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Distinct Roles for the NK Cell-Activating Receptors in Mediating Interactions with Dendritic Cells and Tumor Cells

Lu-En Wai, Jordan A. Garcia, Olivia M. Martinez, and Sheri M. Krams

NK cells are innate immune cells that are important in tumor immunity, but also have the ability to modulate the adaptive immune system through cytokine production or direct cell–cell interactions. This study investigates the interaction of NK cells with dendritic cells (DCs) and tumor cells, and the role of specific NK cell-activating receptors in this process. Primary rat NK cells and an NK cell line produced IFN-γ when cocultured with either DCs or the rat hepatoma cell line McA-RH7777 (McA). This NK cell activation by DCs and McA required cell-cell contact and was dependent on distinct NK-activating receptors. Silencing NK cell expression of NKp46 and NKp30 significantly diminished DC- and McA-mediated NK cell IFN-γ production, respectively. NK cells killed immature and mature DCs independently of NKp46, NKp30, and NKG2D; however, cytotoxicity against McA cells was dependent on NKp30 and NKG2D. Thus, we have shown in this study that NKp30 plays dual activating roles in NK–McA tumor interactions by mediating cytokine production and cytotoxicity. More importantly, NK cells are activated by both DCs and hepatoma cells to produce IFN-γ, but require distinct NK cell-activating receptors, NKp46 and NKp30, respectively. Our data suggest that therapeutics could be developed specifically to target NK–DC interactions without compromising NK tumor immunity. *The Journal of Immunology*, 2011, 186: 222–229.

Natural killer cells are effector cells of the innate immune system, and have the ability to kill virus-infected or tumor cells, secrete cytokines, and regulate both innate and adaptive immune responses (1). NK cell function is controlled by a balance of negative and positive signals transmitted via inhibitory and activating receptors on their cell surface. Although inhibitory receptors such as the human killer cell Ig-like receptors have been well studied (2), less is known about the activating receptors. NKG2D, the best characterized activating receptor on NK cells, has been shown to be important for triggering NK cell cytotoxicity. NKG2D binds to MHC class I-related ligands, including stress-induced MICA/MICB and UL16-binding proteins in humans (3, 4), RAE-1 and H60 in mice (5), and RAE-1–like transcript in rats (6). However, NKG2D expression is not NK cell specific, as NKG2D is also expressed on several T cell subsets and NKT cells.

The natural cytotoxicity receptors (NCRs) are a family of activating receptors that are expressed almost exclusively by NK cells and include NKp30, NKp46, and NKp44 (7). These receptors play a major role in triggering NK cell cytolytic activity. However, ligands for the NCRs are not well established. NCR ligands are likely to be non-MHC class I molecules because NCRs can mediate killing of both MHC I-negative and MHC I-positive cell lines (8).

There is in vitro evidence that NKp44 and NKp46, but not NKp30, recognize viral hemagglutinins and mediate killing of virus-infected cells (9–11). Candidates for NKp30 ligands include human CMV viral protein pp65 (12), HLA-B–associated transcript 3 (13), and the recently identified B7 family member B7-H6 (14). The nature of the human NKp30 ligand is, however, controversial, with evidence to both support and disprove that it is a heparan sulfate glycosaminoglycan (15, 16).

NKp30 also mediates NK cytotoxicity against tumor cells, either independently or in cooperation with NKp44, NKp46, and/or NKG2D (17). Interestingly, NKp30 has been reported to have a unique role in determining the fate of immature DCs (iDCs) during their interactions with NK cells. Under certain conditions, NK cells can kill autologous iDCs via ligation of NKp30 and activation of PI3K (18, 19), thereby altering the number of DCs that reach complete maturation, and thus affecting T cell priming. Alternately, instead of inducing iDC killing, NK cells can also mediate the maturation of iDCs via engagement of NKp30 and the release of TNF-α and IFN-γ (20). The mechanism controlling the dual roles of NKp30 is not clear, although the ratio of NK cells to DCs is thought to be important (21). And whereas it has been shown that NK cells and DCs reciprocally activate one another during an immune response (22), DC-mediated NK activation may involve NKp30, NKp46, and NKG2D, or cytokines like IL-12 and type I IFNs depending on the type of DC and NK activation readout used (11, 18, 23–25).

The NCR NKp46 has been shown to be involved in innate immune defense. For example, NKp46-deficient mice show increased susceptibility to lethal influenza injection (26) and aberrant tumor immunosurveillance (27). Recently, several groups have identified a distinct subset of mouse intestinal lymphocytes that express NKp46 and retinoic acid-related orphan receptor γt and secrete IL-22 (28–30), and suggest that these cells play a role in immune defense against intestinal pathogens. Other than being important for innate immunity, NKp46 also helps modulate adaptive immunity by working in cooperation with NKp30 and DNAX accessory molecule-1 to kill iDCs (18, 19, 31).

Whereas NKp46 and NKG2D are expressed in humans and rodents, NKp30 is a nonexpressed pseudogene in inbred mouse strains (32). NKp30, however, is expressed in the rat and functions as an activation receptor on a subset of rat NK cells (33, 34). In this work, we report, to our knowledge, the first study to investigate the combined role of NKp30, NKp46, and NKG2D in mediating rodent NK cell interactions with DCs and tumor cells. We found that DCs and the hepatoma cell line McA stimulate IFN-γ production from NK cells in a contact-dependent manner, but via different NK-activating receptors, NKp46 and NKp30, respectively. NKp30 and NKG2D are also important for NK-mediated killing of McA tumor cells. Our data suggest that it is possible to manipulate NK–DC interactions while maintaining tumor responsiveness.

Materials and Methods

NK cells

RNK-16-2B3 (2B3) is a rat NK lymphoma line stably transfected with NKp30 (34), and was maintained in complete RPMI (RPMI 1640 [Mediatech, Manassas, VA], 10% FBS [Serum Source International, Charlotte, NC], and 1% penicillin/streptomycin [Mediatech]) supplemented with 50 μM 2-ME (Sigma-Aldrich, St. Louis, MO). Primary rat NK cells were purified from Lewis rat spleens. Spleens were removed from euthanized rats, 6–8 wk of age (Charles River Laboratories International, Wilmington, MA), and mononuclear cells were isolated by density gradient centrifugation with Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). Cells were washed and resuspended to 20×10^6 cells/ml in PBS plus 1% FBS, incubated with NK cell-specific NKp46 mAb [clone WEN23; gift from E. Dissen, University of Oslo, Oslo, Norway (35)] at 0.5 μg/million NK cells for 20 min at 4°C, washed twice, then incubated with *Pan* Mouse IgG Dynabeads (Invitrogen, Carlsbad, CA) at 1 bead/cell for 20 min at 4°C, before removing beads and attached cells with a magnet (Dynal MPC; Invitrogen, Carlsbad, CA). Cells were resuspended at a concentration of 125,000 cells/ml in complete RPMI supplemented with 50 μM 2-ME (Sigma-Aldrich), 1% sodium pyruvate (Life Technologies, Invitrogen), 0.5% HEPES (Life Technologies), and 1000 IU/ml human rIL-2 (Hoffmann-LaRoche, Nutley, NJ). Cell purity was determined using flow cytometry on the day of isolation and again 7 d later.

Dendritic cells

Bone marrow-derived DCs were obtained by culturing rat bone marrow cells in DC RPMI (RPMI 1640, 5% FBS, 1% penicillin/streptomycin, 1% HEPES, 1% L-glutamine [Life Technologies]) supplemented with 5 ng/ml GM-CSF, IL-4, and Flt3-ligand (R&D Systems, Minneapolis, MN) for 8 d, adding media on days 3 and 7, and changing media on day 5 of culture. Mature DCs were obtained by adding 10 μg/ml polyinosinic:polycytidylic acid [poly(I:C)] to DC cultures 24 h before cell isolation, and washed thoroughly before use in cocultures. The adherent DCs were isolated by removing media, rinsing once with sterile PBS, incubating with Versene (Life Technologies) at 37°C, and gently scraping with a cell scraper. The cells were pooled and rinsed with complete RPMI before use. DC cultures expressed a DC-specific marker, integrin α CD103 (OX62). Propidium iodide was used to label dead cells that were gated out from the analyses. Live DCs expressed a high level of CD11b/c and moderate levels of DC maturation markers MHC class II and CD86 (Supplemental Fig. 1A). Poly (I:C)-treated DCs expressed significantly higher levels of MHC class II and CD86 on their cell surface as detected using flow cytometry, and secreted more IL-12 than untreated DCs as measured by ELISA (Supplemental Fig. 1A, 1B).

Tumor cells

Rat hepatoma cell line McA-RH7777 (McA) was maintained in DMEM (Life Technologies) supplemented with 10% horse serum (American Type Culture Collection, Manassas, VA), 5% FBS (Serum Source International), and 1% penicillin/streptomycin (Mediatech). The adherent McA cells were isolated by removing media, rinsing once with sterile PBS, and incubating with trypsin EDTA (Mediatech), before pooling and rinsing with complete RPMI before use.

NK-DC coculture

A total of 2×10^5 NK cells was incubated with 2×10^5 DCs or McA hepatoma cells in 24-well plates in a total volume of 400 μl complete RPMI for 24 or 6 h, respectively, in a 37°C, 5% CO_2, humidified incubator. To prevent cell contact between NK cells and DCs or McA, DCs or McA were added to 0.4-μm Transwell inserts (Corning Life Sciences, Lowell, MA) instead of directly to the wells. After coculture, supernatants were collected and frozen for subsequent cytokine analyses. To obtain DC- and McA-conditioned media, supernatants were collected from DCs and McA cells cultured at 1×10^6 cells/ml for 24–48 h.

RNA interference

NK cell receptor expression was silenced using RNA interference. NK cells (4×10^6 cells) were transfected with either a medium guanine-cytosine control oligo or short interfering RNA (siRNA) oligos specific for NKp46, NKp30, or NKG2D (Invitrogen), using a Nucleofector system (Lonza, Visp, Switzerland). NKp46 knockdown required a second transfection after 24 h. After transfection, cells were cultured at 10^6 cells/ml in complete RPMI for 48 h prior to use in experiments.
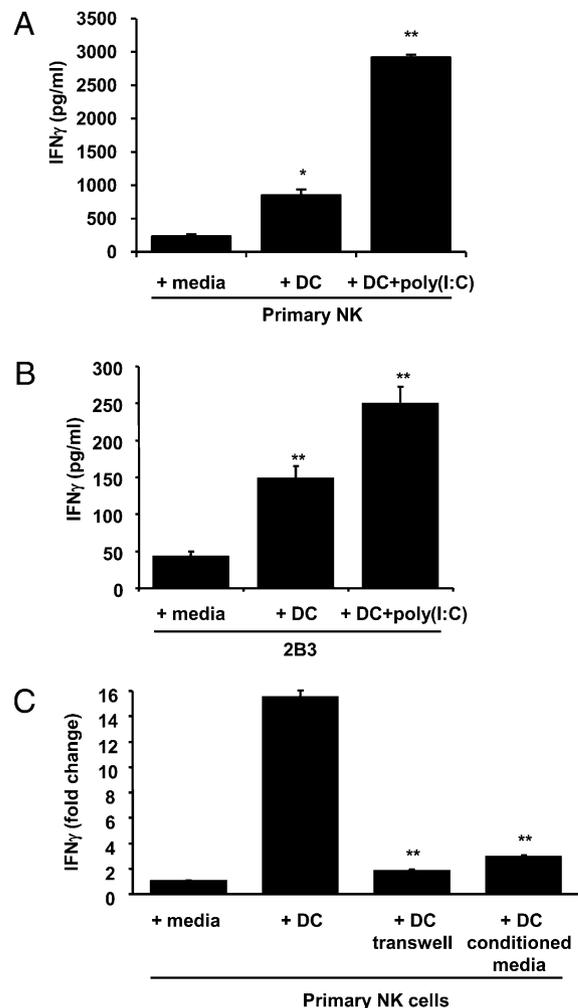

FIGURE 1. DCs stimulate NK cells to produce more IFN-γ in a cell contact-dependent manner. *A* and *B*, Primary NK cells (*A*) or 2B3 NK cells (*B*) were cultured in media or with DCs that were previously cultured in the absence or presence of poly(I:C). After 24 h of incubation, levels of IFN-γ in the supernatants were quantitated by ELISA and expressed as pg/ml. The data shown are representative of three individual experiments. Values are the means of triplicates ± SEM. *C*, Primary NK cells were cultured in media alone, with DCs, DCs placed in a Transwell insert, or supernatant taken from day 8 DC cultures (DC-conditioned media). Supernatants were obtained 24 h after coculture, and IFN-γ levels were quantitated by ELISA and expressed as fold change over NK plus media control. The data shown are representative of three individual experiments. Values are the means ± SEM. **$p < 0.01$.

Detection of cytokines

IL-12 levels from bone marrow DC cultures were measured with a rat IL-12 plus p40 CytoSet (Invitrogen) using a mouse anti-rat IL-12–coating Ab and a mouse anti-rat IL-12 biotin Ab. IFN-γ levels from cocultures were measured with a rat IFN-γ CytoSet (Invitrogen) using a rabbit anti-mouse/rat IFN-γ–coating Ab and a mouse anti-mouse/rat IFN-γ biotin (clone DB-1) Ab. IL-12 and IFN-γ ELISAs were carried out according to manufacturer protocols, and samples were plated in triplicate. ELISA data were reported as the mean fold change over controls ± SEM. Data were analyzed using a two-tailed Student *t* test. The *p* values <0.01 were considered significant. Luminex analysis of cytokines was carried out using a Panomics rat 21-plex panel (Affymetrix, Santa Clara, CA) and analyzed using a Luminex 200 System (Luminex, Austin, TX). Cytokines and chemokines tested were as follows: eotaxin, G-CSF, GM-CSF, ICAM, IFN-γ, IL-10, IL-12p40, IL-1α, IL-1β, IL-4, IL-6, KC (CXCL1), MCP-1 (CCL2), MCP-3 (CCL7), MIP1α (CCL3), nerve growth factor, RANTES (CCL5), TGF-β, TNF-α, VCAM, and vascular endothelial growth factor.

Flow cytometry

Cells were washed with cold FACS buffer (PBS, 1% FBS, 0.1% sodium azide) before immunofluorescent staining. NK cells were incubated with anti-rat NKp46 mAb ($0.5 \mu g/10^6$ cells of WEN23 mAb), anti-rat NKp30 mAb [$1 \mu g/10^6$ cells of clone CLH3 (34); Santa Cruz Biotechnology, Santa Cruz, CA], mouse anti-rat NKG2D-Fc mAb ($1 \mu g/10^6$ cells of clone 11D5F4) (36), or isotype controls mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or mouse IgG1 (DakoCytomation, Carpinteria, CA) for 30 min on ice. R-phycoerythrin (RPE)-conjugated donkey anti-mouse F(ab')₂ Abs (1:200; Jackson ImmunoResearch Laboratories) or RPE-conjugated goat anti-mouse IgG1 (1:40; AbD Serotec, Oxford, U.K.) were used as secondary reagents. DCs were stained for a DC-specific marker using FITC-conjugated anti-rat integrin α ($2 \mu g/10^6$ cells of clone OX62; Cedarlane Laboratories, Burlington, NC) or FITC-conjugated mouse IgG1 (DakoCytomation). Other DC markers were detected using PE-conjugated anti-rat CD11b/c (clone OX42), MHC class II (clone OX6), CD86, or isotype control mouse IgG1 (all from AbD Serotec). Cells were washed in FACS buffer, resuspended in 0.5 μg/ml propidium iodide, and analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Ashland, OR). To detect putative NK cell-activating receptor ligands, activating receptor fusion proteins (NKp30-Fc, NKp46-Fc, NKG2D-Fc) were constructed according to a previous protocol (34, 36). CD30-Fc was used as a negative control. Cells were incubated with $4 \mu g/10^6$ cells of fusion protein for 30 min on ice, washed twice, and incubated with RPE-conjugated goat anti-human Fc Ab (1:200; Jackson ImmunoResearch Laboratories) for 30 min on ice, and propidium iodide was added to cells before flow analysis.

Cytotoxicity assays

To detect cytotoxicity against DCs, a flow cytometry-based killing assay was adapted from the ACT 1 cytotoxicity assay (Cell Technology, Mountain View, CA). Briefly, DCs were incubated with CFSE for 15 min at room temperature, resuspended in complete RPMI for 30 min at 37°C, and washed twice. A total of 5×10^4 CFSE-labeled DCs (100 μl of 5×10^5 cells/ml) was added to each FACS tube. NK cells (5×10^5 cells/ml) were added to the tubes at ratios varying from 32:1 to 0.5:1 (i.e., 3.2 ml to 50 μl). Cells were cocultured in a 37°C humidified incubator for 18 h. Cells were pelleted and resuspended in 400 μl complete RPMI before incubation with 1:200 7-aminoactinomycin D (7AAD) for 15 min on ice. Cells were analyzed using flow cytometry, and dead target cells were identified as $CFSE^+ 7AAD^+$. Percentage increase in dead DCs was calculated by first subtracting background death from sample death, and then dividing by background death and multiplying by 100.

To detect cytotoxicity against tumors, the JAM method to measure DNA fragmentation was used (37). Target McA-RH7777 cells were subcultured into 24-well plates at 2.5×10^5 cells/ml, 0.5 ml/well, for 24 h before labeling with [³H]thymidine (5 μCi/ml; PerkinElmer, Boston, MA) for another 24 h. Washed target cells were plated with and without effector cells for 4 h at 10^4 target cells/well. Cells were harvested onto a glass fiber filtermat (PerkinElmer) using a Tomtec Harvester 96 Mach II (Tomtec, Hamden, CT), and a Wallac 1205 betaplate reader (PerkinElmer) measured radioactivity. Percentage of specific killing was calculated as follows: (cpm of spontaneous killing without effector cells − cpm of experimental killing)/(cpm of spontaneous killing) × 100.

FIGURE 2. NKp46 knockdown significantly prevents NK cell stimulation by DCs. *A*, 2B3 cells were treated with control siRNA or siRNA specific for NKp46, NKp30, and NKG2D. Surface expression of NKp46, NKp30, and NKG2D on cells treated with control siRNA (solid line) or receptor-specific siRNA (dashed line) was analyzed using flow cytometry. Isotype controls are shown in gray. The data shown are a representative experiment demonstrating knockdown of NKp46, NKp30, and NKG2D expression by 80, 95, and 80%, respectively. *B*, 2B3 NK cells treated with control siRNA or a combination of siRNA specific for NKp46, NKp30, and NKG2D were cultured in media or with DCs. Supernatants were obtained 24 h after coculture and analyzed for IFN-γ secretion by ELISA. Fold change in IFN-γ was determined with respect to specific NK plus media controls. *C*, Primary NK cells treated with control siRNA or NKp46 siRNA were cultured in media or with DCs. Supernatants were obtained 24 h after coculture, and IFN-γ levels were analyzed as in *B*. The data shown are representative of three separate experiments. Values are the means ± SEM. *D*, DCs were incubated with NKp46-Fc, NKp30-Fc, NKG2D-Fc (solid lines), or an unrelated fusion protein CD30-Fc (gray histograms). The level of fusion protein binding to the cell surface was detected with a PE-conjugated anti-Fc Ab and analyzed using flow cytometry. **$p < 0.01$.
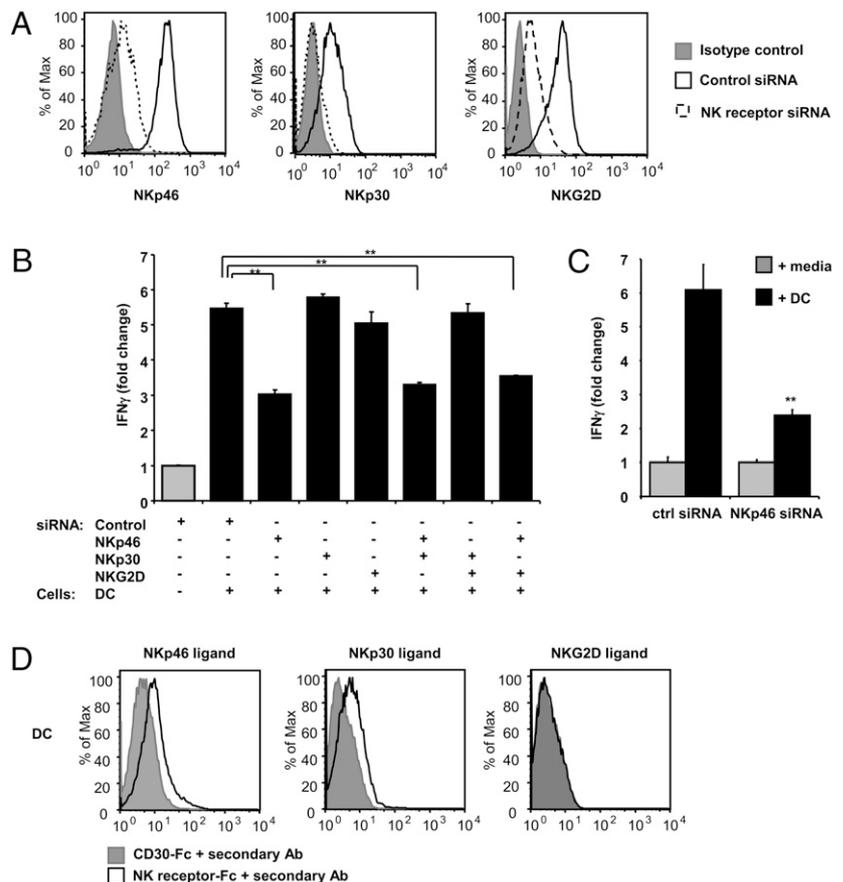
Results

NK cell stimulation by DCs is cell contact dependent

To determine whether NK cells produce IFN-γ in the presence of DCs, rat primary NK cells and NK cell line 2B3 were cultured with an equal number of DCs for 24 h, and IFN-γ production was assessed. Primary NK cells cocultured with iDCs and mature poly(I:C)-treated DCs produced 4-fold ($p = 0.0208$) and 12-fold ($p = 0.0003$) more IFN-γ, respectively, compared with NK cells cultured in media alone (Fig. 1A). Similar results were seen with 2B3 NK cells (Fig. 1B). Using intracellular flow cytometry, we confirmed that NK cells were the source of the IFN-γ (data not shown). These data suggest that both iDCs and mature DCs can stimulate NK cells to produce IFN-γ.

NK cells are activated to produce IFN-γ when stimulated with cytokines such as IL-12 or when NK cell-activating receptors are engaged by ligands expressed on other cells. To determine whether DCs stimulate NK cells through soluble factors or cell-cell contact, a Transwell system was used to separate DCs from NK cells during coculture. Primary NK cells that were physically separated from DCs by a Transwell produced 90% less IFN-γ ($p = 0.0015$) than primary NK cells cultured in direct contact with DCs (Fig. 1C). Similarly, primary NK cells cultured with supernatants from DC cultures produced 80% less IFN-γ than primary NK cells cultured with DCs ($p = 0.0017$). Similar results were obtained with the NK cell line 2B3 (data not shown). Furthermore, cells cultured in the Transwell plates remained healthy and viable, confirming the Transwell plates were not toxic to the cells (data not shown). Taken together these data suggest that direct cell-cell contact is necessary for DC-mediated IFN-γ production by NK cells.

DC stimulation of NK cells is mediated through NKp46

Because NK cells express a variety of cell surface receptors, we sought to determine which NK cell receptor–ligand interaction mediates contact-dependent NK cell stimulation by DCs. We observed that NK cell activation occurred in the presence of both strain-matched and mismatched DCs, suggesting that this interaction is not MHC restricted or repressed by NK inhibitory receptors (data not shown). Turning our attention to NK-activating receptors, we first demonstrated that NKp46, NKp30, and NKG2D were expressed on 2B3 cells and primary NK cells (Fig. 2A, Supplemental Fig. 2). Next, to determine the requirement for NKp46, NKp30, and NKG2D in NK–DC interactions, expression of these activating receptors on NK cells was knocked down using RNA interference. The 2B3 NK cells and primary NK cells treated with receptor-specific siRNA consistently showed 70–95% receptor downregulation on the cell surface as analyzed by immunofluorescence and flow cytometry (Fig. 2A, Supplemental Fig. 2).

The 2B3 NK cells treated with siRNA were cultured with DCs for 24 h, and the levels of IFN-γ in the supernatant were analyzed by ELISA. The 2B3 NK cells treated with control, NKp30, or NKG2D siRNA produced similar levels of IFN-γ in response to DC stimulation (Fig. 2B). In contrast, 2B3 NK cells treated with NKp46 siRNA produced 50% less IFN-γ ($p = 0.0076$) after DC stimulation, suggesting that NKp46 plays a role in DC-mediated NK cell stimulation. To test whether there was cooperation between receptors, 2B3 NK cells were treated with a combination of NKp46, NKp30, and NKG2D siRNAs. Combined knockdown of NKp30 and NKG2D did not alter IFN-γ production. In contrast, combined knockdown of NKp46 with either NKp30 or NKG2D resulted in 50% decrease in IFN-γ after DC-mediated stimulation, similar to the effect of NKp46 knockdown alone. This suggests that NKp30 or NKG2D is not additive with NKp46, and that NKp46 is sufficient to stimulate IFN-γ expression. Similarly, knockdown of

NKp46 on primary NK cells significantly diminished IFN-γ production in response to DCs (Fig. 2C). However, downregulation of NKp46 expression on either 2B3 NK cells or primary NK cells did not completely abrogate the ability of DCs to activate NK cells, either because of residual low levels of NKp46 expression or because an unidentified NK cell receptor also participates in IFN-γ production. In addition, DCs express putative NKp46 and NKp30 ligands, but not NKG2D ligands (Fig. 2D).

IL-12 production by rat DCs was not affected by coculture with NK cells for 24 h, regardless of NK receptor expression (Supplemental Fig. 3A). In contrast, DCs produced significantly more soluble ICAM after coculture with NK cells (Supplemental Fig. 3B). Other cytokines and chemokines were either undetectable (G-CSF, GM-CSF, IL-1α, IL-1β, IL-4, nerve growth factor, TGF-β, vascular endothelial growth factor, eotaxin, VCAM) or showed no significant change after NK-DC coculture (IL-10, IL-6, TNF-α, KC, MCP-1, MCP-3, MIP-1α, RANTES) (data not shown).
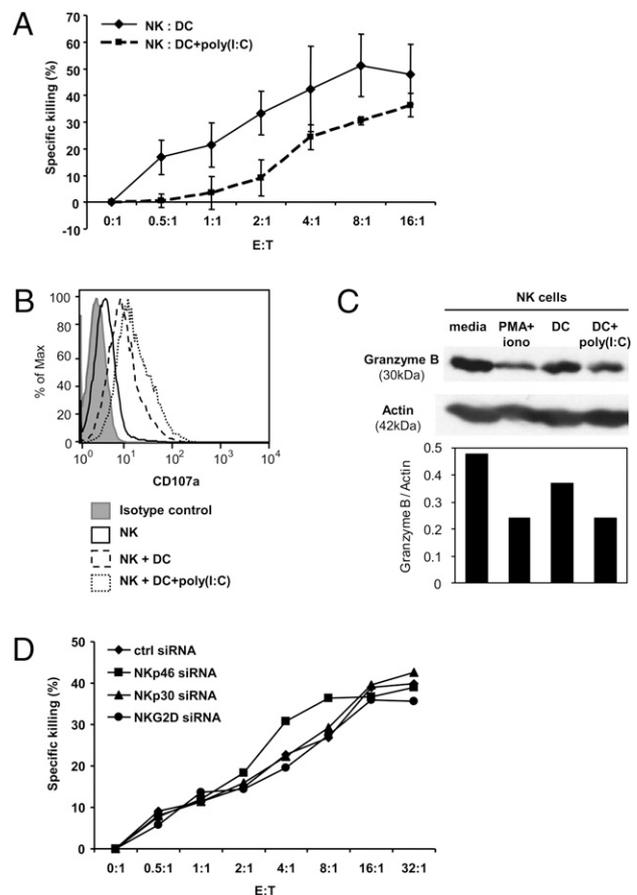

FIGURE 3. NK cells degranulate upon coculture with DCs and kill DCs independently of NKp30, NKp46, and NKG2D. *A*, The ability of 2B3 NK cells to kill iDCs and mature DCs was assessed using a flow cytometry-based cytotoxicity assay. The data shown are the mean of two experiments ± SEM. *B*, The surface expression of degranulation marker CD107a on RNK-16 NK cells cultured with iDCs and mature DCs for 24 h was analyzed using flow cytometry. *C*, Intracellular granzyme B levels in RNK-16 NK cells decreased after coculture with iDCs or mature DCs. PMA plus ionomycin is a positive control for NK cell degranulation. *D*, 2B3 NK cells treated with control or receptor-specific siRNA were cultured with CFSE-labeled DC target cells at E:T ratios shown. After 18-h coculture, cells were stained with 7AAD and specific killing was determined using flow cytometry. The data shown are representative of three separate experiments.

NK cells kill DCs independently of NKp30, NKp46, and NKG2D

Next, we examined the ability of NK cells to kill iDCs and mature DCs using a flow cytometry-based cytotoxicity assay. The 2B3 NK cells killed iDCs more effectively than they killed mature DCs at E:T ratios ranging between 0.5:1 and 16:1 (Fig. 3A). Degranulation of 2B3 NK cells and primary NK cells after NK-DC coculture was detected as an increase in CD107a expression and decreased intracellular granzyme B levels (Fig. 3B, 3C, Supplemental Fig. 4). To determine the NK-activating receptors important in NK cell-mediated DC killing, 2B3 cells were treated with NKp46, NKp30, and NKG2D siRNA. Decreased expression of these receptors was confirmed by flow cytometry (Fig. 2A). The 2B3 NK cells with downregulated NKp46, NKp30, and NKG2D expression killed DCs with the same efficiency as control siRNA-treated NK cells, suggesting that none of these receptors individually mediates DC killing (Fig. 3D). Combined knockdown of the receptors also did not affect DC killing (data not shown), suggesting that other contact-dependent or soluble factors are important in mediating NK killing of DCs.

NK cell stimulation by hepatoma cells is contact dependent and mediated by NKp30

We determined that NK cell-mediated IFN-γ production in the presence of tumor cells is entirely contact dependent (Fig. 4A). To determine whether NKp46 was also important in NK activation by tumor cells, NKp46, NKp30, and NKG2D expression on 2B3 NK cells was knocked down prior to coculture with rat hepatoma McA. The 2B3 NK cells cocultured with McA hepatoma cells produced 3-fold more IFN-γ compared with NK cells alone, re-gardless of whether the cells were treated with control, NKp46, and NKG2D siRNA alone or in combination (Fig. 4B). Strikingly, when NKp30 alone was knocked down, the IFN-γ increase upon McA tumor cell stimulation was completely abrogated. Knockdown of NKp30 in combination with NKp46 and NKG2D did not further decrease IFN-γ production. In addition, when analyzing the expression of putative rat NK-activating receptor ligands on tumor cell lines using a rat NKp30-Fc fusion protein, we found that McA cells expressed high levels of NKp30 ligands (Fig. 4C). These data suggest that the NKp30–NKp30 ligand interaction is sufficient for McA cells to stimulate NK cells to produce IFN-γ.

NK cell cytotoxicity against McA-RH7777 is mediated by NKp30 and NKG2D

We demonstrated that 2B3 NK cells and primary NK cells kill McA hepatoma cells over a wide range of E:T ratios (Fig. 5A). Because NKp30 mediated NK cell stimulation by McA, we determined whether NKp30 was also required for killing of hepatoma cells. In a representative experiment, 2B3 NK cells treated with control or NKp46 siRNA killed similar percentages of McA tumor cells (39.1 ± 3.4% and 31.68 ± 5.5%, respectively) at an E:T ratio of 25:1 (Fig. 5B). In contrast, 2B3 NK cells treated with NKp30 or NKG2D siRNA killed significantly less McA cells (15.8 ± 0.4% and 11.02 ± 3.1% specific killing, respectively) compared with control-treated NK cells (29.1 ± 3.4%). This suggests that NKp30 and NKG2D are important for NK cytotoxicity against McA cells. Correspondingly, McA cells express high levels of both NKp30 ligands and NKG2D ligands (Fig. 4C). In contrast, McA cells do not express NKp46 ligands. Thus, combining these data, we conclude that NKp30 plays important activating roles in NK–tumor interactions as a mediator of both NK stimulation and cytotoxicity.
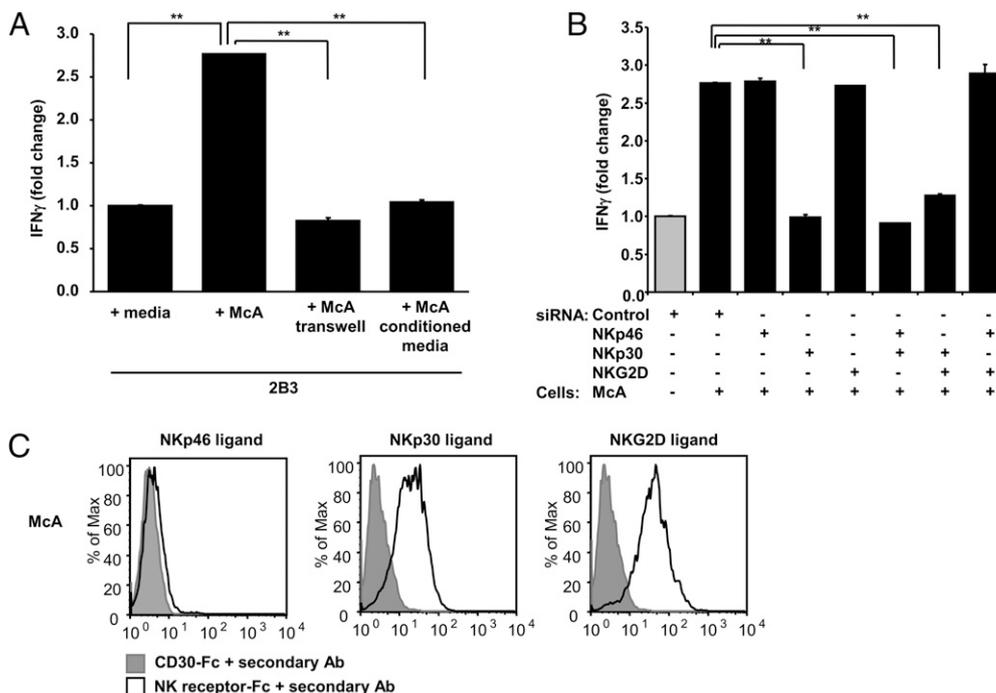

FIGURE 4. NKp30 is required for hepatoma-mediated activation of NK cells. *A*, 2B3 NK cells were cultured in media alone, with McA cells, McA cells placed in a Transwell insert, or supernatant taken from McA cultures (McA-conditioned media). Supernatants were obtained 6 h after coculture, and IFN-γ levels were quantitated by ELISA and expressed as fold change over NK plus media control. The data shown are representative of three separate experiments. Values are the means ± SEM. **$p < 0.01$. *B*, 2B3 NK cells treated with control siRNA or a combination of receptor-specific siRNA were cultured in media or with McA cells. Supernatants were obtained 6 h after coculture and analyzed for IFN-γ secretion. Fold change in IFN-γ was determined with respect to specific NK plus media controls. The data shown are representative of three separate experiments. Values are the means ± SEM. **$p < 0.01$. *C*, McA cells were incubated with NKp46-Fc, NKp30-Fc, NKG2D-Fc (solid lines), or an unrelated fusion protein CD30-Fc (gray histograms). The level of fusion protein binding to the cell surface was detected with a PE-conjugated anti-Fc Ab and analyzed using flow cytometry.
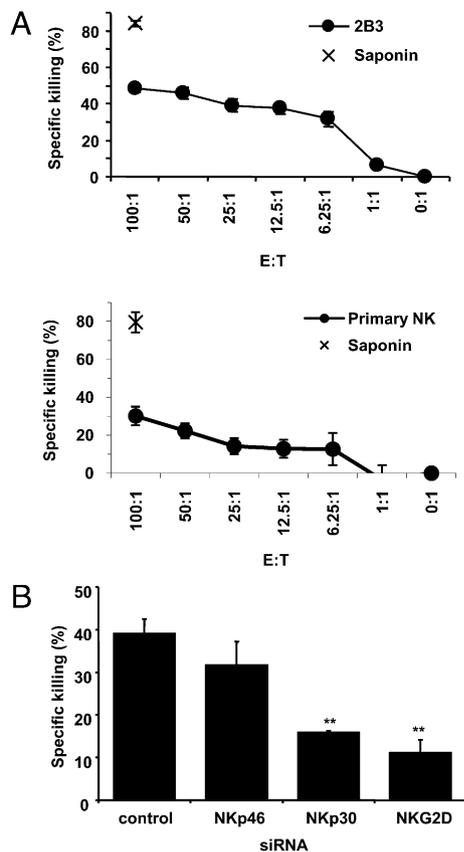
FIGURE 5. NK cell cytotoxicity against McA-RH7777 is mediated by NKp30 and NKG2D. *A*, 2B3 NK cells and primary NK cells were cultured with McA cells at a range of E:T ratios, with 10% saponin-treated McA cells as a positive control. Cells were harvested 4 h after culture to measure radioactivity remaining and to calculate percentage of specific killing, as described in *Materials and Methods*. *B*, 2B3 NK cells were treated with control siRNA or receptor-specific siRNA 48 h before culture with [^{3}H]thymidine-labeled McA cells at 25:1 E:T ratio, and specific killing was measured. The data shown are representative of two separate experiments. Values are the means of triplicates $\pm$ SEM. **$p < 0.01$.

Discussion

In this study, we investigated the combined role of NKp30, NKp46, and NKG2D in mediating rat NK–DC interactions and provide evidence that NKp46 is important for NK cell stimulation by DCs. Several NK-activating receptors and cytokines have been implicated in the interactions between NK cells and DCs, but a clear understanding of the function of specific molecules is lacking. NKp30 and IL-12 have been shown to be important for DC-mediated IFN-γ production by human NK cells (18, 23, 38). Other studies have shown that NKp46 and NKG2D are necessary for NK cell activation by autologous influenza-infected DCs (11). There is evidence that NKp30 and NKp46 are responsible for NK killing of human DCs (18, 39). However, inbred mice do not express NKp30 (32), and mouse studies have instead suggested that cytokines IL-12, IL-18, and IL-15 and type I IFNs play major roles in DC-mediated NK cell activation (25, 40, 41), whereas DNAX accessory molecule-1 mediates NK killing of iDCs (42). Rats do express NKp30 on a subset of their NK cells (33, 34); however, we found no role for NKp30 in rat NK–DC interactions. Our data would support that NKp46 is important for the activation of NK cells by DCs to produce IFN-γ in agreement with a report that NKp46 and NKG2D are important in human NK interactions with influenza-infected DCs (11). However, nonviral NKp46 ligands remain unknown for both human and rodents. In addition, NKp30 and

NKp46 both signal through CD3ζ (43), suggesting these receptors may be interchangeable in interactions with DCs dependent upon the repertoire of receptors and ligands expressed by the NK cells and DCs.

Our study does demonstrate that NKp30 is an important activating receptor in interactions with tumors. McA hepatoma cells stimulate IFN-γ production from NK cells in an entirely NKp30-dependent manner. Both NKp30 and NKG2D are required for NK cell-mediated cytotoxicity. Whereas NKG2D has been implicated in cytotoxicity against hepatocellular carcinoma (HCC) in humans (44, 45), the role of NKp30 in cytotoxicity or IFN-γ production in the presence of HCC has not been reported. There is, however, recent evidence that higher expression of the NKp30 gene (*NCR3*) and other inflammatory and innate immune genes by HCC tumor-infiltrating leukocytes correlates with longer HCC patient survival (46), suggesting that NK cells may interact and/or kill HCC tumors via NKp30 in humans.

Whereas it is not known whether human HCC tumors express NKp30 ligands, McA hepatoma cells express high levels of putative ligands for NKp30 and NKG2D. Using primers previously described (36), we detected transcription of NKG2D ligand RAE1L in McA hepatoma cells (data not shown). The identity of NKp30 ligands on McA hepatoma cells is unknown, although BAT3 and B7-H6 are potential tumor ligands for human NKp30 (13, 14). Trypsin treatment prevented the binding of NKp30-Fc to the surface of McA cells, although silencing BAT3 expression did not, suggesting the presence of protein-based non-BAT3 ligands (data not shown). Further work is needed to identify the NKp30 ligand on McA hepatoma cells.

Recent reports have revealed that the cell surface expression of NKp30 ligands correlates with the capacity of tumor cells to activate NK cell lysis and/or induce IFN-γ (14, 47). Our data demonstrate that high expression of NKp30 ligands on McA hepatoma cells correlates strongly with NKp30-dependent NK cell activation and lysis. Similarly, FaO rat hepatoma cells have lower NKp30 ligand expression and correspondingly stimulate less IFN-γ production from NK cells. In contrast, rat glioma RT2 and thymoma Nb2 express low to moderate levels of NKp30 ligands, but do not significantly activate NK cells (data not shown). These data support a positive correlation between NKp30 ligand expression and the ability to stimulate NK cells by hepatoma cells, but not other tumor types. One possibility is that there are several NKp30 ligands that activate different responses through NKp30. Alternatively, coreceptors or cofactors are necessary for complete activation. Interestingly, the high expression of NKp30 ligands on McA hepatoma cells activates both NK cell IFN-γ production and lysis, but each is only evident at different NK/tumor ratios; NKp30 mediates NK cell activation at low NK/tumor ratio (1:1) and killing of McA cells at high NK/tumor ratio (25:1). This parallels a mechanism proposed to explain human NK–DC interactions; culture of activated human NK cells with iDCs at low NK/DC ratios increased DC cytokine production, but at high NK/DC ratios increased DC killing (21). Perhaps the important factors determining the kind of NKp30-mediated interactions NK cells have with other cells are the level and type of NKp30 ligand expression, expression of cofactors, and the ratio of interacting cells. Our findings may be particularly pertinent in developing DC-based cancer therapies.

In conclusion, we reported in this study that rat NK cells interact with DCs and hepatoma cells through distinct NK-activating receptors, NKp46 and NKp30/NKG2D, respectively. These new data indicate that the NKp46–NKp46 ligand interaction between NK cells and DCs could be blocked without affecting the ability of NK cells to respond to or kill hepatoma cells. Our findings may be particularly pertinent for the development of therapeutics that

target NK–DC interactions without compromising the ability of NK cells to kill tumor cells.

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Disclosures

The authors have no financial conflicts of interest.

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