Influenza Virus Uses Its Neuraminidase Protein to Evade the Recognition of Two Activating NK Cell Receptors

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Natural Killer (NK) cells play a central role in the defense against viral infections and in the elimination of transformed cells. The recognition of pathogen-infected and tumor cells is controlled by inhibitory and activating receptors. We have previously shown that among the activating (killer) NK cell receptors the natural cytotoxicity receptors, NKp44 and NKp46, interact with the viral hemagglutinin (HA) protein expressed on the cell surface of influenza-virus-infected cells. We further showed that the interaction between NKp44/NKp46 and viral HA is sialic-acid dependent and that the recognition of HA by NKp44 and NKp46 leads to the elimination of the infected cells. Here we demonstrate that the influenza virus developed a counter-attack mechanism and that the virus uses its neuraminidase (NA) protein to prevent the recognition of HA by both the NKp44 and NKp46 receptors, resulting in reduced elimination of the infected cells by NK cells.

Keywords. NK cells; immune evasion; influenza virus; neuraminidase; NKp44; NKp46.

The activity of natural killer (NK) cells is controlled by inhibitory signals derived from binding of NK inhibitory receptors to self ligands such as MHC class I, CEACAM1, PVR, and phosphatidylethanolamine (PE) [1–6] and by activating signals derived from the binding of the NK activating receptors to viral proteins, tumor proteins, stress-induced ligands, and even self ligands [5]. NK cells express several killer receptors, among which are the family of natural cytotoxicity receptors (NCRs), which contain 3 members: 2 (NKp30 and NKp46) that are constitutively expressed and 1 (NKp44) whose expression is up-regulated upon NK cell activation [7–9]. Interestingly, mice express only one of these NCRs, the NKp46 orthologue protein Ncr1 [10, 11].

NKp44 was shown to be involved in many key NK-mediated functions such as tumor immune surveillance [12, 13], production of cytokines and growth factors by decidual NK cells [14], and controlling viral infection [15–17]. Two tumor cell ligands were identified for NKp44: the proliferating cell nuclear antigen (PCNA) that surprisingly inhibits NKp44 activity [18] and the mixed-lineage leukemia-5 (MLL5) that activates it [19]. Interestingly, both NKp44 ligands were reported to be expressed under normal conditions primarily in the nucleolus and in the cytoplasm, and it is still unclear how they get to the cell surface of tumor cells. In contrast, the interaction of NKp44 with several viruses is well characterized. Specifically, it was shown that NKp44 can activate NK cells against the influenza virus [16, 20] and against the new castle disease virus [17] by binding to their HA proteins. It was also demonstrated that NKp44 recognizes cells infected with Kaposi sarcoma herpesvirus (KSHV) [21], dengue virus [22], human immunodeficiency virus (HIV) [23], and West Nile virus [22]. However, in all of these later cases, the mechanisms by which NKp44 recognizes KSHV, dengue, HIV, and West Nile virus are still largely unknown.
NA impairs NKP44 and NKP46 Function • JID 2014:210 (1 August) • 411

NKP44 cooperates with the other NCR receptors, NKP46 and NKP30, to induce NK-mediated cytotoxicity against various target cells [24]. In addition, both NKP44 and NKP46 and the mouse Ncr1 recognize HA on influenza-virus-infected cells [10, 25–29]. The binding of NKP44, NKP46, and Ncr1 to viral HA, which is mediated by specific sialic acids residues found on these receptors, leads to the elimination of the infected cells [26]. In the absence of Ncr1, enhanced sensitivity to influenza virus infection is observed [10]. The other NCR, NKP30, does not bind the HA of influenza virus and therefore does not contribute to the NK-mediated killing of influenza-virus-infected cells. However, this receptor was shown to bind the poxvirus HA; and surprisingly, this interaction inhibits the killing of poxvirus infected cells [30].

We have recently shown that the NA protein is also involved in NK cell recognition of infected cells but in an opposite manner to that of HA. We demonstrated that the influenza virus uses the viral NA protein to evade the NKP46-mediated elimination and that inhibition of NA leads to increased elimination of influenza-virus-infected cells both in vitro and in vivo [31]. It is still unknown, however, whether NA antagonizes the activity of NKP44 and whether the NA-mediated neutralization of the recognition of NKP44 is important for the evasion of influenza from NK-cell-mediated elimination.

MATERIALS AND METHODS

Cells and Viruses

The cell lines used in this study were the human choriocarcinoma cell line Jeg3, the mouse lymphoma cell line EL4, and the murine thymoma BW cell line. The human influenza virus A/Puerto Rico/8/34 H1N1 used in this study was generated as described elsewhere [32].

Antibodies, Fusion Proteins, and Compounds

The monoclonal antibodies (mAbs) used in the present study included the anti-influenza type A monoclonal mAb (Centers for Disease Control, Atlanta, Georgia), anti-influenza virus type A (H1) mAb (H17-L2, the kind gift of Dr Jonathan Yewdell, National Institutes of Health [NIH]), APC conjugated anti-human NKP44 mAb (BioLegend), LEAF Purified anti-human CD336 (NKP44), PE-conjugated anti-human NKP46 (Beckman Coulter), and PE-conjugated anti mouse Ncr1 (R&D systems). Biotin-SP-AffiniPure rabbit anti-human immunoglobulin G (IgG) and anti-human Fcγ polyclonal antibodies were purchased from Jackson ImmunoResearch. NKP44-Ig, NKP46-Ig, Ncr1-Ig, D1-Ig, KIR2DS4-Ig, KIR2DL1-Ig, and HA-Ig fusion proteins were generated in the human embryonic kidney cells 293T and were purified on a protein G column as described elsewhere [26]. For NA inhibition, oseltamivir carboxylate (Santa cruz, sc-208495) and zanamivir (Santa cruz, sc-208495) were used.

Western Blot

NKP44-Ig, NKP46-Ig, Ncr1-Ig, NKP30-Ig, KIR2DL1-Ig, and KIR2DS4-Ig (2 µg) were run on 10% SDS-PAGE gels in reducing conditions and blotted with 0.4 µg/mL of biotinylated HA-Ig or with biotinylated anti-human IgG (0.1 µg/mL) and then incubated with Avidin-HRP (Bio Legend). For NA treatment, NKP44-Ig and NKP46-Ig fusion protein were incubated with NA beads (Sigma) at a ratio of 3.5 µL NA beads for 1 µg fusion protein and were treated or not with 25 µL oseltamivir carboxylate (10 µg-10 mg/mL). Samples (4 µg) were run on 10% SDS-PAGE gels and blotted with 20 µg/mL of biotinylated SNA lectin (Vector laboratories) or with 0.4 µg/mL of biotinylated HA-Ig and then incubated for 30 minutes with Avidin-HRP (Bio Legend).

FACS Staining and Viral Infection

For fluorescence-activated cell sorting (FACS) staining of the influenza coated cells, cells were coated overnight with A/Puerto Rico/8/34 (H1N1) virus strain at 37°C. The cells were then washed and incubated with the appropriate fusion protein (5 µg/well), with the influenza type A monoclonal mAb, or with anti-influenza virus type A (H1) mAb (H17-L2) for 2 hours on ice, washed, and then stained with the appropriate secondary labeled antibody. For NA inhibition assays, the cells were coated overnight with A/Puerto Rico/8/34 (H1N1) virus strain at 37°C, washed, incubated with 10 µg/mL of the indicated NA inhibitors for 1 hour on ice, and then stained with the appropriate fusion proteins or mAb. BW cells were stained with APC-conjugated anti-human NKP44 mAb (BioLegend), PE-conjugated anti-human NKP46 (Beckman Coulter), and PE-conjugated anti mouse Ncr1 (R&D systems) for the detection of the relevant chimeric protein. All staining was analyzed by FACS using the CellQuest software.

BW Assay

The BW transfected cells were prepared as described elsewhere [32]. Fifty thousand BW or BW-transfected cells were incubated with the same amount of irradiated (6000 rad) influenza-virus-coated EL4 cells for 8 hours at 37 °C and 5% CO2. For NA inhibition, target cells were incubates with 25 µg/50,000 cells of NA inhibitor for 1 hour on ice. Following 8 hours incubation, supernatants were collected and the level of interleukin 2 (IL-2) was quantified by enzyme-linked immunosorbtant assay (ELISA) using anti-IL-2 mAb. IL-2 secretion from transfected BW cells was normalized by reducing the background IL-2 secretion of parental BW cells. Student’s t test was used to determine significant differences.

Cytotoxicity Assay and NK Cell Preparation

The cytotoxic activity of NK cells against various targets was assessed in 5-hour 35S release assays as described elsewhere [33]. For NA inhibition, target cells were incubated with 25 µg/well of
the NA inhibitor for 1 hour on ice. For blocking NKp44 and NKp46 activity, NK cells were incubated on ice for 1 hour with 0.5 µg/well of anti-human CD336 (NKp44) antibody (BioLegend) or with 10 µL/well of anti-human NKp46 (Beckman Coulter) prior to the incubation with the targets. The effector-to-target ratio was 15:1. Human NK cells were isolated from peripheral blood using the Human NK Cell Isolation Kit and the autoMACS instrument (Miltenyi Biotec) according to the manufacturer’s instructions and were grown as described elsewhere [33]. Student’s t test was used to determine significant differences.

RESULTS

**NKp44, NKp46, and Ncr1 Directly Interact With Viral HA**

We demonstrated previously that the activating receptors NKp44 and NKp46 recognize viral HA on infected cells and that this interaction (which is dependent on the sialylation of the receptors) leads to the killing of the infected cells by NK cells [25–27]. Furthermore, we showed that the mouse homologous protein of NKp46 called Ncr1 also binds to viral HA [10, 29]. To demonstrate that Ncr1, NKp44, and NKp46 directly interact with viral HA, we prepared fusion proteins composed of the extracellular portions of Ncr1, NKp44, NKp46, and of control NK cell receptors such as NKp30, KIR2DS4, and KIR2DL1 fused to the Fc portion of human IgG1. The proteins were purified on protein G columns (purity was around 95%) and ran on SDS-PAGE gels (Figure 1A). The SDS-PAGE gels were blotted with biotynylated HA-Ig (composed of the extracellular portion of the HA of the influenza virus A/Puerto Rico/8/34 H1N1 fused to the human IgG1 Fc domain and were also blotted with anti-human Fc antibody that was used as control (Figure 1A). Importantly, direct recognition by HA-Ig of NKp46-Ig, Ncr1-Ig, and NKp44-Ig but not of NKp30-Ig, KIR2DS4, and KIR2DL1 was observed (Figure 1A). The ratio between the biotynylated HA-Ig staining and the anti-human Fc antibody staining of each fusion protein is summaries in Figure 1B.

**The Direct Interaction of the Activating Receptors NKp44 and NKp46 With HA Is Impaired by Viral NA**

Viral HA binds terminal N-acetyl neuraminic acid residues (sialic acids) attached to galactose [26, 28]. NKp46, Ncr1, and NKp44 are all sialylated proteins that utilize this property to bind the viral HA protein on the infected cells [26, 28, 29]. To test whether the direct interaction between NKp44-Ig, NKp46-Ig, and the HA protein observed above (Figure 1) is sialic-acid dependent we treated both NKp46-Ig and NKp44-Ig with NA and Western blotted the treated and untreated proteins with SNA lectin (that binds specifically to Neu5NAcα(2,6) sialic acid residues) and with HA-Ig. As can be seen and as we previously reported [31], NA treatment of NKp46 resulted in a reduction in the sialic acids content (Figure 2A, detected by the SNA lectin), which consequently lead to impaired HA-Ig binding (Figure 2B). Importantly, NA treatment of NKp44-Ig also lead to reduced sialic acids content, and a complete abolishment of the SNA lectin binding was observed after overnight incubation with NA (Figure 2C). Consequently, abolishment of HA-Ig recognition was also observed (Figure 2D). The extended incubation of NKp44-Ig at 37 degrees either alone (Figure 2E), or

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**Figure 1.** NKp44, NKp46, and Ncr1 directly interact with the viral HA protein. *A.* The fusion proteins Ncr1-Ig, NKp46-Ig, NKp44-Ig, NKp30-Ig, KIR2DS4-Ig and KIR2DL1-Ig were run on SDS-PAGE gels and Western blotted with biotynylated HA-Ig and with anti-human IgG1 Fc antibody. The Y-axis shows the quantification (in arbitrary units [AU]) of the intensity of the HA-Ig binding (gray columns) relative to the anti-human IgG1 Fc antibody binding (black columns, which was set to be 1). Representative results from 3 independent experiments are shown. *B.* A table summarising the ratio between the biotynylated HA-Ig staining and the anti-human Fc antibody staining of each fusion protein depict in Figure 1A. Abbreviations: HA, hemagglutinin; IgG1, immunoglobulin G1.
with NA (data not shown) did not affect protein integrity. Collectively this indicates that the direct binding of NKp46 and NKp44 to viral HA is sialic-acid dependent and that similar to NKp46 binding, the direct NKp44-binding to viral HA, is also impaired by the viral NA.

**Enhanced Binding of All HA-interacting Proteins to Influenza-virus-infected Cells Following NA Inhibition**

Because we demonstrated above (Figure 2), and previously regarding NKp46/Ncr1 [31], that NA impairs the HA recognition by NKp44, we next tested whether inhibition of NA leads to increased NKp44-Ig, Ncr1-Ig, and NKp46-Ig recognition of infected cells. To address this issue, influenza-virus-infected Jeg3 cells were stained with NKp44-Ig, NKp46-Ig, Ncr1-Ig, and with a truncated NKp46 fusion protein (D1-Ig) lacking the HA-interacting domain [26], serving therefore as a negative control. Staining of the infected cells was performed with and without NA inhibition, which was mediated by oseltamivir carboxylate (O. carboxylate) and zanamivir. In the absence of influenza, little or no binding was observed (data not shown). Upon infection, increased binding of NKp44-Ig, NKp46-Ig, and Ncr1-Ig was seen (Figure 3A). Importantly, when NA was inhibited with O. carboxylate (Figure 3B) or with zanamivir (Figure 3C), increased binding of NKp46-Ig, Ncr1-Ig, and NKp44-Ig to the influenza-virus-infected cells was observed. No D1-Ig binding was seen prior to and following the treatments (Figure 3A–C). To further control these experiments, we stained both treated and untreated influenza-virus-infected cells with an anti-influenza virus type A HA mAb and with anti-influenza type A mAb and observed that the untreated and treated cells were similarly recognized by both mAbs (Figure 3D). Altogether, this indicates that inhibiting the NA activity leads to a significant increase in the binding of Ncr1, NKp46, and NKp44 to influenza-virus-infected cells.

We also tested whether influenza infection of mouse cells would result in increased Ncr1, NKp44, and NKp46 binding to the infected cells following NA treatment. In agreement with the results obtained with human cells, following NA

**Figure 2.** Direct binding of NKp44 and NKp46 to HA is reduced following NA treatment. NKp46-Ig (A and B) and NKp44-Ig (C, D, and E) were incubated with and without NA beads (the NKp44-Ig was incubated for various periods of time, indicated in the x axis of C, D and E), run on SDS-PAGE gels and then Western blotted with SNA lectin (A and C), with biotinylated HA-Ig (B and D) and with anti-human Fc antibody (E). The graphs above the Western blots show the quantification (in arbitrary units [AU]) of the relative intensity of the SNA lectin, the HA-Ig and anti-human Fc antibody binding. The present or absence of NA beads is indicated below the Western blot gels. Representative results from 3 independent experiments are shown. Abbreviations: HA, hemagglutin; NA, neuraminidase.
inhibition with O. carboxylate, increased NKp44-Ig, NKp46-Ig, and Ncr1-Ig binding to EL4-infected cells was observed, but no change in anti-HA mAb binding was detected (Figure 4).

Increased Reporter Cell Activity Following NA Inhibition

We next tested whether the inhibition of NA activity that leads to increased Ncr1, NKp46, and NKp44 recognition of infected cells would result in increased function of these receptors. For that we initially used a cell-based reporter system in which we expressed in-mouse BW cell chimeric proteins composed of the extracellular portion of NKp44, Ncr1, or NKp46 proteins fused to the mouse zeta chain (BW NKp44, BW Ncr1, and BW NKp46, respectively). In this cell-based reporter assay, the BW cells secrete IL-2 upon engagement of the chimeric protein with an appropriate antigen/ligand [1]. After verifying the expression of the chimeric proteins (Figure 5A), the various BW cells were incubated for 8 hours with influenza-virus-infected EL-4 cells that were treated or not with O. carboxylate. Following this incubation period, IL-2 in the supernatants was determined by ELISA. In accordance with the increased NKp44 and NKp46/Ncr1 binding observed following O. carboxylate treatment of the influenza-virus-infected cells (Figure 4), increased IL-2 secretion from the various BW cells was also detected following the O. carboxylate treatment (Figure 5B). No increase in IL-2 secretion by parental BW cells was seen following NA inhibition (Figure 5B).

Blocking of NA Resulted in Increased NKp44 and NKp46-dependent Killing

In our final set of experiments we wanted to determine whether NA impairs the activity of both NKp46 and NKp44. We performed NK cytotoxicity assays using the Jeg3 cell line and
human NK cells. In the absence of infection, only minimal killing of Jeg3 cells was observed (since Jeg3 do not express known ligands for NK cells [31]), and upon influenza infection, NK killing was observed (Figure 6). Importantly, when infected cells were pretreated with O. carboxylate, a significant increase in NK cytotoxicity was observed, and this increased killing was

Figure 4. The binding of NKp44-Ig, NKp46-Ig and Ncr1-Ig to influenza-virus-infected EL-4 cells is enhanced following NA inhibition. EL4 cells were infected with influenza virus and then stained with anti-HA mAb, NKp44-Ig, Ncr1-Ig and NKp46-Ig (all indicated in the x axis) prior to (empty black graph) and following (empty gray graph) NA inhibition with O. carboxylate. The filled gray histogram depicts the staining with the secondary mAb only. In all figure parts the staining is presented after gating of the live cell population only. The figure shows representative staining. Three independent experiments were performed. Abbreviations: mAb, monoclonal antibody; NA, neuraminidase.

Figure 5. NA Inhibition leads to increased activity of reporter cell line. A, FACS staining of BW cells expressing the NKp44-zeta (BW NKp44), Ncr1-Zeta (BW Ncr1) and NKp46-Zeta (BW NKp46) chimeric proteins. In each panel the empty black histogram depicts the staining with the indicated mAb, and the filled gray histogram depicts the staining of parental BW cells with the indicated mAb. B, The various BW cells expressing the chimeric proteins seen in (A) were incubated with influenza-virus-infected EL-4 cells that were treated or not with O. carboxylate. IL-2 secretion was determined by ELISA 8 hrs following the incubation with the infected cells. Shown is the fold increase of IL-2 secretion (treated cells/untreated cells). The mean values and SD derived from triplicates. Statistically significant differences are indicated (* P < .01, ** P < .001). Abbreviations: ELISA, enzyme-linked immunosorbent assay; FACS, fluo- rescence-activated cell sorting; IL-2, interleukin 2; mAb, monoclonal antibody; NA, neuraminidase; SD, standard deviation.
Inhibition of NA activity boosts NKp44 and NKp46 killing. A. Uninfected and influenza-infected Jeg3 cells were tested in killing assays in the presence or in the absence of O. carboxylate, with or without anti-NKp44 and anti-NKp46 blocking mAb (the various treatments are indicated in the x axis). The effector-to-target ratio (E:T) was 15:1. Shown are mean values and SD derived from triplicates. Statistically significant differences are indicated (*P<.05, **P<.01, one-tailed student’s t test). The figure shows the results of one representative experiment out of three performed. Abbreviations: mAb, monoclonal antibody; NA, neuraminidase; SD, standard deviation.

**DISCUSSION**

NK cells have developed multiple strategies to recognize and eliminate virus-infected cells. The significance of this NK activity is illustrated by the variety of mechanisms that viruses have developed to evade the NK cell recognition. For example, human cytomegalovirus (HCMV), in order to achieve long and persistent infection in the host, uses viral proteins and miRNAs to evade the NKG2D activating receptor recognition [34–38].

The activity of the NCRs NKp30, NKp44, and NKp46 is also manipulated by different viruses [21, 30, 32]. It was shown that the viral tegument protein pp65 binds to NKp30 and inhibits its activity [39]. For NKp44 it was demonstrated that KSHV uses the ORF54/dUTPase protein to down-regulate the expression of an unknown NKp44 ligand, expressed on infected cells [21]. For NKp46/Ncr1, we have recently demonstrated that the viral NA protein of influenza virus antagonizes its activity [31].

NKp44 and NKp46/Ncr1 are the major NK receptors responsible for the detection and elimination of influenza-virus-infected or coated cells [26]. This is mediated by the binding of these activating receptors to the viral HA protein on the membrane of the infected cells or on cells that are coated with the virus [26, 31]. HA is a central protein in the virus’s life cycle, as its property to bind sialic acids on the membrane of the target cells enables the infection of these cells [40]. The ability of HA to bind sialic-acid residues is conserved among all influenza viruses, and indeed although approximately 10,000 different sequences of HA proteins are found in the data bank, they all probably share this common feature of sialic-acid recognition. We suggest that NK cells use this general HA property to bind sialic acids in order to recognize the infected cells via NKp46 and NKp44.

Influenza viruses, on the other hand, did not remain passive, and since the sialic acid binding property of the virus could not be changed, the virus developed other strategies to evade the NK cell recognition. As we have recently shown, influenza virus uses the sialidase activity of the viral NA to remove sialic acids from the NKp46 receptor and its mouse orthologue Ncr1 protein in order to impair the recognition of infected cells [31]. Here we demonstrate thatNA impairs the activity of NKp44 as well.

To test whether the NA-mediated immune evasion mechanism is NKp46 specific or whether it can be used by the influenza virus to evade other NK activating receptors, we first demonstrated that NKp46/Ncr1 and NKp44 directly interact with viral HA. The direct binding between the NKp46/Ncr1, NKp44, and the viral HA was seen in Western blot assays under denaturative conditions. This further emphasizes that the binding between NKp46, Ncr1, NKp44, and viral HA is largely dependent on the sialic-acid recognition and that conformational protein epitopes are probably less important for this recognition. Nevertheless, a certain degree of specificity is observed because other sialylated proteins such as NKp30, KIR2DL1, and KIR2DS4 do not interact with viral HA. Indeed, for NKp46 we demonstrated that the sialylated residue Threonine, located in position 225, and not the other 2 glycosylated residues of NKp46, is primarily responsible for the NKp46 interaction with viral HA [26]. The exact binding sites through which Ncr1 and NKp44 binds to HA are still unknown.

Our data indicate that the influenza virus uses its NA protein not only to evade the recognition of NKp46 but also to broadly prevent the activation of NK cells, and that this is achieved by impairing the recognition of NKp44 in addition to NKp46/Ncr1 [31]. We further demonstrate that both killer proteins NKp44 and NKp46 are important in the elimination of influenza-virus-infected cells and that blocking the activity of both receptors significantly reduces the killing of infected cells.
The realization that NA inhibitors not only block virus infection and budding but also boost the NKP44 and NKP46 recognition might lead to the development of new drugs that use the activity of NKP44 and NKP46 to prevent or treat influenza virus infection.

Notes

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