytic activity—were shown to depend differentially on these four ligand-receptor interactions (Fig. 9). Although we cannot rule out the possibility that NKp46 binds additional cellular ligand(s) induced by the virus (75) or that HA may serve as a ligand for other NK receptors, our results are consistent with previous reports suggesting that HA is a ligand for NKp46 (43, 44). Moreover, we extend these original findings by placing potential NKp46-HA interactions in the context of NK-DC cooperation (76).

Although our results have been obtained under in vitro experimental conditions, they contribute to the understanding of the potential mechanisms controlling NK/DC responses during influenza infection. Further in vivo modeling will be required to establish the relative importance of these distinct molecular interactions and their functional outcomes in the course of influenza infections in man. Increased cytolytic activity was principally driven by type I IFNs and was not dependent upon direct contact between NK cells and DC. Type I IFNs were also the main cytokines inducing CD69 expression, but that event in NK cell activation also required direct cell contact and interaction between the NKGD2 and NKp46 NK cell receptors and their flu-induced ligands on the DC. NKGD2 and NKp46 recognition of infected DCs was also essential for enhancing IFN-γ production, but for this aspect of NK cell activation the necessary cytokine was IL-12. Such requirements of DC-derived IL-12 for enhancing IFN-γ production by NK cells and DC-derived IFN-α for increased cytolytic activity, parallel those seen in the mouse model of CMV infection (57, 77, 78). Where the two models appear to differ is in the role of NKGD2. Whereas, we find that NKGD2 is essential for enhanced IFN-γ production by human NK cells responding to influenza, Andoniou et al. (52) find no contribution of NKGD2 in enhancing IFN-γ by mouse NK cells responding to murine CMV. This distinction could be due to evolved differences in the human and mouse immune systems (79), or between influenza and murine CMV, two divergent viruses with unrelated structure, genetics, and pathogenesis (80–82). The latter possibility would suggest that NK-DC cooperation has the potential to be pathogen-specific.

First characterized as cytotoxicity receptors of the effector phase, NKGD2 and NKp46 are shown in this study to function in the activation of NK cells by DCs in response to a viral infection. This occurs in the absence of detectable killing of the flu-infected DCs. Influenza is a cytopathic virus that kills infected DCs within 20–40 h of infection (17, 83). In this circumstance, NK cell killing of infected DCs should be a less effective defense than producing IFN-γ, with its capacity to help infected DCs promote an adaptive Th1 response. Although NK cells activated by flu-infected DCs for 20–24 h did not efficiently kill the infected DCs, they were found to kill uninfected, autologous iDCs at high E:T ratio. At later times when the virus is cleared, this function may prevent further activation of the immune response by DCs (9). The reasons for the resistance of flu-infected DCs to NK lysis are unclear at the present time. Previous work has shown that increase in surface expression of HLA-E during maturation of DCs seems to confer resistance to NK lysis (84). Although up-regulation of total HLA class I was observed in flu-infected DCs, HLA-E expression did not increase, as detected by the HLA-E-specific 3D12 mAb (Ref. 85 and data not shown). This suggests that factors other than inhibition of CD94/NKG2A+ NK cells by HLA-E contribute to the resistance of flu-infected DCs to NK cell lysis. Differential expression of other HLA class I molecules may protect DCs from NK lysis. Alternatively, absence of the ligand for Nkp30 in flu-infected DCs as well as other DC surface molecules (e.g., adhesion molecules) may explain the lack of DC lysis in this context.

In the IFN-γ response of NK cells to flu-infected DCs, the signals generated by IL-12R, NKp46, and NKGD2, following engagement of their ligands, are seen to work in concert. In contrast, NK cell activation by poly(I:C)-stimulated DCs was independent of NKp46, being driven by NKGD2 and the IL-12R. This difference suggests that NKGD2 provides a more general mechanism for NK cell activation by infected DCs, whereas NKp46 is more restricted, for example, to certain viral infections. Indeed, the NK cell response to flu-infected DCs, but not poly(I:C)-treated DCs, was exquisitely sensitive to blocking with anti-NKp46 Ab. Despite this difference, the two modes of activation produced comparable amounts of IFN-γ (data not shown). DCs activated with poly(I:C) produced more IL-12 p40 and p70 than DCs activated by flu infection (data not shown), indicating that increased signaling through the IL-12R may compensate for the absence of signals from NKp46 in the response to poly(I:C).

Expression of ligands for NKGD2 and NKp46 receptors on the surface of infected DCs is a consequence of the virus infection, and their simultaneous coengagement with NKGD2 and NKp46 potentially provides a recognition system that responds to an endogenous class I-like molecule and a viral Ag. It has been long appreciated that NK and T cells have many similarities in function and phenotype (86); the only absolute distinction being the presence or absence of a TCR (87). In responding to influenza, the αβ TCR recognizes composite ligands consisting of viral peptide bound to endogenous MHC molecule. When first proposed this model was called “altered self”; the alternative, in which separate receptors saw viral Ag and MHC, was called dual recognition (88–90). Although dual recognition proved wrong for T cell recognition, it describes the NK cell recognition of influenza virus we report in this study. The fact that NK cells more likely resemble primitive lymphocytes than either T or B cells, points to a dual recognition system of Ag recognition having evolved before altered self.

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