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Costimulation of Multiple NK Cell Activation Receptors by NKG2D

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The activation of NK cells is mediated through specific interactions between activation receptors and their respective ligands. Little is known, however, about whether costimulation, which has been well characterized for T cell activation, occurs in NK cells. To study the function of NKG2D, a potential NK costimulatory receptor, we have generated two novel hamster mAbs that recognize mouse NKG2D. FACS analyses demonstrate that mouse NKG2D is expressed on all C57BL/6 IL-2-activated NK (lymphokine-activated killer) (LAK) cells, all splenic and liver NK cells, and ~50% of splenic NKT cells. Consistent with limited polymorphism of NKG2D, its sequence is highly conserved, and the anti-NKG2D mAbs react with NK cells from a large number of different mouse strains. In chromium release assays, we show that stimulation of NK cells with anti-NKG2D mAb can redirect lysis. Also, enhanced lysis of transfected tumor targets expressing NKG2D ligand could be inhibited by addition of anti-NKG2D mAb. Interestingly, stimulation of LAK cells via NKG2D alone does not lead to cytokine release. However, stimulation of LAK via both an NK activation receptor (e.g., CD16, NK1.1, or Ly-49D) and NKG2D leads to augmentation of cytokine release compared with stimulation through the activation receptor alone. These results demonstrate that NKG2D has the ability to costimulate multiple NK activation receptors.


Natural killer cells constitute a subset of lymphocytes that contributes to the innate immune response against intracellular pathogens (1, 2) and may mediate tumor surveillance (3). Unlike B cells, which produce Abs, NK cells use effector mechanisms similar to those of T cells. They engage in cell-mediated cytolysis by releasing granules containing perforin and granzymes that result in target cell apoptosis (4), and secrete cytokines such as IFN-γ, GM-CSF, and TNF-α that can modulate adaptive immune responses (5).

NK cells were originally characterized (and named) for their ability to lyse target cells without prior sensitization, i.e., natural killing (6, 7). It is now known that NK cells use specific receptors to mediate killing through the recognition of distinct ligands expressed on target cells (8). These NK receptors fall into two functional types, inhibitory and stimulatory (9). Much more is known about the inhibitory receptors, which in turn can be organized into two structural categories: killer Ig-like type I integral membrane receptors, encoded in the leukocyte receptor complex, and lectin-like type II integral membrane receptors, encoded in the NK gene complex (NKC) (10–13). Despite their structural differences, the inhibitory NK receptors share many functional characteristics. Where defined, they interact with MHC class I on the target, and this interaction prevents activation of NK effector function by eliciting a potent inhibitory signal mediated by an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain. When NK inhibitory receptors are engaged, the ITIM is tyrosine phosphorylated and SHP-1 phosphatase is recruited and activated, which presumably then dephosphorylates signaling molecules involved in the activation cascade (14–17).

Comparatively less is known about the nature of NK activation receptors. Individual NK cells express multiple different activating NK receptors of both Ig and lectin-like structural families that lack cytoplasmic ITIMs (18–20). These receptors contain charged amino acids in their transmembrane domains, permitting interaction with distinct signaling chains containing immunoreceptor tyrosine-based activation motifs (ITAMs). NK cells express multiple ITAM signaling chains, including KARAP/DAP12, CD3ζ, and FceRIγ (8). Ly-49D and Ly-49H associate with KARAP/DAP12 (21, 22), NKR-P1 associates with FcεRIγ (23), and CD16 associates with CD3ζ (24, 25) and FcεRIγ (26). Although it is not yet known whether these signaling chains are functionally equivalent, when activating receptors are cross-linked, the ITAMs in the associated signaling chain become tyrosine phosphorylated. These ITAMs then recruit downstream signaling kinases such as Syk and ZAP70, triggering activation of cytotoxic, proliferative, and/or secretory responses (27, 28). Thus, NK cell activation is similar to T cell signaling in that both cells use comparable signaling cascades.

T cell activation is a complex process that involves activation through the TCR and a second receptor, a costimulatory receptor, which by the simplest definition, potentiates signals from the TCR. Experiments using anti-TCR Abs and APCs showed that stimulation through TCR alone is insufficient for activation of naive T cells (29). Full activation requires simultaneous engagement of the TCR and early; sNKG2D, soluble NKG2D; LAK, lymphokine-activated killer; CHO, Chinese hamster ovary.; dhfr, dihydrofolate reductase.

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3 Abbreviations used in this paper: NKC, NK gene complex; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; PI3, phosphatidylinositol 3; PIG, pMX-IRES-GFP; RAε, retinoic acid inducible

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COSTIMULATION BY NKG2D

Like T cells, NK cells may also use costimulatory pathways, but until recently, it has not been feasible to dissect costimulation on NK cells because the nature of activation receptors, the molecules responsible for primary activation, was unclear. For example, the T cell costimulatory receptor, CD28, and its ligands, B7.1 and B7.2, appear to play a role in NK cell activation against tumor targets (39, 40). Despite this, it was unclear which activation receptor on an NK cell was costimulated by CD28.

One recently characterized activation molecule expressed on both human and mouse NK cells is the lectin-like NKG2D molecule (41–44). Human NKG2D recognizes the stress-inducible MIC family (MICA and MICB) (43). In addition, it interacts with the ULBP family of molecules (44). Mouse NKG2D binds the minor histocompatibility molecule H60, as well as the retinoic acid-inducible RAE1 family (RAE1α, -β, -γ, -δ, -ε) (44, 46, 47). Mouse and human NKG2D trigger NK cytolytic killing of target cells transfected to express NKG2D ligands.

NKG2D, however, may not directly stimulate NK cell killing because in previous studies other activating receptors may have contributed to target killing. Moreover, unlike other activating NK receptors, NKG2D does not appear to signal through ITAM-containing signaling chains. Instead, it selectively pairs with KAP/DAP10, which can recruit the p85 subunit of PI3-kinase through the YxxM motif contained in its intracellular domain (8, 49). This signaling motif is related to a similar functional motif contained in the cytoplasmic domain of CD28, suggesting NKG2D may costimulate rather than directly activate effector function. Recent studies indicate that human T cells may use NKG2D for costimulation (43, 50), and biochemical results on human KARAP/DAP12 and KAP/DAP10 have suggested that these two signaling chains may function cooperatively (51). However, other studies on NKG2D suggest that it is a primary activation receptor (47, 52, 53). In addition, whether the signal from NKG2D can cooperate with the signals originating from NK-activating receptors associating with the signals originating from NK-activating receptors associating with ITAM-containing signaling chains.

Materials and Methods

Animals

C57BL/6 (B6), A, BALB/c, DBA/2, and B6.129P2S-FCGR3 TM1SJV (CD16-/-; C57BL6 background) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Armenian hamsters were housed by then, respectively. The resulting chimera sequence was sequenced on both strands and removed from pBluescript using Sall and Xbal restriction enzymes and ligated into pHJAP-renio-neo-dfr vector (54). This chimera construct was transfected into CHO-dfr-/- cells using lipofectamine (Life Technologies), and individual clones were obtained by limiting dilution. Protein expression of the chimera molecule by CHO clones was detected by Western blot analysis of cell lysates obtained from each individual cell clone using the 1298 rabbit antiserum, which recognizes the Ly-49A cytoplasmic domain (57). CHO clone F11 (CHO.F11) expresses a high level of the chimeric Ly-49A-NKG2D molecule, while clone E7 (CHO.E7) does not.

Isolation by RT-PCR and sequencing of NKG2D cDNA alleles

Total RNA was prepared from lymphohime-activated killer (LAK) cells using TRizol (Invitrogen, San Diego, CA), according to the manufacturer’s instructions, and was quantitated by UV spectrophotometer. RT-PCR was conducted using the Superscript One-Step RT-PCR kit (Life Technologies), according to the manufacturer’s instructions, using the following NKG2D-specific primers: EcoRI-containing 5-TAGGAATTTCTATTCCAGGAGACTACGGCA-3' and 5-AGGAATCTATGAGGAGCCGTCACTC-3'. Both PCR fragments were then sequentially cloned into pBluescript (Stratagene, La Jolla, CA) using Sall and EcoRI, and EcoRI and Xbal, respectively. The resulting chimera molecule was sequenced on the 1298 rabbit antiserum, which recognizes the Ly-49A cytoplasmic domain (57). CHO clone F11 (CHO.F11) expresses a high level of the chimeric Ly-49A-NKG2D molecule, while clone E7 (CHO.E7) does not.

Chinese hamster ovary (CHO)-dihydrofolate reductase (dhfr') cells were previously described (54). B cell hybridomas were grown in D10 medium containing 10% FCS, 5% L929 (American Type Culture Collection (ATCC, Manassas, VA)) and grown in D10 medium. The YAC-1 lymphoma line was obtained from ATCC, and the IL-3-producing X63Ag8-653 was kindly provided by H. Karasuyama (Tokyo, Japan). Both cell lines were grown in R10 medium (RPMI containing 10% FCS, 2 mM glutamine, 100 μM penicillin, 100 μg/ml streptomycin, and 100 μM 2-ME (Life Technologies, Carlsbad, CA) at 37°C in 5% CO2. The LL2 Lewis lung carcinoma line and Daudi B cell lymphoma line were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in D10 medium. The YAC-1 lymphoma line was obtained from ATCC, and the IL-3-producing X63Ag8-653 was kindly provided by H. Karasuyama (Tokyo, Japan). Both cell lines were grown in R10 medium (RPMI containing 10% FCS, 2 mM glutamine, 100 μM penicillin, 100 μg/ml streptomycin, and 100 μM 2-ME). BA/F3 cells obtained from M. Thomas (Washington University) were grown in D10 containing 10% supernatant from spent X63Ag8-653 cell cultures. S19 insect cells were kindly provided by G. Broze (Washington University) and grown in medium (Life Technologies) supplemented with 10% FCS, H15 insect cells were obtained from J. Sadler (Washington University) and grown in Express5 medium (Life Technologies) with 9 mM glutamine.

Adult and neonatal splenocytes and thymocytes were prepared by passing spleen and thymus tissues through a 70-μm cell filter (BD Biosciences, Lexington, KY) and lysing the RBCs, as described previously (55). For IL-2-activated NK cells, C57BL/6, B6, BALB/c, A, and DBA/2 mice, nylon wool nonadherent cells were cultured in 1000 U/ml human IL-2 (Chiron, Emeryville, CA) for 3–4 days. Plastic nonadherent cells were discarded, and the remaining adherent cells were cultured in 1000 U/ml IL-2 for another 3–4 days before being harvested with EDTA (Versene; Life Technologies). The resulting culture resulted in an NK cell population that was typically 90% CD3 and NK1.1 and DX5.

CDNAs, expression constructs, and transfections

The NKG2D cDNA was cloned as previously described (42). The expressed sequence tag encoding for RAE1α was obtained from Incyte Genomics (St. Louis, MO; accession A1785864) and cloned into the pMX-ires-GFP (PIG) retrovector (kindly provided by T. Kitamura, University of Tokyo, Tokyo, Japan). Full-length RAE1α was amplified using oligonucleotides (BglII-containing 5'-GGAAGATCTACCATGGCCAAGCCGAGAGTGAG-3' and Xhol-containing 5'-GGAATCTTCAGATCTAGGCTGAGAGTTGAC-3') and cloned into PIG using BamHI and Xhol. Cells were transfected to express RAE1 using FuGENE6 (Roche, Basel, Switzerland), and the retrovector supernatants obtained were used to infect Ba/F3 cells. The resulting cells were analyzed by flow cytometry for green fluorescent protein expression and cloned by limiting dilution.

The chimeric Ly-49A-NKG2D cDNA was created using PCR by amplification of intracellular domain coding sequences, cloning them into pEGFP-C3 (Clontech Laboratories, Palo Alto, CA) containing an IRES-GFP (pIG) (kindly provided by T. Kitamura, University of Tokyo, Tokyo, Japan). Full-length RAE1α was amplified using oligonucleotides (BglII-containing 5'-GGAAGATCTACCATGGCCAAGCCGAGAGTGAG-3' and Xhol-containing 5'-GGAATCTTCAGATCTAGGCTGAGAGTTGAC-3') and cloned into PIG using BamHI and Xhol. The resulting constructs were then sequentially cloned into pBluescript (Stratagene, La Jolla, CA) using Sall and EcoRI, and EcoRI and Xbal, respectively. The resulting chimera molecule was sequenced on both strands and removed from pBluescript using Sall and Xbal restriction enzymes and ligated into pHJAP-renio-neo-dfr vector (54). This chimera construct was transfected into CHO-dfr-/- cells using lipofectamine (Life Technologies), and individual clones were obtained by limiting dilution. Protein expression of the chimera molecule by CHO clones was detected by Western blot analysis of cell lysates obtained from each individual cell clone using the 1298 rabbit antiserum, which recognizes the Ly-49A cytoplasmic domain (57). CHO clone F11 (CHO.F11) expresses a high level of the chimeric Ly-49A-NKG2D molecule, while clone E7 (CHO.E7) does not.
Production of soluble NKG2D (sNKG2D)

The entire extracellular domain of mouse NKG2D was cloned into the pFASTBAC vector using PCR and the sequence-specific primers (EcoRI-containing 5′-CGGAATTCGGATCTAGTACT-3′, 5′-ATGGAGCTGCGGACACGGCAGTCGTTGACACAATACTGGACTGCCGTCCCGACGTCCT-3′, and KpnI-containing 5′-GGGTTAATACGACTCACTATAGGGTGCACAATACTGGACTGCCGTCCCGACGTCCT-3′). The resulting construct contains the gp67 secretion signal, a 6-His tag, and a BirA recognition domain upstream from the intact NKG2D extracellular domain. S9 insect cells were transfected with this construct using CellFectin (Life Technologies). Primary NKG2D baculovirus was produced in S9 insect cells using the Bac-to-Bac system (Life Technologies), and sNKG2D was produced in Hi5 insect cells using secondarily amplified baculovirus. sNKG2D was purified from Hi5 insect cell supernatant using a Ni-NTA column (Qiagen). This sNKG2D was used to immunize Armenian hamsters.

Subsequently, a second sNKG2D was constructed (58). Briefly, this molecule is identical with the first described sNKG2D with a factor X cleavage site inserted between the BirA recognition site and the NKG2D extracellular domain sequence. This sNKG2D was biotinylated with N-hydroxysuccinimide-biotin (Pierce, Rockford, IL), according to the manufacturer’s instructions, tetramerized around streptavidin–PE, and used for cell-staining purposes.

Production of anti-NKG2D Abs

Armenian hamsters were inoculated i.p. with 20–100 μg sNKG2D emulsified in synthetic trehalose dicorynyololate-monophosphoryl lipid A adjuvant (Corixa, Seattle, WA) and boosted three times at 3-wk intervals. Splenic B cell hybridomas were produced by the Washington University Hybridoma Center from one Armenian hamster after FACS analysis showed that antisera from this animal stained CHO.F11, but not CHO.E7 cells. Culture supernatants from ~300 hybridomas were screened by flow cytometry for Ab binding to a cell population containing a 1:3 mix of CHO.E7:CHO.F11 cells in each tube. Culture supernatants that shifted 75% of the cells were presumed to contain Ab that specifically recognized NKG2D. This screen resulted in 17 independent hybridomas, which underwent three rounds of subcloning. Two clones, A10 and C7, were selected for further analysis.

Antibodies

The following Abs were purchased from PharMingen: anti-CD3 APC, anti-CD3 CyChrome, anti-CD3 PE, anti-CD3 PerCP, anti-CD4 PE, anti-CD8 PE, anti-CD4 FITC, anti-γδ FITC, APC PK136, PE PK136 (anti-NK1.1), and anti-TNP-1 biotin (used as hamster IgG control). Hamster IgG was purchased from ICN Biomedical, Costa Mesa, CA and used as Ab control. A10, C7, and 2.4G2 Abs were purified from spent D10 culture supernatants by ammonium sulfate precipitation (55% saturation) and affinity purification on a protein A-Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) using standard methods (59). Purified Abs were biotinylated with N-hydroxyssucinimid-biotin (Pierce), according to the manufacturer’s instructions. FITC-2C1 (anti-CD3) mAb was produced using FITC-celite (FITC isomer 1; Calbiochem, La Jolla, CA) (60).

Flow cytometric analysis

Adherent LAK cells, thymocytes, and splenocytes were washed three times in sortter buffer (HBSS without phenol red (Sigma-Aldrich, St. Louis, MO), 3% FCS, 0.05% NaN3). Thymocytes and splenocytes were then filtered through nylon mesh. Cells were incubated in spent 2.4G2 hybridoma culture supernatant to block nonspecific FcγRIII binding for 20 min before addition of Abs. For Ab staining, ~5–10×104 cells were incubated with 2–10 μg/ml Abs for 20 min, washed twice, and then incubated with secondary reagents, such as streptavidin–PE (0.2 μg/ml). Cells were gated using forward and side scatter and cell marker staining. Dead cells were excluded by gating with propidium iodide staining. A total of 1–10×104 gated events was collected from each flow cytometry sample.

51Cr release assay and redirected lysis assay

51Cr release assay was performed using 51Cr-labeled targets, as previously described (55). YAC-1 tumor targets were used as positive controls for killing. Redirected lysis assay using 51Cr-labeled Daudi cells were performed as previously described (57). Purified Abs were used at a final concentration of 10 μg/ml (redirected lysis) and 30 μg/ml (51Cr release) and incubated with effector cells for 20 min before adding targets.

Cytokine release assay

Stimulation of B6 and CD16+ LAK cells was performed as previously described (61). Briefly, 96-well Immulon-2 plates (Dynatech Laboratories, Chantilly, VA) were coated with 20 μg/ml streptavidin (Sigma-Aldrich) overnight at 4°C. Triplicate wells were then coated with biotinylated purified Ab and incubated at room temperature for 30 min. Blocking buffer (2% BSA (V fraction) in PBS; Sigma-Aldrich) was added and incubated for 1 h at room temperature. Wells were washed three times with PBS. A total of 2×103 washed LAK cells resuspended in R10 was added to each well (200 μl final vol) and incubated at 37°C for 5–6 h. For anti-CD16-blocking studies, cells were first incubated with purified soluble 2.4G2 mAb (10 μg/ml) for 15 min. For PMA/ionomycin stimulation, cells were incubated with PMA at 10 ng/ml and ionomycin at 0.5 μg/ml final concentration. Culture supernatants were harvested, diluted 1/3, and assayed for GM-CSF by ELISA (Endogen, Woburn, MA; R&D Systems, Minneapolis, MN), according to manufacturer’s instructions. In each experiment, ELISA standard curves were derived using recombinant cytokines.

Bioinformatics from Celera Genomics database

Mouse NKG2D sequences from C57BL/6 (42), 129 × 1/SvJ, DBA/1, and AJ mouse strains were compared for putative NKG2D amino acid identity between strains of mice. The data for the 129 × 1/SvJ strain were generated through use of the Celera Discovery System and Celera Genomics’ associated databases (Celera Genomics, Rockville, MD; http://celera.com).

Results

Generation of mAbs specific for mouse NKG2D

To generate reagents that would aid analysis of mouse NKG2D, we produced soluble amino-terminal His-tagged NKG2D ectodomain. The disulfide-linked dimers were purified by nickel affinity
chromatography (data not shown). Armenian hamsters were immunized with sNKG2D, and subsequent antisera stained a stable transfectant (CHO.F11) expressing the NKG2D ectodomain (data not shown). Upon fusion and screening for FACS reactivity with CHO.F11, we generated two novel mAbs, A10 and C7, which specifically recognize the mouse NKG2D transfectant (Fig. 1A). These mAbs do not stain CHO cells expressing distantly related lectin-like NK cell receptors CD94/NKG2A and CD94/NKG2C by FACS analysis (data not shown). In addition, they compete with each other for binding to CHO.F11, as assessed by FACS, indicating that they may share similar epitopes or block each other by steric hindrance (data not shown).

NKG2D is also expressed on C57BL/6 IL-2-activated (LAK) cells, indicating that the native conformation of NKG2D can be recognized by the A10 and C7 mAbs (Fig. 1B). These results are consistent with previously published data using polyclonal antisera that recognized NKG2D, which were performed without controlling for potential FcγRIII-mediated (nonspecific) binding (62).

Expression of NKG2D on all NK cells and a subpopulation of splenic NK T cells

NKG2D is expressed on all splenic and liver NK cells (NK1.1⁺/CD3⁻), ~50% of splenic NK T (NK1.1⁺/CD3⁺) cells, and a minor population of NK1.1⁺/CD3⁻ cells by flow cytometry using mAbs A10 and C7 (Fig. 2A). In the thymus, NKG2D is expressed on NK.1.1⁺ thymocytes, but not on CD4⁺ or CD8⁺ single-positive cells, CD4⁺CD8⁺ double-positive cells, or γδ T cells (data not shown). In addition, NKG2D expression on mouse NK cells can be detected as early as day 1 postpartum (Fig. 2B). This is in contrast to Ly-49 receptors, which have been shown in previous studies (57) to be undetectable in neonatal NK cells wk 1 postpartum, suggesting that the function of NKG2D may differ from that of Ly-49.

**FIGURE 2.** FACS analysis of NKG2D expression on C57BL/6 spleen and liver cells. A, FACS plot of C57BL/6 NK1.1⁺/CD3⁻ (NK) and NK1.1⁺/CD3⁺ (NKT) splenocytes stained with mAb A10. As indicated, the x-axes show control mAb and anti-NKG2D mAb staining, and the y-axes show NK1.1 staining. These results are representative of three independent experiments. B, FACS analysis of day 1 and day 21 postpartum and adult C57BL/6 liver NK cells (NK1.1⁺/CD3⁻) with mAb A10.

**FIGURE 3.** NKG2D expression on NK cells from different strains of mice. A, Splenic NK cells (gated on DX5⁺/CD3⁻), and B, IL-2-activated NK cells from BALB/c mice stained with mAb A10. C, Alignment of putative NKG2D protein sequence translated from cDNA sequences of LAK cells derived from C57BL/6 (42), A/J, and DBA/2J strains of mice and from genomic sequences derived from the 129 × 1/SvJ strain (Celera Genomics database). The NKG2D transmembrane domain is indicated by the line. The extracellular domain of this type II integral membrane receptor spans residues 90–232.
Expression of NKG2D on NK cells from disparate inbred mouse strains

In contrast to other signaling receptors on mouse NK cells, which display significant allelic polymorphism, NKG2D expression was found on BALB/c DX5+CD3- spleen cells (Fig. 3A) and LAKs (Fig. 3B), suggesting that NKG2D is expressed by all NK cells in this strain. Furthermore, the anti-NKG2D mAbs reacted with DX5+CD3- cells from a wide variety of other strains (Table I). It is unlikely that the mAbs are reacting with different molecules in these strains because there is only one NKG2D gene in the mouse genome (42) and there is minimal allelic polymorphism of NKG2D among C57BL/6, 129/cJ, BALB/c, and DBA/2J mouse strains (Fig. 3C and see below). From the full-length NKG2D sequences derived from 129 × 1/SvJ, A/J, BALB/c/J and DBA/2J, we observed little or no amino acid differences in the putative NKG2D protein sequence. For instance, the 129 × 1/SvJ NKG2D sequence is identical with the C57BL/6 sequence. In A/J, BALB/c/J and DBA/2J, there are amino acid changes at residue 10, in which a tyrosine is substituted for a histidine, and at residue 71, in which a leucine is substituted for a valine. Because there is minimal polymorphism in the putative cytoplasmic and transmembrane domains (residues 10 and 71, respectively), and no amino acid differences were observed in the extracellular domains, these data indicate that NKG2D is highly conserved between inbred strains of mice. These results strongly suggest that the molecule reacting with the mAb A10 is likely to be NKG2D for all of the mouse strains tested and that NKG2D functions similarly in different strains (see below).

Activation function of NKG2D

To explore the capacity of the anti-NKG2D mAbs to block NKG2D recognition of its ligands, we infected Ba/F3 cells with retrovirus directing expression of RAE1δ (Ba/F3-RAE1δ). Staining with a SNKGD tetramer revealed that the infected Ba/F3 cells express a high level of RAE1δ, while uninfected Ba/F3 and Ba/F3 cells expressing vector only (Ba/F3-PIG) showed nonspecific staining (Fig. 4A). The surface expression of RAE1δ on Ba/F3 resulted in a significant increase in susceptibility to lysis compared with untransfected Ba/F3 and Ba/F3-PIG in a 51Cr release cytotoxicity assay (Fig. 4B). The addition of mAb C7 blocked lysis of Ba/F3-RAE1δ to levels below that of Ba/F3-PIG by C57BL/6 LAK cells, implying mAb C7 may be interfering with the interaction of another ligand recognized by NKG2D. Meanwhile, mAb A10 did not affect lysis compared with control Ab (Fig. 4C). We obtained similar results using BALB/c LAKs, indicating that NKG2D also serves as an activating receptor in BALB/c that also recognizes RAE1δ (Fig. 4D). These results indicate differences in functional capacities of the anti-NKG2D mAbs. More importantly, mAb C7 can specifically block the NKG2D-RAE1δ interaction that otherwise enhances target killing.

Many tumors constitutively express the ligands recognized by NKG2D, as indicated by a sNKG2D tetramer and flow cytometry (data not shown) (44, 46). One such tumor is LL2, which expresses a high level of RAE1 and H60 on the cell surface. In a chromium release assay, LL2 is lysed well by C57BL/6 LAK cells. This killing is significantly blocked by the addition of mAb C7 (Fig. 5A). Comparable with the functional effects on Ba/F3-RAE1δ (Fig. 4C), the addition of mAb A10 does not block killing compared with control Ab. Interestingly, the blocking effect with mAb C7 was not always observed with other tumors that also express RAE1, such as MLg and YAC-1 (data not shown). Because natural killing involves the interaction of many NK receptors (activation and inhibitory) with their respective ligands, the contribution of NKG2D to tumor lysis may not always be readily apparent. Nevertheless, our data show that NKG2D does have a role in killing by B6 LAKs of certain tumors that constitutively express NKG2D ligands.

In redirected lysis assays, mAb A10 can stimulate NK cells to kill Daudi targets (Fig. 5B). Interestingly, mAb C7 failed to stimulate redirected lysis, again indicating different functional properties of the anti-NKG2D mAbs. Taken together, these data indicate that NKG2D can contribute to NK cell activation in target killing.

NKG2D has costimulatory function

These data and those of published studies on NKG2D function in NK cells do not distinguish between direct activation or costimulation of NK cells by NKG2D recognition of ligand. To address this issue, we stimulated NK cells on immobilized anti-NKG2D mAb. Cytokine release assays revealed that stimulation via NKG2D using immobilized mAb A10 led to production of GM-CSF by KY-1 NK clones and C57BL/6 LAK cells (data not shown and Fig. 6A). Comparable with the redirected lysis assays, neither immobilized mAb C7 nor isotype controls stimulated cytokine production (data not shown), demonstrating the specificity of the mAb A10 effect. However, the addition of affinity-purified 2.4G2 mAb inhibited mAb A10 stimulation of B6 LAKs, suggesting that stimulation by A10 may require concomitant CD16 engagement. To test this hypothesis, we were not able to produce F(ab’)2, because digestion of hamster Ig is problematic. However, when we stimulated LAKs from CD16–/– mice with immobilized anti-NKG2D alone, we failed to induce GM-CSF production (Fig. 6A). This result was not due to the inability of CD16–/– LAKs to be stimulated because the level of GM-CSF production stimulated by B6 LAKs, again reflecting differences between the anti-NKG2D mAbs in terms of functional effects in these assays and ruling out a nonspecific effect simply due to mAb binding to NK cells. In addition, similar results

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Table I. Ab staining of splenic NK cells from various mouse strains*

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* NK cells were determined by their reactivity to DX5 and CD3 Ab. +, Positive staining; −, negative staining; n/a, not available.
were obtained in analysis of Ly-49D cross-linking using mAb 4E4. Stimulation of CD16+/H11002/LAK cells using mAbs 4E4 and A10 led to significantly higher levels of GM-CSF production compared with stimulation with 4E4 alone. Thus, our data indicate that NKG2D synergizes with various activating NK receptors such as NKR-P1C, Ly-49D, and CD16, to produce a potent stimulatory signal.

Discussion

To fully activate T cells, signals must be delivered through the T cell Ag receptor and the costimulatory molecule, CD28 (35). The TCR is coupled to ITAM-associated signaling chains, which recruit and activate the Syk family tyrosine kinase ZAP-70. The relatively nonpolymorphic receptor, CD28, through recruitment of PI3-kinase, provides additional costimulatory signals through the Akt pathway. In this study, we have determined that all mature NK cells express the NKG2D receptor and that NK cell activation is potentiated by costimulation through NKG2D. In contrast to T cells, NK cells express multiple different ITAM signaling chains, and both Syk family tyrosine kinases (ZAP-70 and Syk). Whereas the contributions of the Syk family tyrosine kinases are incompletely understood, we have shown in this work that NKG2D can costimulate signals delivered by NK cell activation receptors that are coupled to KARAP/DAP12, CD3ζ, and FcεRIγ. Thus, these data strongly suggest that NKG2D may potentiate signals delivered by all known NK cell-expressed ITAM-associated signaling chains. Together with the expression of NKG2D on virtually all NK cells, our data suggest that NKG2D represents a global costimulatory receptor for NK cells.

In terms of the role of this costimulatory function in natural killing, we found that the C7 anti-NKG2D mAb blocks enhanced Ba/F3 target killing by transfection of the NKG2D ligand RAE1β. In addition, C7 partially blocks the killing of the LL/2 tumor target that constitutively expresses NKG2D ligands. These data suggest that NKG2D synergizes with the action of a primary NK cell activation receptor in the killing of Ba/F3-RAE1β and LL/2 tumors. In contrast, because mAb C7 did not always block lysis of other tumor targets expressing RAE1, it is possible that these particular tumors express ligands to trigger other primary activation NK receptors. Alternatively, in light of the known function of CD28 on T cells, CD28 may also costimulate NK cell activation, particularly because previous work has shown that it is expressed on all C57BL/6 NK cells (63). Although the relationship of NKG2D to CD28 remains to be defined, this may explain why mAb C7 only partially blocks LL/2 tumor lysis. Nevertheless, our data show that NKG2D is involved in the natural killing of some tumor targets.

FIGURE 4. RAE1β activates NK cytolysis of Ba/F3 tumor targets via NKG2D. A, sNKG2D tetramer staining (10 µg/ml) of untransfected Ba/F3 cells, Ba/F3 cells transfected with vector only (Ba/F3-PIG), and Ba/F3 transfected with RAE1β (Ba/F3-RAE1β). For each FACS plot, the dark shading indicates unstained cells, the light line indicates staining with streptavidin-PE only, and the heavy line indicates staining with sNKG2D streptavidin-PE tetramer. B, In a 51Cr release assay using C57BL/6 LAKs as effector cells, expression of RAE1β on Ba/F3 cells increased lysis compared with that of untransfected Ba/F3 and Ba/F3-PIG. C, This increase in lysis was inhibited by the addition of mAb C7, but not mAb A10. D, BALB/c LAKs also recognize RAE1β expressed on Ba/F3 cells in a 51Cr release assay.
even though it is not yet known what primary NK cell activation receptor confers specificity.

The functional differences we observed between mAbs A10 and C7 are intriguing. mAb A10 can trigger NKG2D, while mAb C7 cannot, and mAb C7 can block NKG2D recognition of RAE1/H9254, while mAb A10 cannot. These differences could be due to several factors. For example, the epitope recognized by mAb C7 may be similar to the one used by NKG2D to interact with RAE1/H9254. In addition, it is possible that mAb A10 recognizes a triggering epitope on NKG2D or perhaps is a better cross-linker of NKG2D molecules. Further studies mapping the epitope domains recognized by these two Abs should elucidate the functional differences between mAbs A10 and C7.

In contrast to other mouse NK gene complex-encoded lectin-like receptors, NKG2D is unique in that it is relatively nonpolymorphic. Furthermore, it does not belong to the NKG2 family of molecules, despite its name. It forms a homodimer, whereas the other NKG2 molecules heterodimerize with CD94 (64–66). It has just 25% amino acid identity to these molecules, comparable with the relationship of NKRPI molecules with the Ly-49 family or the general relationship of NKC-encoded molecules to the lectin superfamily. Within a family, there is ~85% amino acid identity.

For other NKC-encoded molecules, this homology can lead to significant serologic differences between mouse strains. mAbs specific for a given molecule in one strain may react with a functionally different family member in another mouse strain, as well as allelic forms. For example, Abs against activation receptors NKRPI1C and Ly-49D in C57BL/6 mice react with related inhibitory receptors in other mouse strains (67, 68), while Abs against inhibitory Ly-49A in C57BL/6 react with related activation receptors in C57L and 129/J (68, 69). These studies highlight the distinct repertoire of NK receptors expressed within a strain as well as allelic polymorphisms between strains. This is not surprising given that the known ligands for these receptors are MHC class I molecules, which include several family members and are among the most polymorphic molecules encoded in the genome.

Strikingly, despite the evolving polymorphism that characterizes the NKC genetic regions flanking the NKG2D gene (M. G. Brown, L. Nadler, A. A. Scalzo, and W. M. Yokoyama, manuscript in preparation), there is little polymorphism observed for NKG2D. NKG2D does not appear to belong to a family of related molecules because there is evidence for only one NKG2D gene by Southern analysis of genomic clones from the NKC and by bioinformatics analysis of the Celera Genomics database. Between strains, NKG2D shows minimal amino acid sequence differences between four mouse strains. These molecules most likely represent alleles with the same function because of conservation of the transmembrane and cytoplasmic domains. The charged amino acid responsible for NKG2D interaction with its signaling chain DAP10 (arginine at residue 69) is conserved in all four mouse strains. Finally, our mAbs react with all NK cells from all strains with similar levels of expression, and the extracellular domain sequences are identical. Thus, NKG2D is unique among NKC-encoded molecules in showing little polymorphism.

With respect to its own ligands, the limited polymorphism of NKG2D is somewhat surprising because the ligands for NKG2D are numerous and heterogeneous, and display significant allelic polymorphism. NKG2D binds MICA, MICB, and several ULBP molecules, whereas mouse NKG2D binds H60, and several RAE1 family members (45, 46, 52, 70). Other than an apparent null allele of H60, significant allelic polymorphisms of its ligands have not been described in the mouse (71). By contrast, the human MICA and MICB genes are the most highly polymorphic of the MHC class I loci (72, 73). Although there are differences in ligand-binding affinities to mouse NKG2D, the affinities do not correlate with ligand sequence relationships (58). One possible explanation may be that NKG2D possesses multiple ligand-binding sites. This has been suggested by the recent crystal structure of NKG2D (74).

**Figure 5.** NKG2D has activating function on NK cells. A, Lysis of RAE1-expressing LL/2 tumor target can be partially inhibited with mAb C7. B, Daudi targets can be lysed by mAb A10, but not mAb C7 in a redirected lysis assay.
COSTIMULATION BY NKG2D

Each one of these domains may provide an evolutionary advantage, again highlighting the functional importance of NKG2D.

Acknowledgments

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References


FIGURE 6. NKG2D costimulates CD16, NK1.1, and Ly-49D activation. A, C57BL/6 LAKs are stimulated by plate-bound mAb A10 (1 μg/ml final concentration). This activation is inhibited by adduction of soluble anti-CD16 (2.4G2) mAb. B, CD16−/− LAKs are not activated with A10 or C7 Ab alone (5 μg/ml). Addition of mAb PK136 (anti-NK1.1) with mAb A10 (5 μg/ml) leads to GM-CSF production at a significantly higher level compared with that of PK136 or PK136 with control Ab alone. mAb 4E4 (anti-Ly-49D)-mediated activation of CD16+/− LAKs is also augmented by addition of mAb A10, but not by mAb C7 or control Ab. For this particular experiment, the GM-CSF produced by cells stimulated with control Ab alone in the PK136 assay (left panel) was unusually high. Usually, control mAb leads to GM-CSF production at background or below (as seen in the 4E4 assay (right panel)).


