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NKG2D Is Required for NK Cell Activation and Function in Response to E1-Deleted Adenovirus

Jiangao Zhu,* Xiaopei Huang,* and Yiping Yang*†

Despite high transduction efficiency in vivo, the application of recombinant E1-deleted adenoviral vectors for in vivo gene therapy has been limited by the attendant innate and adaptive immune responses to adenoviral vectors. NK cells have been shown to play an important role in innate immune elimination of adenoviral vectors in vivo. However, the mechanisms underlying NK cell activation and function in response to adenoviral vectors remain largely undefined. In this study, we showed that NK cell activation upon adenoviral infection is dependent on accessory cells such as dendritic cells and macrophages and that cell contact-dependent signals from the accessory cells are necessary for NK cell activation. We further demonstrated that ligands of the NK activating receptor NKG2D were upregulated in accessory cells upon adenoviral infection and that blockade of NKG2D inhibited NK cell activation upon adenoviral infection, leading to a delay in adenoviral clearance in vivo. In addition, NKG2D was required for NK cell-mediated cytolytic activity on adenovirus-infected targets. Taken together, these results suggest that efficient NK cell activation and function in response to adenoviral infection is critically dependent on the NKG2D pathway, which understanding may assist in the design of effective strategies to improve the outcome of adenovirus-mediated gene therapy.

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Recombinant E1-deleted adenoviruses have been developed for gene therapy due to their high transduction efficiency in vivo. However, significant problems of attendant innate and adaptive immune responses to adenoviral vectors have limited their applications for in vivo gene therapy (1). Although the CD8+ and CD4+ T cell responses against both the viral Ags and the transgene contribute significantly to the adaptive immune elimination of adenoviral vectors (2–4), emerging evidence has suggested a critical role for NK cells in innate immune elimination of adenoviral vectors (5–7), leading to the loss of adenoviral genome and the reduction of transgene expression (7). Although our recent work shows that type I IFNs are important for the regulation of NK cell activation (7), how NK cells are activated in response to adenoviral infection remains largely unknown. Delineation of this process may assist in the design of effective strategies to improve the outcome of adenovirus-mediated gene therapy.

NK cells represent an important component of the innate immune system and play a critical role in innate immune defense against various viral infections in vivo (8, 9). The activation of NK cells is tightly controlled by both inhibitory and activating receptors (10). During murine CMV (MCMV) infection, NK cell activation is mediated by the NK cell activating receptor Ly49H, which specifically recognizes the m157 gene product of MCMV expressed on the surfaces of infected cells (11, 12). Similarly, recognition of influenza virus hemagglutinin on virus-infected cells by another activating receptor, Nkp46, activates lysis by human NK cells (13), and the murine Nkp46 equivalent, NCR1, is required for protection against lethal influenza infection (14). In addition to these pathogen-specific NK cell activation pathways, the NK activating receptor NKG2D has been shown to recognize host stress proteins induced upon viral infections and to play an important role in the control of human CMV and MCMV infections (15, 16).

In this study, we showed that NK cell activation upon adenoviral infection was also dependent on accessory cells such as dendritic cells (DCs) and macrophages and that the NK cell–accessory cell contact is necessary for NK cell activation in vitro. We further demonstrated that accessory cells upregulated NKG2D ligands upon adenoviral infection and that NKG2D blockade inhibited NK cell activation both in vitro and in vivo, leading to a delay in the clearance of adenoviral vectors. In addition, NKG2D was critical for NK cell-mediated cytolytic activity on adenovirus-infected targets. Collectively, these results suggest that efficient NK cell activation and function in response to adenoviral infection depends on the NKG2D pathway.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). RAG-2 deficient (RAG-2−/−) mice were purchased from Taconic (Hudson, NY). Mice used were between 6 and 10 wk of age. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Duke University (Durham, NC).

Recombinant E1-deleted adenovirus

Recombinant E1-deleted adenovirus encoding LacZ (Ad-LacZ) under the control of the CMV promoter was generated and purified as described previously (17). For in vivo studies, mice were infected by i.v. injection of 2 × 106 PFU Ad-LacZ in 100 μl PBS. After 24 h, splenocytes or intrahepatic lymphocytes were harvested for further analyses. For in vitro stimulation, cells were infected with Ad-LacZ at multiplicity of infection (MOI) of 400 at 37°C for 18 h.

Preparation of DCs, macrophages, and B cells

Plasmacytoid DCs (pDCs) were generated as described (17). Briefly, bone marrow cells were harvested from femurs and tibiae of C57BL/6 mice and cultured in the presence of 100 ng/ml Flt-3 ligand (R&D Systems,
NK cell and accessory cell coculture system

NK cells were enriched from spleens of C57BL/6 mice by positive selection with PE-conjugated anti-DX5 and anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were stained with PE-Cy5-conjugated anti-CD3ε and then sorted for NK cells (DX5+CD3ε). The purity was more than 95%. Sorted NK cells (1×10^6) were cultured in 96-well U-bottom tissue culture plates in 100 μl RPMI 1640 medium supplemented with 10% FCS, 2 mM l-glutamine, 10 mM HEPES, 10 μM 2-mercaptoethanol, and 100 IU/ml penicillin/streptomycin. Then, 2×10^3 accessory cells (pDCs, cDCs, or macrophages) in 100 μl were added to the same wells in the presence or absence of Ad-LacZ at MOI of 400 for 18 h.

For blocking experiments, 10 μg/ml anti-mouse NKG2D mAb (CX5; eBioscience) or anti-mouse CD226 (10E5; eBioscience) or 30 μg/ml recombinant mouse Nkp46/NCR1/Fc chimera (R&D Systems) was added to the culture as described (20–22).

Intracellular IFN-γ and granzyme B staining

For ex vivo experiments, splenocytes were restimulated for 4 h in the presence of 5 μg/ml brefeldin A (Sigma-Aldrich), 40 ng/ml PMA (Sigma-Aldrich, St. Louis, MO), and 100 ng/ml ionomycin (Sigma-Aldrich). After staining of NK cell surface markers (DX5+CD3ε), the cells were fixed with CytopermCytofix (BD Biosciences) for 20 min and incubated for 30 min with aliphosphocyanin-conjugated rat anti-mouse IFN-γ and FITC-conjugated anti-granzyme B. The cells were then subjected to flow cytometry event collection, which was analyzed using FACSDiva software (Becton Dickinson).

FIGURE 1. Accessory cells are required for NK cell activation in response to adenoviral infection. Purified splenic NK cells (DX5+CD3ε) were either cultured alone (NK alone) or cocultured with peritoneal macrophages (+MΦ), pDCs (+pDCs), cDCs (+cDCs), or B cells (+B cells) in the presence (Ad) or absence (Medium) of Ad-LacZ. Eighteen hours later, brefeldin A was added into each well and incubated for an additional 4 h. A, NK cells were analyzed for the expression of the early activation marker, CD69, by flow cytometry. The MFI of CD69 staining is indicated. Events were gated on DX5+CD3ε NK cells. B and C, NK cells were stained intracellularly for IFN-γ and granzyme B and analyzed by flow cytometry. FACS plots of granzyme B production by NK cells with the percentage of granzyme B+ cells among DX5+CD3ε NK cells indicated (B). The mean percentage ± SD of granzyme B+ or IFN-γ+ cells among DX5+CD3ε cells is indicated (n = 5 per group) (C). The data shown are representative of four independent experiments. MFI, mean fluorescence intensity.
**In vivo Ab blocking**

Mice were injected twice with 100 μg anti-NKG2D mAb (CX5; eBio-science) or a control Ab (rat IgG1) 24 and 6 h before adenoviral infection.

**NK cell cytotoxicity assay**

NK cell cytotoxicity assay was performed as described (7). Briefly, peritoneal macrophages infected with Ad-LacZ at an MOI of 100 for 18 h were used as target cells. Target cells (1 × 10^7) were labeled with 100 μCi Na^251^ClO_4, ^51^CrCl_3 for 1 h at 37°C. NK cells were enriched from spleens by positive selection twice with PE-conjugated anti-DXS and anti-PE microbeads. NK cells were incubated with target cells (1 × 10^7/well) labeled with ^51^Cr for 4 h at various effector to target ratios as indicated in triplicate wells. The supernatants were obtained after the incubation and subjected to gamma counting. The maximum or spontaneous release was defined as counts from samples incubated with 1% SDS or medium alone, respectively. Cytolytic activity was calculated with the following formula: percentage specific lysis = (experimental − spontaneous) release × 100/ (maximum − spontaneous) release.

**Detection of adenoviral genomic DNA by real-time PCR**

Total genomic DNA was isolated from the liver tissue as described previously (7). Real-time quantitative PCR was used to measure the amount of adenoviral genomic DNA in liver tissues using primers located in the fiber gene from adenovirus and mouse β-actin gene. Amounts of adenoviral DNA were normalized to β-actin gene within each sample. The sequences of the forward and reverse primers for adenoviral fiber gene were 5'-CCACGGATAGCACTACCTT-3’ and 5'-GACACGTGTCTACGTCAAA-3’, respectively. Normalized value for adenoviral DNA in each sample was calculated as the relative quantity of adenoviral DNA divided by the relative quantity of β-actin gene.

**X-gal staining**

The sections of fresh-frozen liver tissue (5 μm) were fixed in 0.5% glutaraldehyde for 10 min, rinsed twice for 10 min in PBS containing 1 mM MgCl_2, and incubated in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM K_3Fe(CN)_6, 5 mM K_4Fe(CN)_6, and 1 mM MgCl_2 in PBS for 2 h at 37°C. Sections were counterstained in hematoxylin and examined microscopically.

**β-Galactosidase activity in liver tissues**

This assay was performed as described (7). Briefly, liver tissues were homogenized in reporter lysis buffer on ice. Homogenates were then clarified by centrifugation, and supernatant was recovered. Total protein concentration was measured in liver homogenate using a standard Bradford protein assay (Bio-Rad, Hercules, CA). β-Galactosidase activity was evaluated using β-galactosidase enzyme assay (Promega) under conditions recommended by the manufacturer. The absorbance was read at 420 nm in a plate, and the results were expressed as units per gram of liver protein.

**Statistical analysis**

Results were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Student t test.

**Results**

**NK cell activation upon adenoviral infection is dependent on accessory cells**

Accessory cells, such as DCs and macrophages, have been implicated in NK cell activation (23). To study whether accessory cells are required in the activation of NK cells in response to adenoviral infection, freshly sorted DX5^+^CD3^−^ NK cells were stimulated with Ad-LacZ in the presence or absence of macrophages, pDCs, cDCs, or B cells. NK cells were then harvested 18 h later and assayed for the expression of the activation marker CD69, as well as the production of effector molecules, granzyme B and IFN-γ. NK cells stimulated with Ad-LacZ alone did not significantly upregulate CD69 (Fig. 1A) or produce granzyme B (Fig. 1B, 1C) and IFN-γ (Fig. 1C) compared with that of the medium control (Fig. 1A, 1B). However, addition of macrophages, pDCs, and cDCs, but not B cells, stimulated NK cells to produce significantly (p < 0.001) higher levels of CD69 (Fig. 1A), granzyme B (Fig. 1B, 1C) and IFN-γ (Fig. 1C). These results indicate that accessory cells such as macrophages and DCs are required for the activation of NK cells in response to adenoviral infection.

**NKG2D is required for efficient NK cell activation in response to adenovirus**

What are the underlying mechanisms responsible for the accessory cell-dependent NK cell activation to E1-deleted adenovirus? Accessory cells may provide indirect (soluble) and/or direct (contact dependent) signals to NK cells necessary for their activation. Indeed, we have shown that adenovirus can induce pDCs, cDCs, and macrophages to produce type I IFNs (17) and that type I IFNs are critical for NK cell activation and function in response to adenoviral infection (7). To examine whether accessory cells also provided direct contact-dependent signals for NK cell activation upon adenoviral infection, purified NK cells were cocultured with macrophages either mixed together or separated by a Transwell, followed by stimulation with Ad-LacZ for 18 h. Our data showed that the expression of CD69 (Fig. 2A) and the production of IFN-γ and granzyme B (Fig. 2B, 2C) by NK cells were significantly (p < 0.001) reduced in the Transwell culture compared with those of
the mixed culture control. These results suggest that NK cell activation in response to adenovirus also requires contact-dependent signals from the accessory cells.

We next determined what contact-dependent signal(s) was required for adenovirus-induced NK cell activation. The NK cell receptors, such as NKG2D, NKp46, and DNAM-1 (CD226), have been shown to play an important role in NK cell activation and function (14–16, 24). We thus investigated the role of NKG2D, NKp46, and DNAM-1 in NK cell activation upon adenoviral infection using a blocking anti-NKG2D or anti-CD226 mAb or a blocking NKp46-Fc fusion protein. We showed that addition of anti-NKG2D mAb, but not anti-CD226 mAb or NKp46-Fc, to the NK cell–macrophage coculture markedly inhibited the expression of CD69 (Fig. 3A) and the production of IFN-γ and granzyme B (Fig. 3B, 3C) by NK cells compared with those of the control rat IgG-treated group. These results suggest that NKG2D signaling is critical for NK cell activation upon adenoviral infection in vitro.

**NKG2D ligands are upregulated in accessory cells upon adenoviral infection**

We then investigated whether NKG2D ligands were induced upon adenoviral infections. Murine NKG2D recognizes nine ligands: Rae-1α, Rae-1β, Rae-1γ, Rae-1δ, Rae-1ε, Mult1, H60a, H60b, and H60c (25). We first investigated if adenoviral infection upregulated the expression of NKG2D ligands. RNA samples from naive or Ad-LacZ–infected mice were analyzed for the expression of NKG2D ligands by semiquantitative PCR technology (Table I).

All sequences are presented in the 5′–3′ direction.

**Table I. Oligonucleotide primer pairs for semiquantitative PCR analysis**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rae-1α</td>
<td>GGCCGCTGTAGTGTAGTTACC</td>
<td>GAGTGCTCCCAGCTGTTTTC</td>
</tr>
<tr>
<td>Rae-1β</td>
<td>GCCATGCAGCCAGCTGTTTACG</td>
<td>CCAGATGAGGAGGCTGTTTTC</td>
</tr>
<tr>
<td>Rae-1γ</td>
<td>TCTCTGATCCACCTGTTTTCAG</td>
<td>TGGTAGGAGGAGGCTGTTTTC</td>
</tr>
<tr>
<td>Rae-1δ</td>
<td>CTTCTGATCCACCTGTTTTCAG</td>
<td>TGGTAGGAGGAGGCTGTTTTC</td>
</tr>
<tr>
<td>Rae-1ε</td>
<td>GCTTATGCTGATCAGGTTTTCAG</td>
<td>TGGTAGGAGGAGGCTGTTTTC</td>
</tr>
<tr>
<td>Mult1</td>
<td>CATGCCATCTGTGCTTTTACG</td>
<td>GAGGAGGAGGCTGTTTTC</td>
</tr>
<tr>
<td>H60a</td>
<td>ACCAAGCTGATCGTAGTTTTCAG</td>
<td>GAGGAGGAGGCTGTTTTC</td>
</tr>
<tr>
<td>H60b</td>
<td>GGCGCTGATCGTAGTTTTCAG</td>
<td>GAGGAGGAGGCTGTTTTC</td>
</tr>
<tr>
<td>H60c</td>
<td>CCTACTGATCGTAGTTTTCAG</td>
<td>GAGGAGGAGGCTGTTTTC</td>
</tr>
<tr>
<td>β-Actin</td>
<td>CTTCTGATCTGAGGTTTTCAG</td>
<td>GAGGAGGAGGCTGTTTTC</td>
</tr>
</tbody>
</table>

As shown in Fig. 4A, the mRNA levels for Rae-1α, Rae-1β, Rae-1δ, and Mult1 were markedly increased in Ad-LacZ-infected splenocytes, whereas mRNAs coding for Rae-1β, Rae-1δ, and Mult1 were not detected in Ad-LacZ-infected splenocytes, whereas mRNAs coding for Rae-1β, Rae-1δ, and Mult1 were not detected in Ad-LacZ-infected splenocytes.
H60b, and H60c were not detected. To determine further whether accessory cells upregulate the expression of NKG2D ligands in response to adenovirus in vivo, splenic macrophages (CD11b+F4/80+) and cDCs (CD11c+B220) from naive or Ad-LacZ–infected mice were analyzed for the expression of Rae-1 and Mult1 by flow cytometry. Indeed, in Ad-LacZ–infected mice, the expression of Mult1 on the surfaces of macrophages and cDCs was uniformly upregulated compared with that of the uninfected naive controls (Fig. 4B). Although the expression of Rae-1 was also uniformly upregulated on macrophages of the infected mice, it appears only a subpopulation of cDCs upregulated Rae-1 (Fig. 4B). This Rae-1+ subpopulation increased in size that is proportional to increasing doses of Ad-LacZ infection in vivo (data not shown), suggesting that induction of Rae-1 in cDCs by adenovirus requires a higher viral load. Collectively, these results suggest NKG2D ligands Mult1 and Rae-1 are induced in accessory cells upon adenoviral infection in vivo.

**NKG2D is critical for NK cell activation and function upon adenoviral infection in vivo**

We next examined the role of NKG2D signaling in NK cell activation in vivo. To address this question, mice were treated with the blocking NKG2D Ab or a control Ab, followed by infection with $2 \times 10^9$ PFU Ad-LacZ administered i.v. Twenty-four hours postinfection, splenic NK cells were analyzed for their activation. NK cells from the control rat IgG-treated mice produced large amounts of IFN-γ and granzyme B compared with those of the naive mice (Fig. 5A,5B). However, the production of IFN-γ and granzyme B by NK cells was significantly ($p < 0.05$) reduced in the anti-NKG2D mAb–treated group (Fig. 5A,5B). These results indicated that the NKG2D pathway is also important in the activation of NK cells upon adenoviral infection in vivo.

The critical role of NKG2D in NK cell activation in response to adenoviral infection suggested the blockade of NKG2D may...
cytolysis on adenovirus-infected target cells. "Impaired NKG2D is critical for NK cell-mediated cytolysis on adenovirus-infected targets. C57BL/6 mice were injected intravenously with 2 × 10^3 PFU Ad-LacZ or left uninfected as the control. Twenty-four hours postinfection, splenic NK cells were enriched from Ad-LacZ-infected (Activated NK) or naive (Naive NK) mice and assayed for NK cell lytic activity on Ad-LacZ-infected macrophages in the presence of anti-NKG2D mAb (+Anti-NKG2D) or the rat IgG control (+Rat IgG) for 4 h at different effector to target ratios. The mean percentage ± SD of specific lysis is indicated (n = 3 per group). Data shown are representative of two independent experiments.

FIGURE 7. NKG2D is critical for NK cell-mediated cytolysis on adenovirus-infected targets. C57BL/6 mice were injected intravenously with 2 × 10^3 PFU Ad-LacZ or left uninfected as the control. Twenty-four hours postinfection, splenic NK cells were enriched from Ad-LacZ-infected (Activated NK) or naive (Naive NK) mice and assayed for NK cell lytic activity on Ad-LacZ-infected macrophages in the presence of anti-NKG2D mAb (+Anti-NKG2D) or the rat IgG control (+Rat IgG) for 4 h at different effector to target ratios. The mean percentage ± SD of specific lysis is indicated (n = 3 per group). Data shown are representative of two independent experiments.

Discussion
NK cells play an important role in innate immune control of adenoviral infection in vivo (5–7). We have previously shown that type I IFN, induced upon adenoviral infection, acts directly on NK cells to regulate their activation and function (7). However, how NK cells are activated upon adenoviral infection remains to be further defined. In this study, we provided evidence that the NKG2D pathway is critical for NK cell activation and function in response to adenoviral infection. This is supported by upregulation of NKG2D ligands in accessory cells upon adenoviral infection and a requirement of the accessory cell–NK cell contact for NK cell activation. Furthermore, NKG2D blockade inhibits NK cell activation and function and delays adenoviral clearance in vivo.

The NKG2D activating receptor has been shown to play an important role in NK cell activation in response to human and murine CMV infections (15, 16). NKG2D recognizes host stress proteins induced upon viral infections (10). The stress-induced NKG2D ligands are Rae1, Mult1, and H60 classes in mice and the ULBP and MIC classes in humans. In this study we showed that both Rae-1 and Mult1 are induced upon adenoviral infection and that NK cell activation and NK cell-mediated control of adenoviral infection in vivo are critically dependent on the NKG2D pathway. Furthermore, NKG2D is required for the recognition of adenovirus-infected target cells for NK cell-mediated cytolyis. This is in line with the previous observation that NK cell-mediated cytolyis on adenovirus-infected primary human fibroblasts is also dependent on NKG2D (26). Our results may have important implications for the design of effective strategies to reduce NK cell-mediated early toxicity and improve the outcome of adenovirus-mediated gene therapy. Specifically, the observation that NKG2D blockade significantly blunts innate immune elimination of adenoviral vectors suggests a feasible strategy to reduce early phase of hepatotoxicity and prolong transgene expression.

NKG2D is not only expressed by most NK cells but also by subsets of T cells including activated CD8+ T cells (27). This could complicate the interpretation of the NKG2D blocking experiments in immunocompetent mice in vivo. To address this question, we have used RAG-2−/− mice. Indeed, our data in RAG-2−/− mice clearly demonstrated a critical role of NKG2D in NK cell-mediated clearance of adenovirus in vivo. We further demonstrated that NKG2D plays an important role both in the activation of NK cells and in NK cell recognition of target cells.

What triggers the expression of NKG2D ligands on accessory cells upon adenoviral infection remains to be determined. Previous studies in other models have shown that NKG2D ligands can be induced in response to cellular stress, such as the response to DNA damage (28), infection (29), as well as TLR stimulation (30). In addition, it has been shown that wild-type adenovirus E1A protein can regulate the expression of Rae-1, which sensitizes tumor cells to NKG2D-dependent NK cell lysis and promotes tumor rejection (31). It remains to be determined what component(s) of the E1-deleted adenovirus or which mechanism(s) is responsible for the induction of NKG2D ligands upon adenoviral infection. Understanding this process will help us design effective NK cell-based strategies to improve adenovirus-mediated gene therapy in vivo.

In conclusion, we have shown that the NKG2D pathway is critical for NK cell activation in response to adenoviral infection in vitro and in vivo. We have further demonstrated that NKG2D is required for the recognition of virus-infected targets and that it plays an important role in elimination of adenoviral vectors. These results may suggest potential strategies to improve the outcome of adenovirus-mediated gene therapy in vivo.

Disclosures
The authors have no financial conflicts of interest.

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