

Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages

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Natural killer (NK) cells attack tumor and infected cells, but the receptors and ligands that stimulate them are poorly understood. Here we report the expression cloning of two murine ligands for the lectin-like receptor NKG2D. The two ligands, H-60 and Rae1 β , are distant relatives of major histocompatibility complex class I molecules. NKG2D ligands are not expressed by most normal cells but are up-regulated on numerous tumor cells. We show that mouse NKG2D is expressed by NK cells, activated CD8⁺ T cells and activated macrophages. Expression of either NKG2D ligand by target cells triggers NK cell cytotoxicity and interferon- γ secretion by NK cells, as well as nitric oxide release and tumor necrosis factor α transcription by macrophages. Thus, through their interaction with NKG2D, H-60 and Rae1 β are newly identified potent stimulators of innate immunity.

It has long been established that natural killer (NK) cells attack tumor cells and cells infected with viruses and some bacteria¹. However, the molecular basis of the specific interaction between NK cells and their targets remains largely unknown. NK cell reactivity is controlled by inhibitory and stimulatory receptor interactions. Inhibitory receptors specific for major histocompatibility complex (MHC) class I molecules endow NK cells with the capacity to attack self cells that have down-regulated or extinguished expression of class I molecules². Three such inhibitory receptor families have been discovered³: the killer cell immunoglobulin-like receptors (KIR) in primates, the Ly49 lectin-like receptors in rodents and the CD94-NKG2A lectin-like receptors shared by primates and rodents. Members of these receptor families are typically expressed by overlapping subsets of NK cells. Although inhibitory receptors play a key role in regulating NK cells, stimulatory receptor interactions are believed to be crucial in initially activating them. The outcome of an NK cell–target cell interaction would be finally determined by the balance of inhibitory and stimulatory receptor interactions. Indeed, each of the aforementioned MHC-specific receptor families includes MHC-specific stimulatory isoforms³. In addition, three immunoglobulin-like receptors identified in humans, NKp30, NKp44 and NKp46, have been implicated in the recognition of various tumor and normal target cells^{4–6}. The identity of the ligands for these receptors is not known. The Lag-3 receptor, which may interact with MHC class II molecules, is implicated in the recognition of a subset of the tumor cell lines that NK cells attack⁷. Other stimulatory or costimulatory receptors, such as NKR-P1A⁸ and 2B4⁹, have also been identified but their physiological function remains to be defined.

The lectin-like NKG2D receptor has also been implicated in the activation of NK cells and some T cells. cDNA clones of the gene encoding NKG2D were first characterized by Houchins^{10,11}. The *NKG2D* gene is located next to the *NKG2A*, *NKG2C*, *NKG2E* (and *NKG2F* in human only) genes in both the human and mouse *NK* gene complex. These

other NKG2 isoforms show a high degree of sequence identity to one another and pair with a unique subunit, CD94, to form receptors specific for the nonclassical class I molecules HLA-E (human) or Qa-1 (mouse)^{3,12}. In contrast, NKG2D is only distantly related to the other NKG2 isoforms and apparently does not pair with CD94¹³. Recent evidence indicates that human NKG2D forms a homodimer that associates with the signaling subunit DAP10 on the surface of NK cells and CD8⁺ T cells¹⁴. Human NKG2D has been shown to bind to MICA on target cells. The MHC-encoded proteins MICA and the closely related MICB are distantly related to MHC class I proteins¹⁵. MICA and MICB are normally expressed on a subset of intestinal epithelial cells, but their expression is up-regulated by cellular stress¹⁶. MICA and MICB expression were strongly up-regulated on a large number of different human epithelial tumor cell isolates¹⁷. These results suggest that human NKG2D represents one of the stimulatory receptors that NK cells employ to attack tumor cells and stressed cells.

Although mouse NKG2D is very similar to human NKG2D, no MIC homologs have been identified in mice. Here we report the isolation and functional characterization of two distinct ligands for mouse NKG2D, both of which are encoded on mouse chromosome 10 and are distantly related to MHC class I molecules. They are not expressed by most normal cells but are up-regulated on numerous tumor cells. Furthermore, we show that mouse NKG2D is expressed on freshly isolated and activated NK cells, on activated macrophages and on activated CD8⁺ T cells. Engagement of NKG2D by either ligand is a potent activation signal for NK cells and macrophages.

Results

Expression of mouse NKG2D ligands

To identify the ligand(s) for mouse NKG2D (mNKG2D), we generated fluorescently labeled, soluble tetrameric complexes of the extracellular domain of mNKG2D. The tetrameric mNKG2D stained the standard

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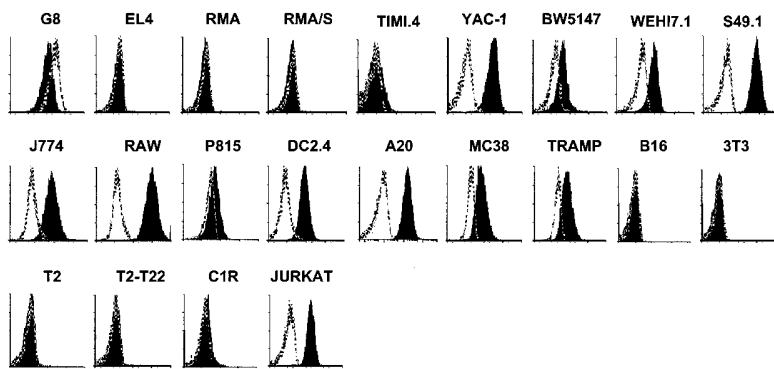


Figure 1. Numerous tumor cells express ligands for mNKG2D. A panel of mouse (upper two rows) and human cell lines (lower row) were analyzed by flow cytometry for their expression of ligands for mouse NKG2D. Cell lines were stained with tetrameric NKG2D (filled histogram) or with a control T22-tetramer (dashed histogram). The BW5147-derived G8 hybridoma expresses (as does BW5147) a ligand for NKG2D, and also binds the T22 tetramer as it expresses a T22-specific $\gamma\delta$ T cell receptor. One representative experiment of three is shown.

NK target cell line YAC-1 brightly (**Fig. 1**). The tetramers also stained three other T lymphomas (BW5147, WEHI7.1 and S49.1), both macrophage lines tested (J774 and Raw309Cr.1), the P815 mastocytoma cell line (weakly), a dendritic cell line (DC2.4), a B lymphoma (A20), a colon carcinoma (MC38) and a prostate carcinoma (TRAMP). In addition, the mNKG2D tetramers were bound by the human Jurkat T cell line, but did not react with the human T2 and C1R cell lines (**Fig. 1**). No significant staining was observed in the case of T lymphomas from B6 mice (EL4, RMA and RMA/S), the B16 melanoma line or 3T3 fibroblasts. No staining of any of these cell lines was observed with a control tetramer, prepared from the class Ib molecule T22¹⁸. As a positive control the T22 tetramer, however, did stain a T hybridoma (G8) that expresses a T22-specific T cell receptor (**Fig. 1**)¹⁹. G8 was also stained by the NKG2D tetramer, as it is derived from the BW5147 T cell line.

T22 had been considered a good candidate for an mNKG2D ligand, due to similarities in its structure to MICA and MICB molecules²⁰. However, we observed no reactivity of tetrameric NKG2D with T22-transfected T2 cells that expressed high levels of T22 (**Fig. 1**). We also could not detect any binding of the T22 tetramer to stable transfectants

expressing high levels of mNKG2D or to various mNKG2D⁺ NK cell populations, that is NK1.1⁺ splenocytes, lymphokine-activated killer (LAK) cells and different murine NK clones (data not shown).

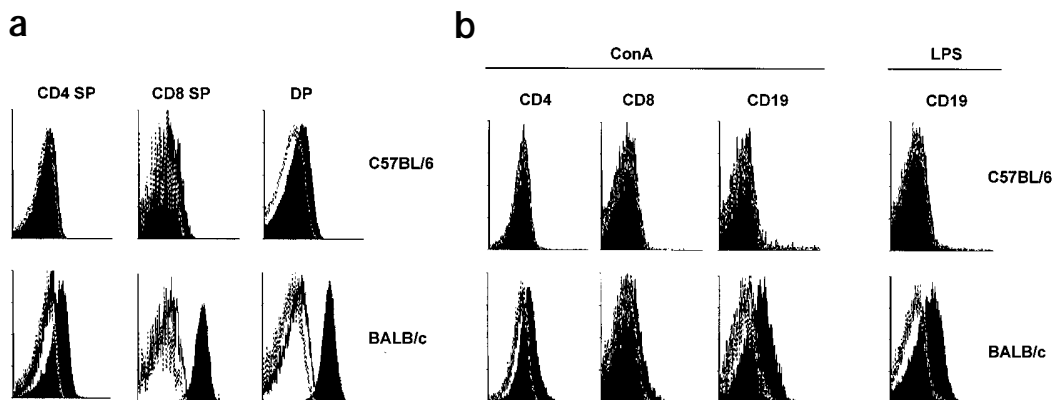
Among normal cells, mNKG2D tetramers stained most thymocytes from BALB/c mice, but did not significantly stain thymocytes from C57BL/6 mice (**Fig. 2a**). The strongly positive cells in BALB/c mice represented most CD4⁺CD8⁺ and CD4⁺CD8⁻ thymocytes, whereas CD4⁺CD8⁻ thymocytes stained weakly. Staining of BALB/c thymocytes was blocked by inclusion of an excess of unlabeled NKG2D tetramers in the staining reaction, demonstrating the specificity of the interaction (**Fig. 2a**). In contrast, freshly isolated splenic T and B cells from both strains failed to stain significantly with NKG2D tetramer (data not shown). After stimulation of BALB/c splenocytes with either ConA or lipopolysaccharide (LPS), CD19⁺ B cells were significantly stained with the NKG2D tetramer (**Fig. 2b**). CD4⁺ T cells from BALB/c also up-regulated NKG2D ligands after stimulation with ConA whereas CD8⁺ T cells did not detectably

bind NKG2D tetramer (**Fig. 2b**). This staining could be blocked by preincubation of the lymphoblasts with unlabelled NKG2D tetramer. We did not detect any staining of these lymphoblast populations with the control tetramer (**Fig. 2b**). C57BL/6 lymphoblasts were not stained by the NKG2D tetramer (**Fig. 2b**). Thus, CD4 single positive (SP) and double positive (DP) thymocytes and activated lymphoblasts (CD4⁺, CD19⁺) from BALB/c mice express ligands for NKG2D whereas C57BL/6 cells do not.

Expression cloning of two mouse NKG2D ligands

To clone the ligand(s) for mNKG2D, we screened a cDNA expression library prepared from a positively staining cell line, the BALB/c-derived J774 macrophage line²¹. Five confirmed positive clones were isolated. These five clones encoded two distinct and previously described cDNAs of unknown function: two clones encoded H-60²², a minor histocompatibility antigen, and three clones encoded the retinoic acid early transcript (Rae)-1 β ^{23,24}. The gene encoding Rae1 β , *Rae1b*, is one member of a family of three highly related genes, *Rae1a*, *Rae1b* and *Rae1c*, isolated based on the inducibility of one member (*Rae1a*) in F9 embryocarcinoma cells by retinoic acid.

Figure 2. BALB/c thymocytes and stimulated splenocytes express ligands for mNKG2D. (a) Freshly isolated thymocytes from C57BL/6 and BALB/c mice were stained with monoclonal antibodies to CD4 and CD8 and with the streptavidin-phycoerythrin (PE)-complexed NKG2D tetramer (filled histogram). Staining with an irrelevant T22-tetramer (solid line) and blocking of the NKG2D-tetramer staining (dashed line) with an excess (molar ratio 5:1) of unlabeled tetramer (multimerized with streptavidin alone) was performed to show the specificity of the staining. The histograms show electronic gating on the designated cell populations. One representative experiment out of three is shown. (b) Splenocytes from C57BL/6 and BALB/c mice were stimulated for 72 h with ConA or LPS and stained with monoclonal antibodies to CD4, CD8 and CD19 and with streptavidin-PE-complexed NKG2D tetramer (filled histogram). Staining with an irrelevant T22-tetramer (solid line) and blocking of the NKG2D-tetramer staining (dashed line) with an excess (molar ratio 5:1) of unlabelled tetramer (multimerized with streptavidin alone) was performed to show the specificity of the staining. The histograms show electronic gating on the designated cell populations. One representative experiment out of four similar experiments is shown.



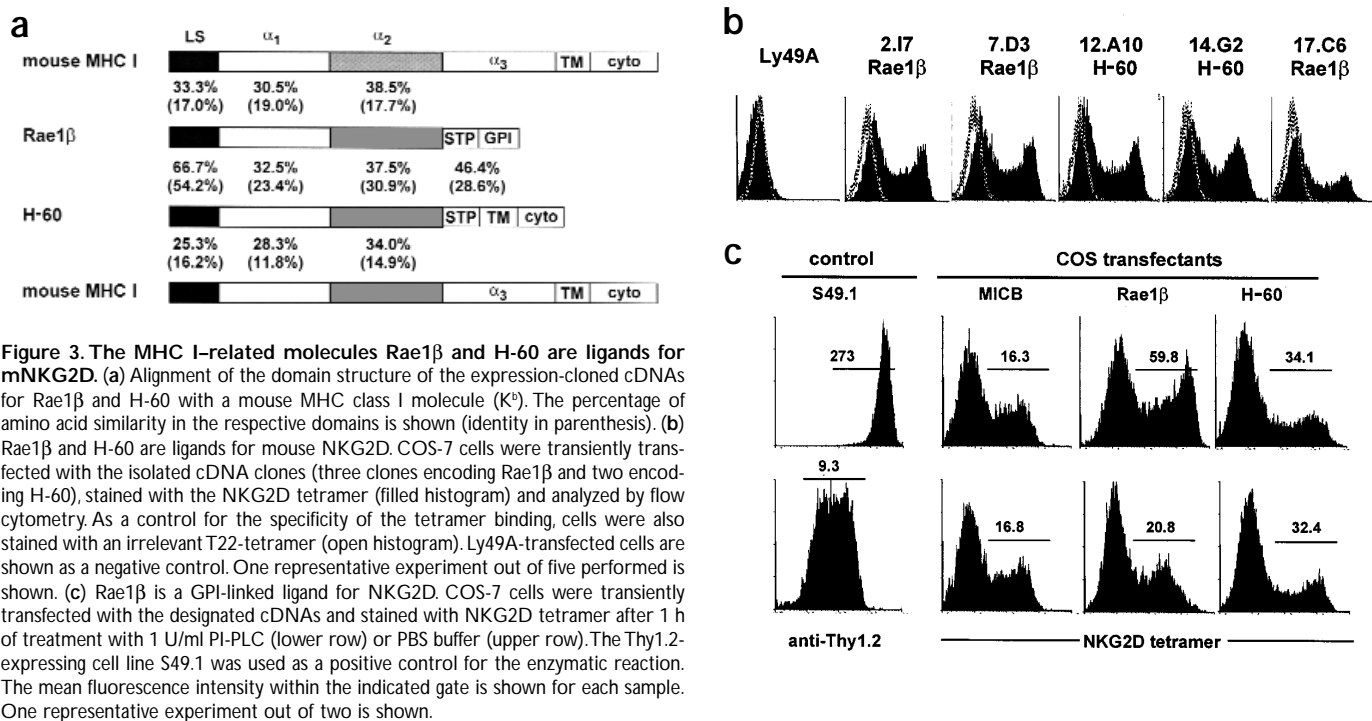


Figure 3. The MHC I-related molecules Rae1 β and H-60 are ligands for mNKG2D. (a) Alignment of the domain structure of the expression-cloned cDNAs for Rae1 β and H-60 with a mouse MHC class I molecule (K^b). The percentage of amino acid similarity in the respective domains is shown (identity in parenthesis). (b) Rae1 β and H-60 are ligands for mouse NKG2D. COS-7 cells were transiently transfected with the isolated cDNA clones (three clones encoding Rae1 β and two encoding H-60), stained with the NKG2D tetramer (filled histogram) and analyzed by flow cytometry. As a control for the specificity of the tetramer binding, cells were also stained with an irrelevant T22-tetramer (open histogram). Ly49A-transfected cells are shown as a negative control. One representative experiment out of five performed is shown. (c) Rae1 β is a GPI-linked ligand for NKG2D. COS-7 cells were transiently transfected with the designated cDNAs and stained with NKG2D tetramer after 1 h of treatment with 1 U/ml PI-PLC (lower row) or PBS buffer (upper row). The Thy1.2-expressing cell line S49.1 was used as a positive control for the enzymatic reaction. The mean fluorescence intensity within the indicated gate is shown for each sample. One representative experiment out of two is shown.

The Rae1 mRNAs were not detected in various adult tissues²³. Rae1 transcripts, most of which were identified as Rae1 β and Rae1 γ mRNAs, were detected throughout the early embryo (at day 9 days post coitus) and at significant levels in the brain/head region of 10- to 14-day-old embryos^{24,25}. This pattern of expression, along with the induction of Rae1 α transcripts in F9 cells by a differentiation inducing signal, retinoic acid, led to the suggestion that these proteins play a role in cellular differentiation.

H-60 represents a dominant minor histocompatibility (H) antigen in the response of C57BL/6 (also called B6) T cells to BALB.B cells²². The relevant T cell epitope has been identified as the LTFNYRNL peptide located near the amino-terminus of the protein, presented by the K^b class I molecule. H-60 mRNA was detected in ConA+LPS-activated lymphoblasts from BALB.B mice but was not detected in unstimulated splenocytes. No mRNA was detected in lymphoblasts from B6 mice, raising the possibility that the gene is not expressed in this strain²². This would account for its activity as a minor H antigen in the B6-anti-BALB.B combination. Alternatively, substitutions in the region of the B6 allele that encodes the antigenic epitope could also account for the minor H activity of the BALB.B H60 allele (N. Shastri, unpublished data).

Previous studies have mapped both the H60²² and Rae1²⁴ genes to murine chromosome 10, raising the possibility that the genes comprise a related gene family. The deduced H-60 and Rae1 protein sequences are distantly related to each other, exhibiting an overall amino acid sequence identity of 25.2% and a similarity of 39.1% (Fig. 3a). Both proteins contain a hydrophobic leader segment, an extracellular segment with similarity to the α_1 and α_2 domain of MHC class I molecules (Fig. 3a), and a serine, threonine and proline (STP)-rich domain (most apparent for Rae1) preceding a hydrophobic domain. Rae1 β lacks a cytoplasmic domain whereas H-60 appears to have a short cytoplasmic tail. Both Rae1 β and H-60 are distantly related to mouse class I molecules (~30% similarity; Fig. 3a).

COS-7 cells transiently transfected with each of the five isolated cDNA clones stained brightly with mNKG2D tetramers, but not with

control T22 tetramers (Fig. 3b). Control COS-7 cells transfected with Ly49A did not stain with the NKG2D tetramers. COS-7 cells transfected with the human NKG2D (hNKG2D) ligand, MICB, also stained with mNKG2D tetramers (Fig. 3c). Jurkat cells have been shown previously to express MIC proteins, suggesting a possible explanation for the positive staining of these cells with mNKG2D tetramers (Fig. 1). Thus, Rae1 β and H-60 bind mouse NKG2D.

It has been suggested that Rae1 may be a glycosyl-phosphatidylinositol (GPI)-linked protein²⁴. To examine the GPI linkage of Rae1 and H-60, COS-7 cells were transfected with each cDNA expression construct, or with a MICB expression construct as a control. Transfected cells were incubated for 1 h with PBS buffer or with glycosylphosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme that cleaves GPI-linked proteins from the cell surface. Subsequent staining of the cells with NKG2D tetramers revealed that PI-PLC treatment of Rae1 β -transfectants reduced the staining intensity by 65.2%, whereas the enzyme had no observed effect on the H-60-transfectants or MICB-transfectants (Fig. 3c). The S49.1 cell line expressing high levels of the GPI-linked surface protein Thy1.2 was included in the assay to show the efficacy of the PI-PLC treatment. These results confirm that most Rae1 β is GPI-linked, though it remains possible that a fraction of the protein is in a transmembrane form. H-60 and MICB, in contrast, may not be GPI-linked proteins.

Cellular distribution of the mNKG2D receptor

To analyze the functional interactions of NKG2D with these newly identified ligands, we examined the expression of mNKG2D on hematopoietic cells. We prepared an antiserum to mNKG2D (anti-mNKG2D) by immunizing rats with chinese hamster ovary (CHO) cells that had been stably transfected with a tagged version of NKG2D, followed by extensive absorption of the antiserum with untransfected CHO cells. To establish the specificity of the antiserum for mNKG2D, we stained stable CHO cell transfectants expressing high levels of NKG2D or two other lectin like NK receptors (NKG2A-CD94 and

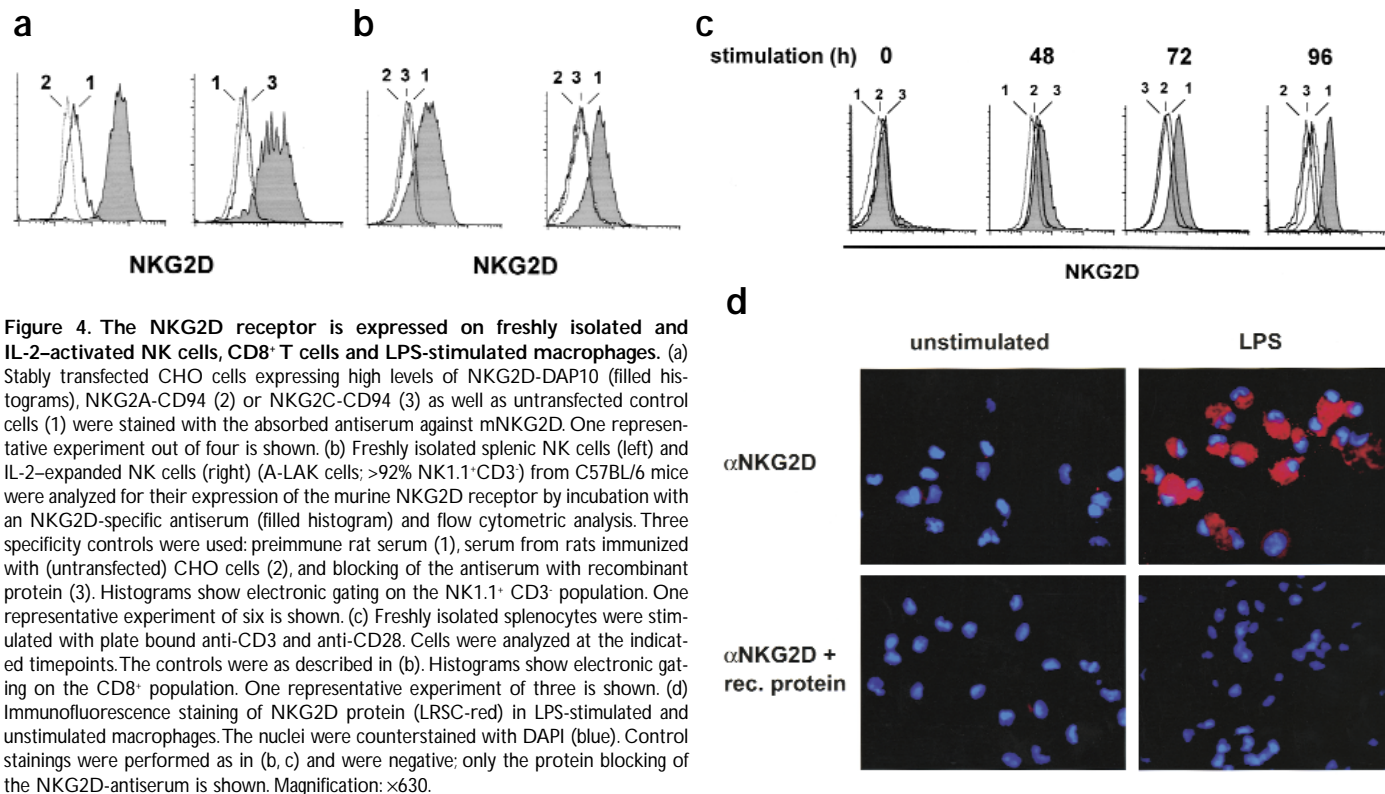


Figure 4. The NKG2D receptor is expressed on freshly isolated and IL-2-activated NK cells, CD8⁺ T cells and LPS-stimulated macrophages. (a) Stably transfected CHO cells expressing high levels of NKG2D-DAP10 (filled histograms), NKG2A-CD94 (2) or NKG2C-CD94 (3) as well as untransfected control cells (1) were stained with the absorbed antiserum against mNKG2D. One representative experiment out of four is shown. (b) Freshly isolated splenic NK cells (left) and IL-2-expanded NK cells (right) (A-LAK cells; >92% NK1.1⁺CD3⁺) from C57BL/6 mice were analyzed for their expression of the murine NKG2D receptor by incubation with an NKG2D-specific antiserum (filled histogram) and flow cytometric analysis. Three specificity controls were used: preimmune rat serum (1), serum from rats immunized with (untransfected) CHO cells (2), and blocking of the antiserum with recombinant protein (3). Histograms show electronic gating on the NK1.1⁺CD3⁺ population. One representative experiment of six is shown. (c) Freshly isolated splenocytes were stimulated with plate bound anti-CD3 and anti-CD28. Cells were analyzed at the indicated timepoints. The controls were as described in (b). Histograms show electronic gating on the CD8⁺ population. One representative experiment of three is shown. (d) Immunofluorescence staining of NKG2D protein (LRSC-red) in LPS-stimulated and unstimulated macrophages. The nuclei were counterstained with DAPI (blue). Control stainings were performed as in (b, c) and were negative; only the protein blocking of the NKG2D-antiserum is shown. Magnification: $\times 630$.

NKG2C-CD94). The absorbed antiserum specifically stained NKG2D-transfected CHO cells, but did not stain CHO cells transfected with either of the distantly related NK receptors, or untransfected control cells (**Fig. 4a**). It is unlikely that the antiserum reacts with non-NKG2D proteins because the closest known relative of NKG2D, CD94, shares only 24% amino acid identity and is apparently not stained by the antiserum (**Fig. 4a**).

Anti-NKG2D stained most, if not all freshly isolated mouse NK cells, as well as interleukin 2 (IL-2)-activated NK cells (LAK cells). No staining of NK cells was observed with a preimmune serum, or with a control antiserum raised against untransfected CHO cells. The staining of NK or LAK cells by the antiserum was specific because it was blocked by inclusion of an excess of soluble recombinant NKG2D in the staining reaction (**Fig. 4b**). We also evaluated the expression of NKG2D mRNA by quantitative, competitive polymerase chain reaction (PCR). Freshly isolated and sorted NK1.1⁺CD3⁺ cells expressed high levels of NKG2D mRNA (**Fig. 5a**). We could not detect any significant up-regulation of NKG2D transcription by the NK cell-activating cytokines IL-2, IL-12 or interferon- α and interferon- β (IFN α/β) or by prolonged culture in IL-2 (**Fig. 5a**). The data suggest that NKG2D is constitutively expressed by all mouse NK cells and its transcription is not significantly regulated by the activating cytokines IL-2, IL-12 or IFN- α/β .

The antiserum to NKG2D did not stain freshly isolated CD8⁺ (**Fig. 4c**) or CD4⁺ (data not shown) T cells. However, when CD8⁺ T cells were activated with plate-bound antibodies to CD3 and CD28 they were uniformly stained with the NKG2D antiserum, particularly after four days of culture (**Fig. 4c**). Activated CD4⁺ T cells stained poorly, if at all, with anti-NKG2D (data not shown). Analysis of NKG2D mRNA expression in sorted CD4⁺ and CD8⁺ T cells revealed the same pattern: NKG2D transcription was strongly up-regulated in CD8⁺ T cells by

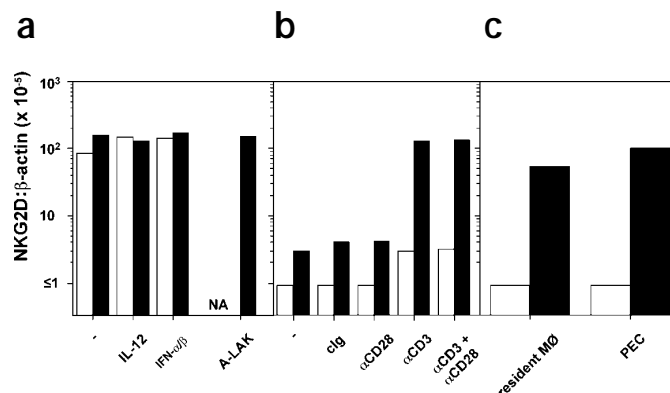
stimulation with plate-bound anti-CD3 alone or in combination with anti-CD28, whereas CD4⁺ T cells only expressed very low levels of NKG2D mRNA (**Fig. 5b**). A control antibody and anti-CD28 alone had no effect on the expression of NKG2D.

Peritoneal macrophages that were cultured on slides in the presence of LPS also stained specifically with the anti-NKG2D, and again staining could be blocked by soluble NKG2D (**Fig. 4d**). No staining was observed in unstimulated macrophage cultures (**Fig. 4d**) or in LPS-stimulated macrophages stained with either of the control sera (preimmune and CHO serum; data not shown). The results of these controls as well as the absence of staining of unstimulated cells represent strong evidence that the staining with antisera is not due to unspecific interactions with Fc receptors. NKG2D mRNA levels, determined by a quantitative, competitive reverse transcription (RT)-PCR assay in two different macrophage populations (resident peritoneal and peritoneal exudate), exactly mirrored the cell surface staining data. Unstimulated macrophages did not express detectable levels of NKG2D mRNA, whereas transcription was strongly up-regulated after 48 h of stimulation with LPS (**Fig. 5c**). Thus the data suggest that mouse NKG2D is expressed on the surface of NK cells, activated CD8⁺ T cells and LPS-activated macrophages.

Cellular activation by engagement of mNKG2D ligands

Like hNKG2D, mNKG2D lacks the inhibitory motif (ITIM) characteristic of inhibitory receptors^{26,27}. As with other lymphocyte receptors shown to provide stimulatory signals, NKG2D contains a conserved charged residue in the transmembrane region. It has been shown that hNKG2D associates with DAP10, a newly identified adapter protein involved in stimulatory signaling¹⁴. To determine whether NKG2D ligands stimulate NK cell function, COS-7 cells were transiently transfected with *Raet1b* or *H60*, stained with mNKG2D tetramers and sort-

Figure 5. NKG2D mRNA is expressed in naive NK cells, activated CD8⁺ T cells and LPS-stimulated macrophages. (a) Sorted NK1.1⁺CD3⁻ NK cells (>98% purity) were incubated with the designated cytokines for 24 h. NKG2D mRNA was quantified by competitive RT-PCR. One representative experiment of four is shown. (Filled bars, IL-2; open bars, control; NA, not applicable) (b) Sorted CD4⁺CD3⁺ or CD8⁺CD3⁺ T cells were stimulated with plate-bound antibodies for 48 h. NKG2D mRNA levels were quantified by competitive RT-PCR. One representative experiment of two. (Filled bars, CD8; open bars, CD4) (c) Resident and peritoneal exudate macrophages (MØ) were stimulated with LPS for 24 h. The levels of NKG2D mRNA were quantified using competitive RT-PCR. One representative experiment of three is shown. (Filled bars, LPS added; open bars, unstimulated.)



ed to obtain positively and negatively staining populations. As a control, COS-7 cells were transfected with Ly49A and, using an antibody to Ly49A, sorted into positive and negative fractions. After overnight culture, the various cell populations were tested as target cells for adherent LAK cells (>92% NK1.1⁺CD3⁺) and polyI:C-treated RAG-1^{-/-} splenocytes (25–35% NK1.1⁺CD3⁺). Ly49A-transfected COS-7 cells were lysed inefficiently by both NK populations (**Fig. 6a**), as were the negatively staining populations (ligand negative) from the Rae1 β and H-60 transfections (data not shown). Positively staining cells (ligand positive) from the transfections with H-60 or Rae1 β were lysed very efficiently by A-LAK cells and, to a lesser extent, by RAG-1^{-/-} splenocytes (**Fig. 6a**). In both cases, lysis was blocked to the level of the control transfectants if anti-NKG2D was included in the killing assays. Control antiserum specific for CHO cells (**Fig. 6a**) or a preimmune serum had no effect on target cell lysis (data not shown). These data indicate that both Rae1 β and H-60 induce NK cell killing via the NKG2D receptor.

To examine the effects of these newly identified ligands for NKG2D on cytokine production, activated LAK cells (A-LAK cells) and splenocytes from polyI:C-treated RAG-1^{-/-} mice were stimulated with COS-7 cells that had been transiently transfected with cDNA expression vectors for either H-60, Rae1 β or control Ly49A. Stimulation with Ly49A-transfected COS-7 cells resulted in little or no induction of interferon γ (IFN- γ). In contrast, both Rae1 β and H-60-transfected COS-7 cells stimulated substantial levels of IFN- γ secretion by A-LAK cells and RAG-1^{-/-} splenocytes (**Fig. 6b**). In both cases, IFN- γ secretion was blocked to control levels if anti-NKG2D was included in the stim-

ulation cultures. Thus, both H-60 and Rae1 β interact with NKG2D to stimulate target cell killing and cytokine production by NK cells.

To determine whether NKG2D engagement by the newly defined ligands also activates macrophages to produce nitric oxide or tumor necrosis factor α (TNF- α), we prepared ligand-expressing cell lines to use as stimulator cells. We transduced the RMA T lymphoma cell line with retroviruses that directed the synthesis of Rae1 β or H-60. The retroviruses also directed the synthesis of green fluorescent protein (GFP) from an internal ribosome entry site in the same transcription unit. Staining with the NKG2D tetramer demonstrated that transfected GFP⁺ cells also expressed high levels of NKG2D ligands compared to cells transduced with a retrovirus that encoded GFP alone (data not shown). In each case, transduced RMA cells were sorted to high purity based on GFP expression and the sorted cells were expanded for two days before using them to stimulate macrophages. Peritoneal macrophages were prestimulated for 24 h with graded doses of LPS to induce NKG2D expression and subsequently cocultured for 2 days with transduced RMA cells. In the absence of LPS activation, neither H-60-RMA cells nor Rae1 β -RMA cells stimulated nitric oxide release or TNF- α transcription. Low levels of nitric oxide production or TNF- α transcription were induced with low doses of LPS (0.02, 0.2 or 2 ng/ml), whether or not macrophages were stimulated with GFP-RMA stimulator cells (**Fig. 7a,b**). In contrast, low LPS doses (0.2 and 2 ng/ml) together with H-60-RMA or Rae1 β -RMA stimulator cells

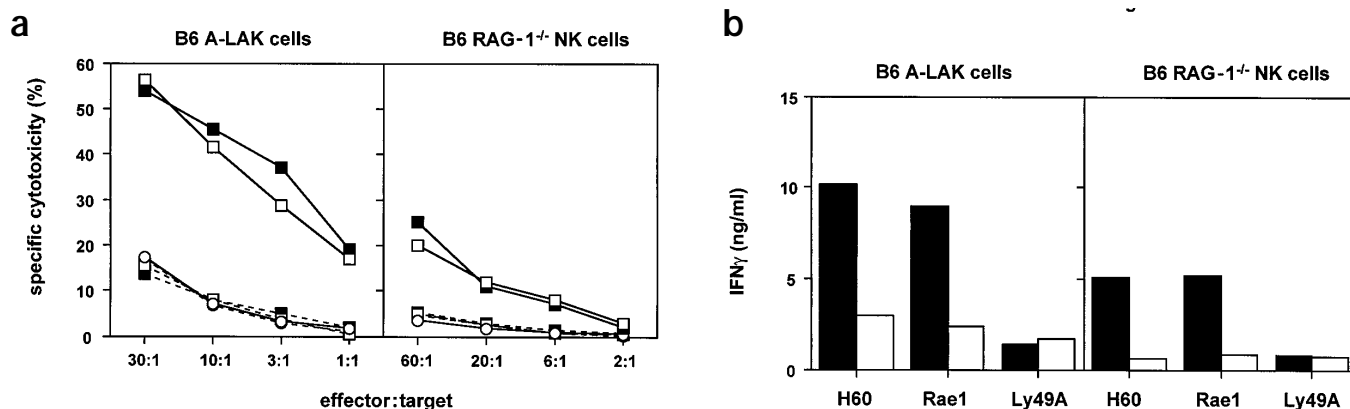


Figure 6. Expression of Rae1 or H-60 induces target cell killing and IFN- γ production by NK cells. (a) NK cell cytotoxic activity of C57BL/6 A-LAK cells (>92% NK1.1⁺CD3⁺) and freshly isolated splenocytes from RAG-1^{-/-} mice (25–35% NK1.1⁺CD3⁺) against COS-7 cells transfected with Rae1 β (filled squares), H-60 (open squares) or Ly49A (open circles). The effector cells were incubated with anti-CHO serum (solid lines) or NKG2D antiserum (dashed lines) before the standard ⁵¹Cr-release assay. One representative experiment of three is shown. (b) IFN- γ release in the culture supernatants of A-LAK cells and RAG-1^{-/-} splenocytes after 24 h of coculture with COS-7 cells transfected with Rae1 β , H-60 or Ly49A. The assay was performed in the presence of NKG2D antiserum (open bars) or CHO antiserum (filled bars) as a control. One representative of three is shown.

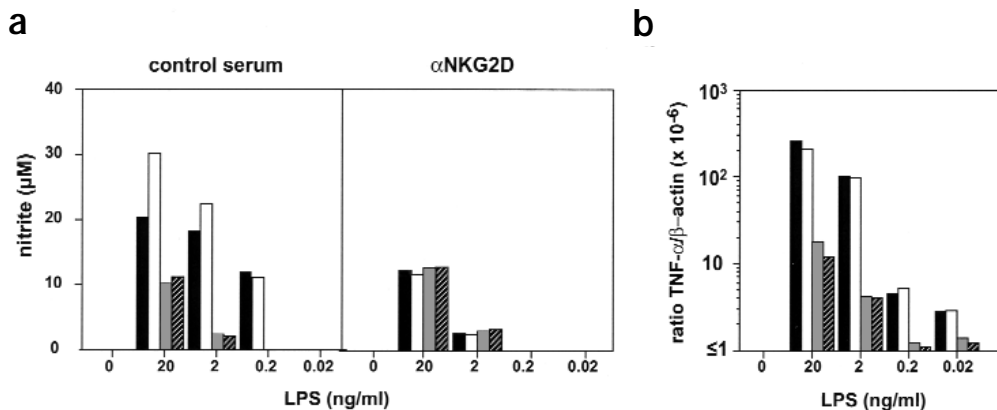


Figure 7. Expression of Rae1 or H-60 induces nitric oxide production and TNF- α transcription in stimulated peritoneal macrophages. Peritoneal exudate macrophages were stimulated with LPS (to allow for NKG2D receptor expression) and cultured alone (hatched bars) or cocultured for 48 h with RMA cells that had been retrovirally transduced with Rae1 β (filled bars), H-60 (open bars), GFP (shaded bars) (a) Nitrite was determined in the cell culture supernatants using the Griess reagent. The experiment was performed in the presence of NKG2D antiserum or a CHO control serum. One representative experiment of three is shown. (b) The induction of TNF- α mRNA expression in macrophages was determined by quantitative competitive RT-PCR. One representative experiment of two is shown.

resulted in higher nitric oxide production, which was blocked completely by the anti-NKG2D (Fig. 7a). At high doses of LPS (20 ng/ml), significant levels of nitric oxide production occurred even without NKG2D-ligand stimulation, though the levels were modestly enhanced by H-60-RMA or Rae1 β -RMA stimulator cells. Similarly, TNF- α transcription was strongly induced by H-60-RMA or Rae1 β -RMA stimulator cells, but not by GFP-RMA stimulator cells (Fig. 7b). In this case a substantial enhancement of TNF- α transcription was induced by NKG2D ligands even at high LPS doses (20 ng/ml). The RMA transductants themselves did not produce significant levels of nitric oxide or TNF- α mRNA in the absence of macrophages under any of the tested stimulation conditions (data not shown). These data show that engagement of the NKG2D receptor by either H-60 or Rae1 β synergizes with LPS to stimulate macrophage effector functions.

Discussion

Mouse NKG2D is expressed on all freshly isolated or activated NK cells, LPS-activated macrophages and activated CD8⁺ T cells. The two ligands we identified, H-60 and Rae1 β , stimulate cytotoxicity and IFN- γ production by NK cells, and TNF- α and nitric oxide production by activated macrophages. These features suggest a general role of the NKG2D-ligand system in innate immunity. The expression of NKG2D ligands by many tumor cell lines, but not most normal lymphohematopoietic cells, suggests that cellular transformation may induce NKG2D ligands. It will be interesting to investigate whether cellular infection with viruses or intracellular parasites also up-regulates NKG2D ligands. The expression of NKG2D by activated CD8⁺ T cells suggests a role for the receptor in T cell responses as well.

It is striking that NKG2D interacts with two ligand families (three, including MICB) with such disparate sequences. H-60 and Rae1 β are clearly related as they share ~40% overall amino acid sequence similarity to each other (allowing for conservative substitutions) and a similar primary structure that shows similarity to the α_1 and α_2 domains of MHC class I molecules. In addition, both genes encoding these ligands are located on chromosome 10. On the other hand, the two genes are only 25% identical at the amino acid level, and Rae1 β is GPI-linked to the cell membrane whereas H-60 may not be. In addition, the two lig-

ands clearly differ in their cell, tissue and strain distributions^{22,24}. These different properties raise the possibility that the two genes are functionally distinct in some respects. Although we consider it highly unlikely, it is formally possible that mNKG2D interacts indirectly with one or more of those ligands. In any case, it is interesting to point out that the interaction of a cell surface receptor with multiple disparate ligands is not without a precedent: an individual MHC class I molecule can interact with a T cell receptor, a CD8 coreceptor and an NK receptor. It will be useful to determine whether the various mNKG2D ligands interact with the same binding site or with different ones, as in the case of MHC proteins. Rae1 β is induced by retinoic acid in F9 carcinoma cells²³ and it

could be speculated that induction of NKG2D ligands may in some cases contribute to the well documented chemotherapeutic activities of retinoic acid²⁸. Studies have shown that Rae1 transcripts are present in the brain and other regions of early embryos, but not detectable in the normal adult²⁴. Whether the genes function at this early stage of development, and if so how, remains to be established.

The *H60* gene may be expressed in a strain-specific fashion as transcripts were found in activated spleen cells from BALB.B but not in B6 mice, whereas *H60* genomic sequences were found in both strains²². It remains possible that the gene in B6 mice is expressed in a specific tissue or is induced by yet unknown stimuli. In BALB.B mice H-60 is expressed by peripheral lymphocytes only after activation²². We show here that H-60-transduced RMA cells and LPS act synergistically to induce nitric oxide production and TNF- α transcription by macrophages. The expression of H-60 by activated lymphocytes raises questions regarding its function. One possibility is that H-60 on activated T cells is one means by which T cells can activate macrophages. This property of the NKG2D-H-60 interaction may account for why H-60 is an immunodominant antigen in the CD8⁺ T cell response of B6 mice to BALB.B grafts (N. Shastri, unpublished data).

The relationship of the new mNKG2D ligands to the hNKG2D ligands is of obvious interest. The human MICA and MICB proteins, like H-60 and Rae1 β , are distant relatives of class I MHC molecules and evidence herein indicates that mNKG2D binds to human MICB. However, the MIC proteins exhibit few other similarities to H-60 or Rae1 β . MICA and MICB proteins contain a longer membrane proximal extracellular domain, which is unrelated to those of H-60 and Rae1 β . Furthermore, although the *MICA* and *MICB* genes are encoded within the human MHC, the syntenic region of the mouse MHC contains fewer genes²⁹ and it is possible that no functional mouse orthologs of the *MIC* genes exist in this region. The *H60* and *Rae1b* genes reside relatively near each other on a different chromosome (chromosome 10) from the mouse MHC (chromosome 17). Interestingly, however, the human sequences syntenic to *H60* and *Rae1b* are located on chromosome 6 on which also resides the human MHC. Most interesting is the recent preliminary report of human genes in this region, called ULBP genes, which interact with hNKG2D. Like Rae1 β and H-60, the ULBP

proteins exhibit similarities to the α/α_2 domains of MHC class I proteins^{30,31}. The data suggest that the genes encoding Rae1 β , H-60 and ULBP may represent a newly identified subtype of functionally related genes distantly related to the genes encoding MHC. It appears likely that all these genes play significant roles in the regulation not only of NK cells but of CD8⁺ T cells and macrophages in innate immunity against transformed and infected cells.

Methods

Synthesis of NKG2D tetramers. A recognition site for enzymatic biotinylation using biotin protein ligase (BirA) was engineered to the 5' end of the entire extracellular domain of mouse NKG2D using PCR and a full length NKG2D cDNA as a template²⁶. The oligonucleotide primers were ctgagCTGAACGACATCTTCGAGGCTCAAAAAGATCGAGTG GCACAACAAGGAAGTCCAGTTTCC (the inserted recognition site for enzymatic biotinylation is underlined) and ctgagTTACACCGCCCTTTTCATGCAGATGTAC (XhoI sites in small letters). The digested PCR fragment was cloned into pET-15b (Novagen, Madison, WI). After verification of the sequence, the protein was expressed in *E. coli* BL21 (DE3)pLysS. The recombinant protein was purified from inclusion bodies and refolded *in vitro* as described^{32,33}. The refolded protein was biotinylated using BirA-Ligase (Avidity, Denver). After purification on a Pharmacia Superdex 200 gel filtration column (Pharmacia, Piscataway, NJ) to remove unbound biotin, the protein was multimerized with streptavidin-R-phycoerythrin (Molecular Probes Inc., Eugene, OR) or unlabelled streptavidin (Gibco-BRL, Gaithersburg, MD) in a 4:1 molar ratio. The MHC class Ib tetramer T22 was a kind gift of Y.H. Chien (Stanford University). For staining, the tetramers were used at a concentration of 1–10 μ g/ml.

Expression cloning and transfection. COS-7 cells were transfected with a pJFE14 expression vector³⁴—based cDNA library derived from the BALB/c macrophage cell line J774 (gift from K. Moore, DNAX)²¹ using the Lipofectamine PLUS reagent (Gibco-BRL) following the manufacturer's protocol. 48 h after transfection cells were stained with the NKG2D tetramer and the upper 0.1–2% of tetramer-binding cells were sorted on an ELITE cytometer (Beckman Coulter, Fullerton, CA). The extrachromosomal DNA was extracted from the sorted cells as described³⁵ and was used to transform competent bacteria (*E. coli* XL-1 blue). After five rounds of transfection and enrichment, COS-7 cells were transfected with pools consisting of ten individual colonies. Individual cDNA clones coding for the mouse NKG2D ligands were identified from the positive staining pools by transfecting COS-7 cells with plasmid DNA derived from single colonies. The cDNA inserts were sequenced using the following primers: pJFE-5'-ACTTCTAGGCTGTACGGA and pJFE3'-TCACTGCATTCTAGTTGTGG. The open reading frames of the isolated clones were a complete match of previously published sequences^{22,25}.

Mice, *ex vivo*-derived cell populations, cell lines, antibodies and flow cytometry. C57BL/6, B6.RAG-1^{-/-} and BALB/c mice were bred and housed under specific pathogen-free conditions in the UC Berkeley animal facilities. Thymocytes and splenocytes were prepared as described³⁶. For the lymphoblast experiments, splenocytes were incubated for 24–72 h with ConA (2.5 μ g/ml; Sigma) or LPS (25 μ g/ml; *E. coli* O55:B5; Calbiochem, La Jolla, CA). A-LAK cells were prepared from splenocytes from C57BL/6 mice, which were incubated for 4–5 days with medium containing 1 μ g/ml recombinant human IL-2 (Chiron, Emeryville, CA). Only the adherent cell population was used in the experiments and contained >92% NK1.1⁺CD3⁺ cells. Groups of five to eight B6.RAG-1^{-/-} mice were treated with intraperitoneal injections of 150 μ g poly:I:C (Sigma). Splenocytes were prepared 24 h after injection and used as effector cells in the NK cell activation assays. Peritoneal exudate macrophages (PECs) and resident macrophages were prepared as described³⁷. For *in vitro* activation of T cells, splenocytes from C57BL/6 mice were incubated on tissue culture plates with plate bound anti-CD3 (clone 500A2) and anti-CD28 (clone 37.51)—gifts of J.P. Allison, UC Berkeley—at 1 and 10 μ g/ml, respectively. Stimulated cell populations were collected after 24, 48, 72 and 96 h and analyzed by flow cytometry. The monoclonal antibodies (mAb) to CD4 and CD8 α and fluorescein isothiocyanate (FITC)-CD8 α were purchased from Caltag (San Francisco); the mAbs to mouse NK1.1, CD19, and Thy1.2 were purchased from Pharmingen. For testing the GPI-linkage of the identified clones, transfected cells were detached and washed five times in PBS buffer and then incubated for 1 h at 37°C in PBS buffer containing 1 U/ml phosphatidyl-inositol-specific phospholipase C (PI-PLC; Sigma) before staining and flow cytometric analysis. Three-color flow cytometry was performed essentially as described³⁶. The flow cytometric profiles were analyzed using the WinMDI program (John Trotter, Salk Institute, San Diego) and FloJo (Tree Star Inc., San Carlos, CA).

Preparation of the NKG2D antiserum. The antiserum to mNKG2D and the control CHO serum was generated by injecting rats with CHO cells or with CHO cells expressing DAP10 and a hemagglutinin-tagged version of mNKG2D. The rats were immunized at two week intervals a total of four times. Three days after the last boost the rats were killed and serum collected by heart bleed. The antiserum to mNKG2D was extensively absorbed on CHO cells by incubating at 4°C with 1×10^9 CHO cells, four times overnight. The specificity of the antiserum was confirmed by blocking the staining of mNKG2D by preincubation of the antiserum with recombinant soluble NKG2D protein for 1 h at 4°C.

Immunofluorescent staining of macrophages. Peritoneal exudate cells were adhered for 2 h to eight-well chamber glass slides (Nunc, Naperville, IL). Nonadherent cells were removed by extensive washing with PBS buffer. Macrophages (>98% Mac1⁺ and F4/80⁺ cells) were stimulated or not with 20 ng/ml LPS for 48 h. Cells were fixed with acetone at -20°C. Slides were blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and consecutively incubated with the NKG2D antiserum or control serum and lissamine-rhodamine conjugated donkey-anti-rat F(ab')₂ fragments (Jackson ImmunoResearch Laboratories Inc.). Nuclei were counterstained with DAPI (Molecular Probes Inc.). The slides were examined with a Zeiss Axioplan 2 using a Spot 2 camera, a $\times 63$ oil immersion objective and standard rhodamine and DAPI filters (Zeiss, Thornwood, NY). The images were analyzed and processed with the Slide Book software (Intelligent Imaging Innovations, Denver).

Quantification of NKG2D mRNA. A competitor for mNKG2D was generated by using the linker primer technique as described³⁸ using the following primers: TCCTATCACTGGGGACTGGTC (corresponding to bp 493–516 of the mNKG2D sequence²⁶; GGTTGTCTGCTGAGATGGGTAATG (corresponding to bp 701–723); and the linker primer GATGGGTAATGACCTTGACCCATAGACAGCACAGG (corresponding to bp 605–628; adapted linker site underlined). For RNA preparation, NK and T cell populations were sorted to high purity (>95%) and RNA was prepared as described before³⁷. Total cell RNA was reverse transcribed and the cDNAs were analyzed by quantitative competitive PCR as described³⁷.

Preparation of target cells. COS-7 cells were transiently transfected with expression vectors containing cDNAs encoding Ly49A (pME18S)³⁹, Rae1 β or H-60 (pJFE14) and sorted (using the Ly49 mAb, JR9 or NKG2D tetramer) to obtain positively and negatively staining populations. After overnight expansion of the sorted cell populations the stimulator cells were used in the different assays.

For the macrophage experiments, cDNA for Rae1 β and H-60 were subcloned into a MSCV-IRES-GFP retroviral vector (gift of B. Sha, UC Berkeley)⁴⁰. The GFP-encoding vector was used as a control. High-titer helper-free retroviral stocks were produced using the BOSC packaging cell line⁴¹ and were used to infect RMA cells. In initial experiments, GFP⁺ cells were shown to also stain brightly with the NKG2D tetramer. GFP⁺ RMA cells were sorted to high purity (>98%) and expanded for two days before their use as stimulators.

Determination of NK cell activity. For stimulation of NK cells, the sorted stimulator cell populations were labeled with ⁵¹Cr and tested as target cells against A-LAK cells and poly:I:C-stimulated RAG-1^{-/-} splenocytes in a standard 4 h ⁵¹Cr-release assay. Spontaneous lysis never exceeded 10%. To determine IFN- γ secretion, the sorted cell populations were seeded into 96-well plates and incubated for 24 h with the different NK cell populations. IFN- γ was determined in the cell culture supernatants by enzyme-linked immunosorbent assay (ELISA) as described³⁷. The detection limit of the ELISA was between 25–50 pg/ml.

Determination of macrophage activation. Peritoneal exudate macrophages were stimulated for 24 h with graded doses of LPS to induce the expression of the mNKG2D receptor. Retrovirally transduced stimulator cells were cocultured (at a 2:1 ratio) with the peritoneal macrophages for 48 h in 48-well (nitric oxide assay) and six-well tissue culture plates (TNF- α mRNA determination). Initial experiments showed that the transduced RMA cells alone did not produce significant levels of nitric oxide or TNF- α mRNA under any of the stimulation conditions used. After 48 h of cocultivation, cell culture supernatants were collected for the determination of NO₂⁻ using the Griess reagent as described³⁷. RNA was prepared from the macrophage populations and TNF- α mRNA was quantified by competitive RT-PCR as described³⁷ using the pMUS2 competitor plasmid (gift of M. Kopf, Basel Institute of Immunology).

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Corrigendum: T cell killing does not require the formation of a stable mature immunological synapse

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In the legend to Figure 4, the last sentence should be replaced with the following: “Curves in **b** and **d** represent the average of at least eight CTL-APC conjugates each.” The authors also regret having omitted reference to M. Faroudi *et al.* Lytic versus stimulatory synapse in cytotoxic T lymphocyte/target cell interaction: manifestation of a dual activation threshold. *Proc. Natl. Acad. Sci. USA* **100**, 14145–14150 (2003), which showed that with a human cytotoxic T lymphocyte line, lower concentrations of the antigenic peptide than were needed for mature synapse formation could nonetheless induce cytotoxicity. This should be cited on page 528, column 2, at the end of the first paragraph.

Corrigendum: Selective associations with signaling proteins determine stimulatory versus costimulatory activity of NKG2D

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Although the major conclusions of this paper, that the NKG2D immunoreceptor in mice exists in two splice isoforms that associate differentially with DAP10 and DAP12 and contribute to distinct signaling properties of the receptor in CD8 T cells as opposed to NK cells, are not affected, the authors wish to alert the community that recent experiments with macrophages do not bear out our conclusion that macrophages express easily detectable amounts of NKG2D on the cell surface. In recent repetitions of the staining experiments, we have failed to obtain clear-cut staining of activated bone marrow or peritoneal macrophages with antibodies to NKG2D. Similarly, efforts to elicit a functional response from activated macrophages by cross-linking NKG2D with antibodies to NKG2D yielded either no response or a questionable response. Low expression is still possible, because we confirmed earlier studies that activated macrophages do contain detectable mRNA for NKG2D, and it is known that these cells express the necessary adapter proteins for surface expression of the receptor.

The new data also bear on one conclusion of an earlier paper from Diefenbach, A., Jamieson, A.M., Liu, S.D., Shastri, N. & Raulet, D.H. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nature Immunology* **1**, 119–126 (2000). The conclusion in that paper that activated peritoneal macrophages express readily detectable NKG2D at the cell surface is now also in question, but the conclusions concerning natural killer cells and CD8 T cells remain unchanged.