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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 was 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 cytolysis 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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Abbreviations used in this paper: cDCs, conventional DCs; DCs, dendritic cells; MΦ, macrophages; MCMV, murine CMV; MFI, mean fluorescence intensity; MOI, multiplicity of infection; pDCs, plasmacytoid DCs.

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Minneapolis, MN) for 9 d. For generation of conventional DCs (cDCs), bone marrow cells were cultured in the presence of mouse GM-CSF (1000 U/ml) and IL-4 (500 U/ml) (R&D Systems) for 5 d as described (18). pDCs and cDCs were stained with anti-B220–FITC and anti-CD11c–PE and purified by FACS sorting. The purity of cells was 95–97%. Macrophages were isolated from the peritoneal cavity of mice 2 d after i.p. injection of 1.5 ml 3% thioglycolate as described (19). B220+ B cells were purified from the spleen by double selection with B220 microbeads.

**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 × 10^5 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).

**Intracelular 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 allophycocyanin-conjugated rat anti-mouse IFN-γ and FITC-conjugated anti-granzyme B. The cells were then subjected to flow cytometry analysis.

**Transwell experiments**

Transwell experiments were performed in 24-well plates (Corning, Acton, MA). Peritoneal macrophages were plated in the lower wells (2 × 10^5), and NK cells (1 × 10^5) were added to the upper wells in the presence or absence of Ad-LacZ (MOI of 400). After culturing for 18 h, cells were harvested for intracellular cytokine or cell surface marker staining.

**Abs and flow cytometry**

The following Abs were used for flow cytometry: PE-conjugated anti–Rae-1 (CX1), Multi1 (SD10), and anti-CD49b (DX5); PE-Cy5–conjugated anti-CD3ε (145-2C11); FITC–conjugated anti-granzyme B (16G6), anti-CD11c (HL3), anti-CD11b (M1/70), and anti-CD69 (H1.2F3); allopurinol-conjugated rat anti-mouse IFN-γ (XMG1.2) and anti-CD11b (M1/70). These Abs were purchased from BD Biosciences (San Diego, CA) or eBioscience (San Diego, CA). Allopurinol-conjugated anti-F4/80 (CI-A3-1) was purchased from AbD Serotec (Raleigh, NC). FACScan (Becton Dickinson, Franklin Lakes, NJ) was used for flow cytometry event collection, which was analyzed using FACSDiva software (Becton Dickinson).

**Semiquantitative RT-PCR analysis for NKG2D ligand expression**

Total RNA samples were prepared from spleens using TRIzol reagent and treated with DNase I (Roche, Branchburg, NJ). RNA samples (1 μg) were then converted to first-strand cDNAs using oligo(dT)15 primers and Superscript III reverse transcriptase (Promega, Madison, WI). PCR amplification was set up on serial 3-fold dilutions of cDNA using the primer pairs specific for nine NKG2D ligand genes shown in Table I. RT-PCR products were electrophoresed through a 2% agarose gel.

**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 = 3 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, peri-
toneal macrophages infected with Ad-LacZ at an MOI of 100 for 18 h were
used as target cells. Target cells (1 × 10⁶) were labeled with 100 μCi
Na₂⁵¹CrO₄ (⁵¹Cr) for 1 h at 37˚C. NK cells were enriched from spleens
by positive selection twice with PE-conjugated anti-DX5 and anti-PE
microbeads. NK cells were incubated with target cells (1 × 10⁵/well) la-
beled with ⁵¹Cr for 4 h at various effector to target ratios as indicated in
tripticate 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 pre-
viously (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′-
CCACCGATAGCAGTACCCTT-3′ and 5′-GACCAGTTGCTACGGTCA-
AA-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% glu-
taraldehyde for 10 min, rinsed twice for 10 min in PBS containing 1 mM
MgCl₂, and incubated in 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-
galactopyranoside (X-gal), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 1 mM
MgCl₂ in PBS at 37°C. Sections were counterstained in hematoxy-
lin 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 Brad-
ford 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 pro-
duction 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

**FIGURE 2.** Accessory cell–NK cell contact is necessary for NK cell activation in vitro. Purified NK cells (DX5⁺CD3⁺) were cocultured with macrophages either mixed together (Mixed) or separated by a Transwell system (Transwell), followed by stimulation with Ad-LacZ for 18 h (Ad) or left unstimulated (Medium). Brefeldin A was added into each well and incubated for an additional 4 h. DX5⁺CD3⁺ NK cells were analyzed for intracellular IFN-γ and granzyme B production with the percentage of IFN-γ⁺ and granzyme B⁺ cells indicated (B). The mean percentage ± SD of IFN-γ⁺ or granzyme B⁺ cells is indicated (n = 5 per group) (C). The data shown are representative of three independent experiments.
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 naïve or Ad-LacZ–infected mice were analyzed for the expression of NKG2D ligands by semiquantitative PCR technology (Table I). As shown in Fig. 4A, the mRNA levels for Rae-1α, Rae-1γ, Rae-1δ, and Mult1 were markedly increased in Ad-LacZ–infected splenocytes, whereas mRNAs coding for Rae-1β, Rae-1ε, H60a,

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**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>GCCCGCTGTAAGTGACATGTTACC</td>
<td>GAGTGTCCTCCACGCTGTTTTCC</td>
</tr>
<tr>
<td>Rae-1β</td>
<td>GCATTACGGCACTGAAATGGAA</td>
<td>CCAAGGCGAGTACAGGTTTTCC</td>
</tr>
<tr>
<td>Rae-1γ</td>
<td>CTCTTACGCAAGAAGATTTCC</td>
<td>TTCTCCTGTTCTGAGAGAACCC</td>
</tr>
<tr>
<td>Rae-1δ</td>
<td>CTCTTACGCAAGAAGATTTCC</td>
<td>TTCTCCTGTTCTGAGAGAACCC</td>
</tr>
<tr>
<td>Rae-1ε</td>
<td>GCCAGTGTAATGACATTGATCC</td>
<td>CGTTTCTCTCTCTGGTTGAGAG</td>
</tr>
<tr>
<td>Mult1</td>
<td>CATGCGATTTGCTCATATG</td>
<td>TGGGCTGTGTCATACAGGATAG</td>
</tr>
<tr>
<td>H60a</td>
<td>ACCAGGGATGTTACCTGCC</td>
<td>CGTTTCTCTCTCTGGTTGAGAG</td>
</tr>
<tr>
<td>H60b</td>
<td>GGGTGCTGTTCTGTCCCTAG</td>
<td>TGGAAAGTACAGCAGCAATTT</td>
</tr>
<tr>
<td>H60c</td>
<td>CTCTTACGCAAGAAGATTTCC</td>
<td>AGCCATGATGGCAGCTTCTCC</td>
</tr>
</tbody>
</table>

All sequences are presented in the 5′–3′ direction.
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 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...
FIGURE 7. NKG2D is critical for NK cell-mediated cytolysis on adenovirus-infected targets. C57BL/6 mice were injected intravenously with 2 × 10⁹ 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.

improve the outcome of adenovirus-mediated gene therapy in vivo. To eliminate the potential influence of the adaptive immunity on the clearance of adenovirus, RAG-2⁻/⁻ mice were used to test this strategy. RAG-2⁻/⁻ mice were treated with either the control rat IgG or the anti-NKG2D mAb 24 and 6 h prior to infusion of Ad-LacZ. Mice treated with the NKG2D blocking mAb showed significantly (p < 0.05) higher levels of LacZ expression evaluated by X-gal histochemistry (Fig. 6A) and a quantitative β-gal assay (Fig. 6B) in the liver 3 d postinfection. Furthermore, the copy numbers of adenoviral genome in the liver were evaluated by real-time quantitative PCR. Significantly (p < 0.05) larger amounts of adenoviral DNA were detected in mice treated with anti-NKG2D mAb than those in the control Ab-treated mice (Fig. 6C). These results indicate that the NKG2D pathway is critical for NK cell-mediated elimination of adenoviral infection in vivo. Previous studies have shown that NKG2D also plays a critical role in recognizing NKG2D ligands expressed on target cells and mediating NK cell-mediated cytolysis in the setting of human CMV and MCMV infections (15, 16). To address whether NKG2D is involved in NK cell-mediated cytolysis on adenovirus-infected targets, NK cells isolated from Ad-LacZ–infected or naive control mice were assayed for their lytic activities on Ad-LacZ–infected macrophages in vitro in the presence of NKG2D blocking mAb. A significant (p < 0.05) reduction in cytolytic activities was observed with the NKG2D blockade (Fig. 7), suggesting that NKG2D is also critical for NK cell-mediated cytolysis on adenovirus-infected target cells.

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 cytolysis. This is in line with the previous observation that NK cell-mediated cytolysis 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.

References
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NKG2D IN NK CELL ACTIVATION TO ADENOVIRUS


