

# The Role of the NKG2D Immunoreceptor in Immune Cell Activation and Natural Killing

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## Summary

Little is known concerning the stimulatory receptors responsible for tumor cell lysis by NK cells. We generated a monoclonal antibody specific for murine NKG2D in order to investigate its function. Blocking of NKG2D inhibited natural cytotoxicity of all tumor cells tested that express ligands for the receptor. Staining analysis showed that NKG2D is also expressed by activated CD8<sup>+</sup> T cells and macrophages, and subsets of TCR $\gamma\delta$ <sup>+</sup> and NK1.1<sup>+</sup> T cells. Contradicting reports that NKG2D is solely a costimulatory receptor, we observed that cross-linking of NKG2D directly stimulates NK cells and activated macrophages. In contrast, NKG2D costimulates activated CD8<sup>+</sup> T cells. Thus, NKG2D engagement directly stimulates NK cells and macrophages, costimulates CD8<sup>+</sup> T cells, and plays a substantial role in natural killing.

## Introduction

Natural killer (NK) cell reactivity with tumor cells and infected cells is determined by a balance between stimulatory and inhibitory receptor interactions. NK cells express inhibitory receptors from three known families that directly recognize MHC class I molecules: the killer cell immunoglobulin-like receptors (KIRs) in humans, the CD94/NKG2A lectin-like receptors found in both humans and rodents, and the Ly49 receptor family found in rodents (Moretta et al., 1996; Lanier, 1998; Raulet et al., 2001). Interactions between these inhibitory receptors and self class I molecules are important in the regulation and self-tolerance of NK cells.

The frequent downregulation of MHC class I molecules in tumor cells explains, in part, their sensitivity to NK cells. However, numerous NK-susceptible tumor target cell lines express normal levels of MHC class I molecules (Dennert et al., 1988; Correa et al., 1994), suggesting the existence of other determinants of NK sensitivity. Recently, evidence has accumulated that NK cells are also regulated by stimulatory receptors, some of which recognize ligands that are upregulated specifically by tumor cells (reviewed in Moretta et al., 2001). The best characterized example to date is the NKG2D receptor and its ligands (Diefenbach and Raulet, 2001).

NKG2D is a lectin-like stimulatory receptor originally identified in NK cells. Various families of cell surface

ligands have been identified, including the MICA/MICB and ULBP proteins (Bauer et al., 1999; Cosman et al., 2001) in humans and the Rae1 and H60 families in mice (Nomura et al., 1996; Malarkannan et al., 1998; Cerwenka et al., 2000; Diefenbach et al., 2000). All of the identified ligands are distantly related to MHC class I proteins. MICA/B and Rae1 proteins are not expressed by most normal cells in postnatal humans or mice, respectively, but are upregulated strongly in many types of tumor cells (Groh et al., 1996; Zou et al., 1996; Cerwenka et al., 2001; Diefenbach et al., 2001). In addition, MICA/B proteins are upregulated in virally infected cells (Groh et al., 2001). The upregulation of MIC and Rae1 in tumor cells suggests a role for NKG2D in stimulating NK cell responses against tumor cells. Indeed, tumor cells transduced to express high levels of NKG2D ligands are attacked by NK cells in vitro (Bauer et al., 1999; Cerwenka et al., 2000; Diefenbach et al., 2000). Furthermore, tumor cells transduced with NKG2D ligands are vigorously rejected by syngeneic mice (Cerwenka et al., 2001; Diefenbach et al., 2001) and stimulate the development of potent protective T cell immunity (Diefenbach et al., 2001). While these studies implicate NKG2D as a receptor involved in tumor cell recognition, direct evidence of its participation in natural killing of untransfected tumor cells is lacking.

NKG2D expression is not limited to NK cells. Initial reports indicated that NKG2D is expressed by all human CD8<sup>+</sup> T cells and  $\gamma\delta$  T cells (Bauer et al., 1999). In mice, studies with a polyclonal antibody and RT-PCR indicated that activated but not resting CD8<sup>+</sup> T cells and macrophages express NKG2D (Diefenbach et al., 2000). RT-PCR experiments suggest expression in murine  $\gamma\delta$  T cells in the skin (Girardi et al., 2001). Expression by various other types of  $\gamma\delta$  and  $\alpha\beta$  T cells has not been investigated. Furthermore, cell surface expression of murine NKG2D by any of these cell types has not been confirmed with monoclonal antibodies.

A key issue with respect to NKG2D function is whether it provides a stimulatory or costimulatory signal to responding cells. Analysis of human NK cells has led to the conclusion that NKG2D pairs exclusively with the signaling subunit DAP10 (Wu et al., 1999, 2000). Other NK cell stimulatory receptors pair with distinct signaling subunits such as DAP12, Fc $\epsilon$ R1 $\gamma$ , and CD3 $\zeta$ , all of which contain one or more immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic domains (Vivier and Daeron, 1997; Moretta et al., 2001). In contrast, DAP10 lacks a discernable ITAM and contains instead a YxxM motif, implicated in binding to the p85 subunit of PI-3 kinase. Indeed, tyrosine phosphorylated DAP10 was shown to coimmunoprecipitate with p85 from lysates of human NK cells (Wu et al., 1999).

The YxxM motif is also found in the cytoplasmic domain of the CD28 and ICOS molecules, where it is thought to impart a costimulatory signal as opposed to the directly stimulatory signal typically transmitted by receptors that contain ITAMs (Chambers, 2001). In line with these findings, studies of human CD8<sup>+</sup> T cells suggest that NKG2D engagement is costimulatory in con-

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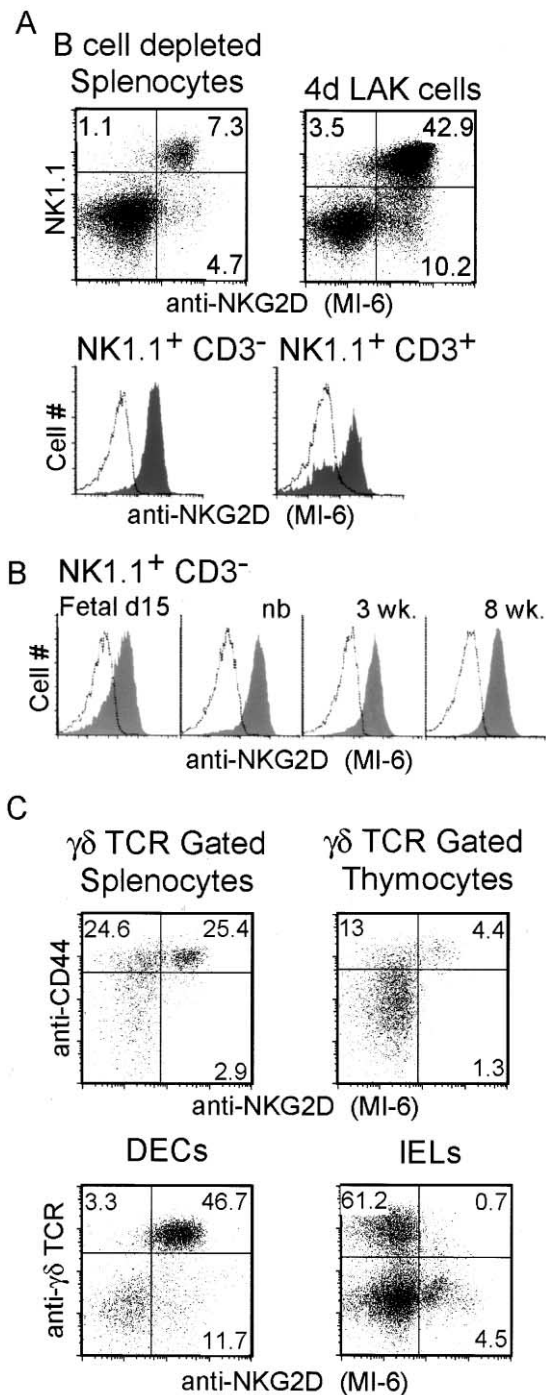


Figure 1. NKG2D Expression by NK Cells, NK1.1<sup>+</sup> T Cells, and  $\gamma\delta$  Cells

(A) Nearly all NK cells express NKG2D. Top: staining, with MI-6 mAb versus anti-NK1.1, of B cell-depleted splenocytes as a source of freshly isolated NK cells (left) or IL-2 activated NK cells (4 day LAK cells, right). Note that after 4 days in LAK culture conditions, some NK1.1<sup>-</sup> cells express NKG2D; a separate analysis (data not shown) showed that these cells were CD8<sup>+</sup>. Bottom: comparison of gated splenic NK cells (NK1.1<sup>+</sup> CD3<sup>-</sup>) and gated thymic NK1.1<sup>+</sup> CD3<sup>+</sup> cells. Representative analyses of six performed are depicted.

(B) NKG2D is expressed early in NK cell ontogeny. Gated NK cells from the E15 fetal thymus or from the spleen of newborn (nb), 3-week-old, and 8-week-old B6 mice were compared. In all cases,

junction with T cell antigen receptor engagement (Groh et al., 2001). The situation is less clear-cut in the case of NK cells. Transfection of NKG2D ligands into several NK-resistant target cell lines was sufficient to render the cells sensitive to NK cells (Bauer et al., 1999; Cerwenka et al., 2000; Diefenbach et al., 2000, 2001). Similarly, such transfected tumor cell lines were able to stimulate nitric oxide synthesis and TNF $\alpha$  transcription by activated macrophages (Diefenbach et al., 2000). NKG2D engagement may therefore signal differently in NK cells and macrophages compared to T cells, resulting in direct cellular activation. Alternatively, it is possible that the transfected tumor cells also express unknown ligands for undefined stimulatory receptors expressed by NK cells and macrophages. In this case, NKG2D engagement may provide a costimulatory signal that dramatically amplifies the response.

Here we report the derivation of a monoclonal antibody specific for mouse NKG2D. We have been able to definitively identify the cell types expressing the receptor in mice and address two important questions regarding NK cell function: the contribution of the NKG2D receptor in natural killing of tumor cells and the capacity of the receptor to directly stimulate various NKG2D<sup>+</sup> cell types.

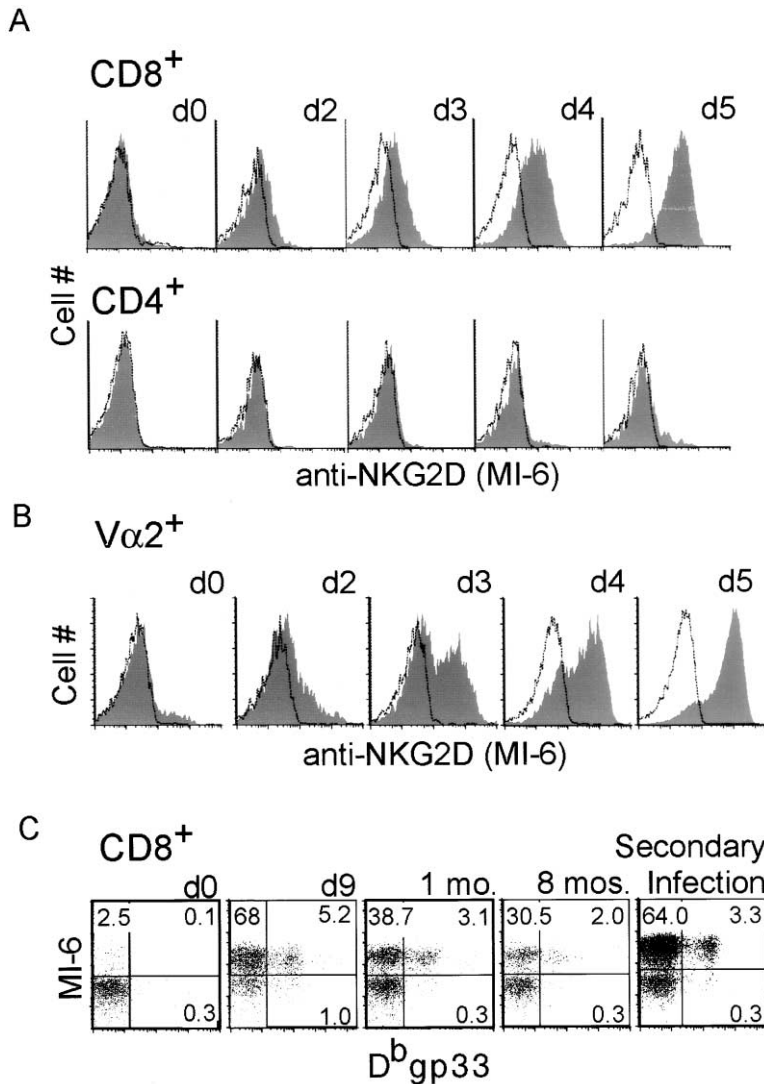
## Results

### NKG2D Expression Pattern

A monoclonal antibody specific for NKG2D, MI-6, was used to examine the cell surface expression of NKG2D on a variety of cell types. Fc receptor binding was prevented by preincubation of the cells with a FcR-specific mAb. Nearly all NK1.1<sup>+</sup> CD3<sup>-</sup> splenocytes and lymphokine activated killer (LAK) cells from adult C57Bl/6J (B6) mice expressed NKG2D (Figure 1A). Small percentages of NK1.1<sup>+</sup> cells present in the NKG2D-negative quadrants appeared, nevertheless, to express low levels of NKG2D compared to the control NK1.1<sup>-</sup> cells. Comparable staining was observed with NK cells from a variety of other mouse strains, including BALB.B, BALB/c, DBA-2/J, DBA-2Ncr, CBA/J, CD-1, 129/Tac, FVB/NJ, B10.A/SgSnJ, and B10.BR/J mice (data not shown). Substantial NKG2D expression was detected as early as day 15 of gestation on NK cells extracted from the fetal thymus, indicating that the receptor is expressed early in NK cell development (Figure 1B). The receptor was also expressed by approximately 70% of NK1.1<sup>+</sup> T cells present in the thymus (Figure 1A) or spleen (data not shown) of B6 mice. NKG2D expression occurred in a similar percentage of CD4<sup>+</sup>, CD8<sup>+</sup>, and CD4<sup>-</sup>CD8<sup>-</sup> NK1.1<sup>+</sup> T cells (data not shown).

NK cells were initially enriched by treatment with anti-CD24 and complement. A representative analysis of three performed is depicted.

(C) Selective expression of NKG2D by TCR $\gamma\delta$ <sup>+</sup> cells. Top: NKG2D expression by a subset of CD44<sup>+</sup> TCR $\gamma\delta$  cells from the spleen (left) or adult thymus (right). Bottom: NKG2D expression by TCR $\gamma\delta$ <sup>+</sup> dendritic epidermal cells (DEC) but not by TCR $\gamma\delta$ <sup>+</sup> intestinal intraepithelial lymphocytes (IEL). The 4.5% of intestinal IELs that express NKG2D but not TCR $\gamma\delta$  also express NK1.1 (data not shown). A representative analysis of five performed is depicted.



**Figure 2. Induced Expression of NKG2D by CD8<sup>+</sup> but Not CD4<sup>+</sup> αβ T Cells**

Representative results of three or four experiments performed are depicted.

(A) Spleen cells were stimulated for the indicated number of days in 96-well plates coated with 5 μg/ml anti-TCRβ mAb before analysis of NKG2D surface expression on gated CD8<sup>+</sup> and CD4<sup>+</sup> T cells.

(B) Splenocytes from OT-1 transgenic mice were stimulated for the indicated number of days with irradiated B6 splenocytes that had been pulsed with ovalbumin peptide (SIINFEKL) before analysis of NKG2D expression on gated transgene-expressing (Vα2<sup>+</sup>) T cells.

(C) At the indicated times after infection of B6 mice with LCMV, gated splenic CD8<sup>+</sup> T cells were analyzed for NKG2D expression as well as for expression of TCRs specific for the GP33/D<sup>b</sup> viral epitope (using the corresponding tetramers). For analysis of secondary infection, virus-immune mice were reinfected with LCMV and analyzed 5 days later.

MI-6 mAb stained approximately 25% of splenic γδ T cells and 5% of thymic γδ T cells (Figure 1C). The NKG2D<sup>+</sup> γδ T cells all expressed the memory/activation marker CD44, but many CD44<sup>+</sup> γδ T cells did not express NKG2D. NKG2D was expressed by a similar percentage of Vγ2<sup>+</sup> and Vγ1.1<sup>+</sup> γδ T cells, the major Vγ-defined subsets in the spleen (data not shown). Interestingly, NKG2D was expressed by essentially all γδ T cells in the skin (dendritic epidermal T cells, or DEC) (Figure 1C). In contrast, intestinal intraepithelial γδ T cells (IEL) did not express NKG2D (Figure 1C).

Freshly isolated CD8<sup>+</sup> and CD4<sup>+</sup> splenic αβ T cells from nonimmune mice did not stain with MI-6 mAb, confirming previous findings that naive T cells in mice do not express NKG2D (Figure 2A) (Diefenbach et al., 2000). After activation *in vitro* with plate-bound anti-TCR antibody, NKG2D expression was detectable on the surface of CD8<sup>+</sup> T cells within 2 days (Figure 2A). The levels continued to increase, reaching high uniform levels by the fifth day of activation. The unimodal staining pattern indicated that NKG2D was upregulated in CD8<sup>+</sup> T cells that did not previously express it. In con-

trast, NKG2D was not significantly expressed by activated conventional CD4<sup>+</sup> T cells at any time point tested (Figure 2A).

NKG2D was also upregulated by CD8<sup>+</sup> T cells activated with antigens *in vitro* or *in vivo*. Stimulation of CD8<sup>+</sup> T cells from ovalbumin peptide-specific OT-1 transgenic mice with peptide-pulsed splenocytes *in vitro* led to NKG2D expression by a subpopulation of transgene-expressing (Vα2<sup>+</sup>) T cells on day 3 of activation and by nearly all the cells by day 5 (Figure 2B). To study NKG2D induction *in vivo*, B6 mice were infected with lymphocytic choriomeningitis virus (LCMV), and antigen-specific CD8<sup>+</sup> T cell populations were examined using fluorochrome-conjugated MHC class I tetramers loaded with viral epitopes. At the peak of the immune response (day 9), nearly all CD8<sup>+</sup> T cells specific for the GP33 or NP396 viral peptides had upregulated NKG2D (Figure 2C, and data not shown). An interesting finding was that NKG2D expression remained high on virus-specific memory T cells even 8 months after infection and was retained on the virus-specific T cells responding to a secondary infection. NKG2D expression

was also induced on many CD8<sup>+</sup> T cells that did not stain with these two tetramers. Most of these cells are probably specific for other viral epitopes, though some may represent "bystander" CD8<sup>+</sup> T cells that are partially activated during LCMV infection.

To examine NKG2D expression by macrophages, we employed F(ab')<sub>2</sub> fragments of MI-6 mAb to prevent interactions with Fc receptors. MI-6 F(ab')<sub>2</sub> failed to stain bone marrow-derived macrophages that had been cultured for 1–3 days in medium alone or with the addition of TNF $\alpha$  (Figure 3A). However, macrophages that had been cultured for 1–3 days in the presence of LPS, IFN- $\gamma$ , or IFN- $\alpha/\beta$  exhibited clear staining with MI-6 F(ab')<sub>2</sub>, as compared to control polyclonal rat F(ab')<sub>2</sub> (Figure 3A). Staining was completely blocked if the MI-6 F(ab')<sub>2</sub> were preincubated with recombinant NKG2D protein. Surface expression of NKG2D was detectable after 1 day in culture and increased on days 2 and 3 of stimulation. Similar results were seen for macrophages isolated from the peritoneum after thioglycollate induction (data not shown). Accordingly, Northern blot analysis revealed that NKG2D mRNA was absent in bone marrow-derived macrophages that had been cultured with TNF $\alpha$  or no addition, but was clearly detectable in macrophages that had been cultured with LPS, IFN- $\gamma$ , or especially IFN- $\alpha/\beta$  (Figure 3B).

#### Cross-Linking of NKG2D with MI-6 mAb Activates NK Cells and Macrophages, but Not CD8<sup>+</sup> T Cells

The capacity of MI-6 mAb to stimulate NKG2D<sup>+</sup> cells was assessed. The addition of MI-6 mAb to FcR<sup>+</sup> Daudi target cells enhanced lysis of these cells by LAK cells, suggesting that the antibody mediates redirected lysis when bridged to a target cell (Figure 4A). Although these data are consistent with direct stimulation of NK cells by NKG2D, it remained possible that additional receptor/ligand interactions between the NK cell and the target cell were necessary for realization of MI-6-mediated cytotoxicity.

To investigate whether NKG2D engagement alone imparts a sufficient stimulatory signal, we determined whether cells could be activated by cross-linking NKG2D with MI-6 mAb. In a similar setting, antibodies specific for the costimulatory CD28 or ICOS receptors on T cells do not provide a sufficient stimulatory signal, but do provide signals that synergistically stimulate the cells in conjunction with engagement of the ITAM-containing T cell receptor complex (Harding et al., 1992; Hutloff et al., 1999). Incubation with MI-6 followed by cross-linking with secondary anti-rat IgG Abs resulted in a Ca<sup>2+</sup> flux in many IL-2 cultured NK cells (LAK cells); control rat IgG or the nonstimulatory DX5 mAb had no effect (Figure 4B, and data not shown). These data suggest that NKG2D cross-linking leads to Ca<sup>2+</sup> mobilization.

For functional analysis of NK cells and macrophages, it was important to avoid the possibility that the MI-6 mAb could engage Fc receptors on the cells, thus imparting a directly stimulatory signal. Therefore, we employed MI-6 F(ab')<sub>2</sub> fragments for these experiments. LAK cells or freshly isolated NK cells were stimulated to produce IFN- $\gamma$  by plate-bound MI-6 F(ab')<sub>2</sub> at coating doses of 1  $\mu$ g/ml or higher, reaching a maximum with

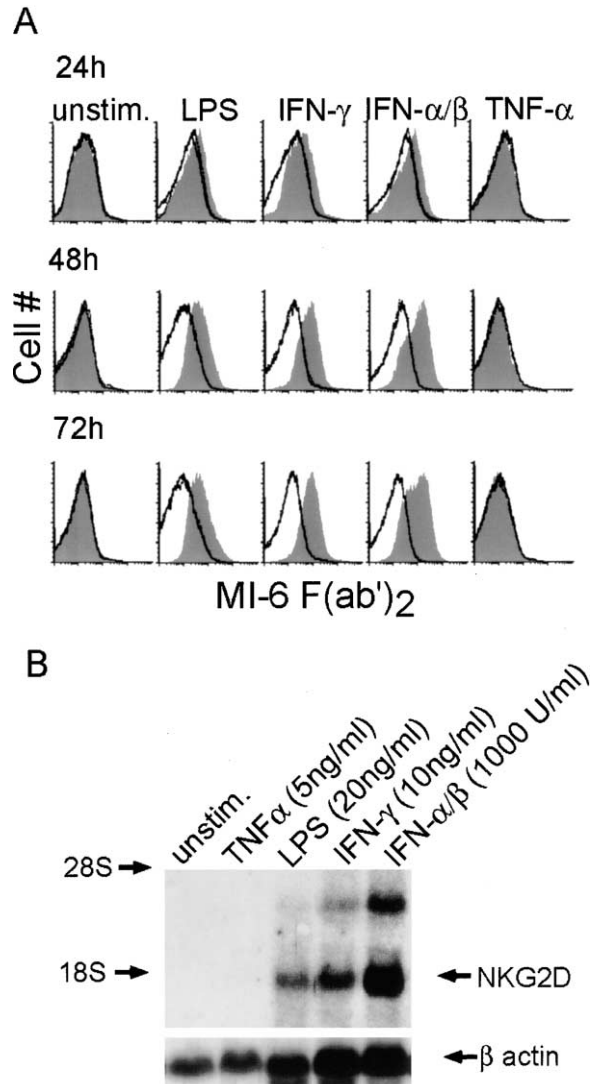
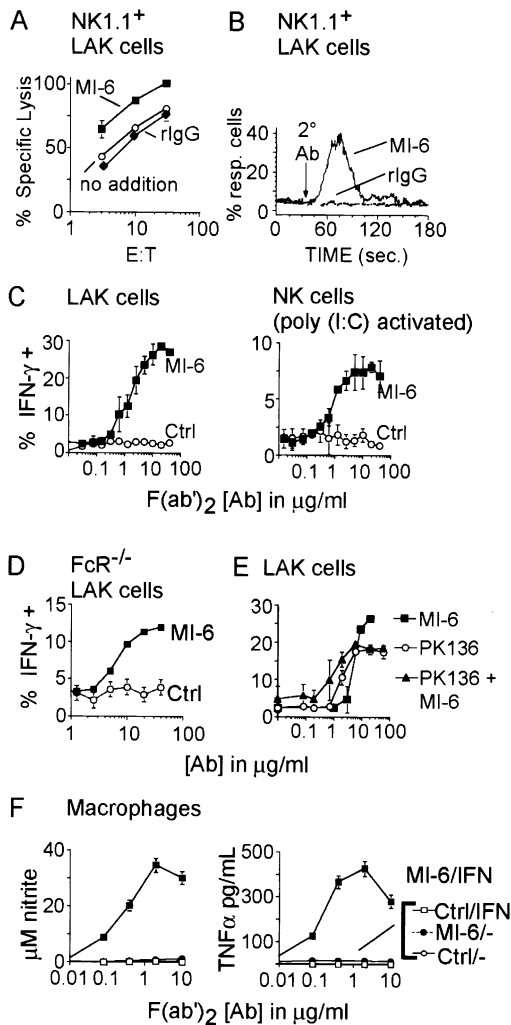


Figure 3. NKG2D Expression by Macrophages Is Induced by Interferons or LPS but Not TNF $\alpha$

(A) Bone marrow-derived macrophages (>98% F4/80<sup>+</sup> and CD11b<sup>+</sup>) were cultured without stimulation or with 20 ng/ml LPS, 10 ng/ml IFN- $\gamma$ , 1000 U/ml IFN- $\alpha/\beta$ , or 1 ng/ml TNF $\alpha$ . After 24–72 hr, the cells were examined for NKG2D surface expression using MI-6 F(ab')<sub>2</sub> fragments. Shaded, MI-6 F(ab')<sub>2</sub>; solid open, control F(ab')<sub>2</sub>; dotted open, MI-6 F(ab')<sub>2</sub> preincubated with recombinant NKG2D protein. (B) Northern blot analysis of NKG2D (top) or actin (bottom) transcripts in bone marrow-derived macrophages that had been cultured *in vitro* for 6 days with L-CSF (to stimulate macrophage proliferation), which was supplemented on the last 2 days of culture with LPS, IFN- $\gamma$ , IFN- $\alpha/\beta$ , TNF $\alpha$ , or no addition as indicated.

~10  $\mu$ g/ml (Figure 4C). Control F(ab')<sub>2</sub> had no effect, even at 40  $\mu$ g/ml. A similar response to MI-6 mAb was observed with LAK cells from mutant mice lacking the NK cell Fc receptor (Fc $\gamma$ R111<sup>-/-</sup> mice), effectively ruling out the possibility that the response was due to residual intact antibody in the F(ab')<sub>2</sub> preparation (Figure 4D).

The response of LAK cells to MI-6 mAb was similar to the response to PK136, a mAb specific for the stimulatory receptor NKR-P1C (Karlhofer and Yokoyama, 1991). Combining PK136 with a limiting dose of MI-6 F(ab')<sub>2</sub>



**Figure 4. Cross-Linking of NKG2D with MI-6 mAb Is Sufficient to Activate NK Cells and Macrophages**

Data are representative experiments of at least three performed. (A) MI-6 mAb enhances lysis of FcR<sup>+</sup> Daudi target cells by NK1.1<sup>+</sup> LAK cells in a redirected lysis assay. (B) Addition of an anti-rat F(ab')<sub>2</sub> cross-linking antibody to NK1.1<sup>+</sup> LAK cells coated with the MI-6 antibody induced rapid calcium flux. No calcium flux was seen with control antibody. (C and D) Plate-bound F(ab')<sub>2</sub> fragments of MI-6 induce IFN- $\gamma$  production by LAK cells, freshly isolated NK cells, and LAK cells from FcR<sup>-/-</sup> mice. Day 4 LAK cells or splenocytes from poly (I:C) pre-treated mice were analyzed. The cells were stained for intracellular IFN- $\gamma$  while gating on NK1.1<sup>+</sup> CD3<sup>-</sup> cells. (E) The level of stimulation with MI-6 mAb is comparable to that with PK136 (anti-NKR-P1C). Addition of limiting (3  $\mu$ g/ml) doses of MI-6 mAb to cells stimulated with PK136 enhanced the response only slightly. (F) Stimulation through NKG2D activates macrophage effector functions. Bone marrow macrophages were precultured for 2 days with IFN- $\alpha/\beta$  to induce NKG2D expression and then stimulated for another 2 days with plate-bound F(ab')<sub>2</sub> fragments of MI-6 mAb or control Ig. Secreted nitrite (indicating nitric oxide) or TNF $\alpha$  was assayed in the culture supernatants.

enhanced the response only slightly compared to the response to PK136 alone (Figure 4E), suggesting that NKG2D engagement does not provide significant costimulatory activity in conjunction with NKR-P1C engage-

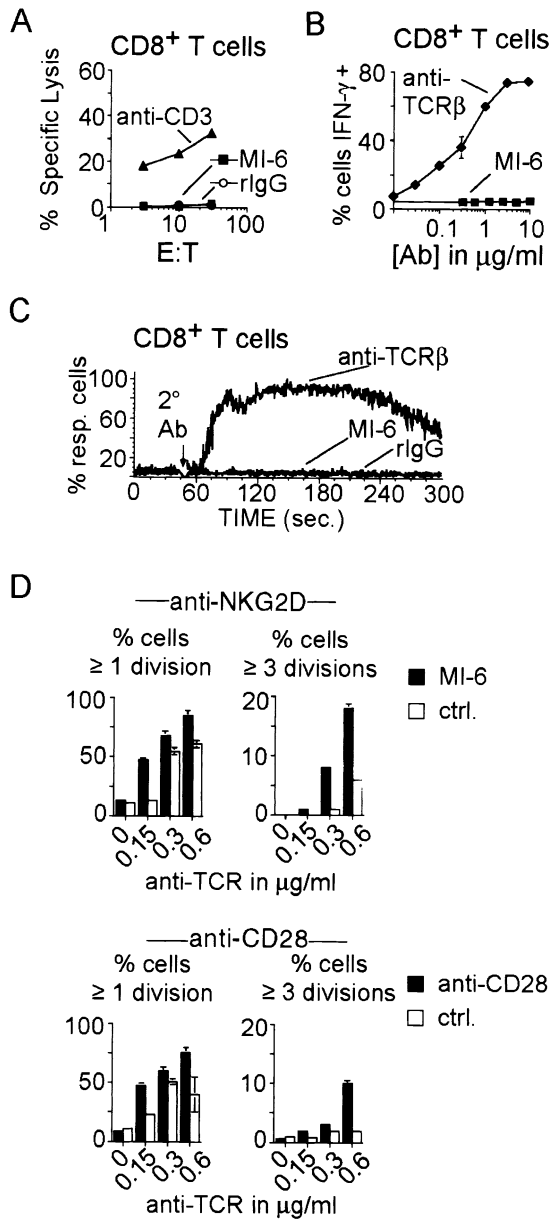
ment. For unknown reasons, the percentage of NK cells that secrete IFN- $\gamma$  in response to PK136 or MI-6 generally reached a maximum of 10%–30% in these experiments (Figure 4), and only 50%–80% of the cells responded even to pharmacologic agents (PMA plus ionomycin) in most experiments (data not shown). A substantial fraction of NK1.1<sup>+</sup>CD3<sup>-</sup> cells are therefore generally hyporesponsive to stimulation with these agents, perhaps because they are immature or anergic.

To examine the responses of activated macrophages to receptor stimulation, bone marrow macrophages were precultured alone or with IFN- $\alpha/\beta$  to induce NKG2D expression and then transferred to wells coated with MI-6 F(ab')<sub>2</sub> or control F(ab')<sub>2</sub>. Macrophage stimulation was assessed 2 days later in terms of either nitric oxide (NO) production or TNF $\alpha$  secretion. NKG2D cross-linking resulted in substantial secretion of nitric oxide and TNF $\alpha$ , whereas wells coated with control F(ab')<sub>2</sub> had no effect (Figure 4F). Control macrophages that had been cultured in the absence of IFN- $\alpha/\beta$ , and therefore did not express NKG2D, did not respond to stimulation by MI-6 mAb (Figure 4F). The requirement for receptor induction argues against the possibility that macrophage activation was due to LPS or other possible contaminants in the anti-NKG2D antibody preparation. Similar results were obtained with thioglycollate-induced peritoneal macrophages (data not shown). Cross-linking with MI-6 F(ab')<sub>2</sub> also enhanced a basal level of NO production that was induced by LPS or IFN- $\gamma$  (data not shown).

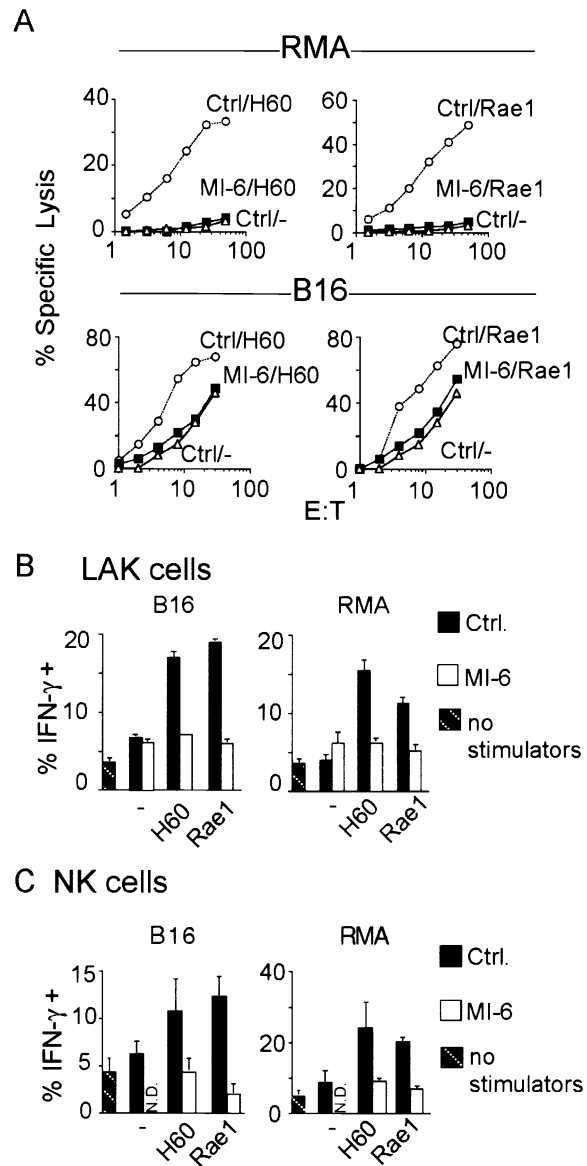
To investigate whether MI-6 mAb stimulates activated CD8<sup>+</sup> T cells, we generated a CD8<sup>+</sup> T cell line specific for the LCMV GP33 peptide. These cells expressed uniformly high levels of NKG2D (data not shown). In contrast to the results with NK cells, MI-6 failed to mediate redirected lysis of Daudi target cells by activated CD8<sup>+</sup> T cells, suggesting that NKG2D does not directly stimulate CD8<sup>+</sup> T cells (Figure 5A). Furthermore, the activated CD8<sup>+</sup> T cells did not respond to receptor cross-linking by producing IFN- $\gamma$  or by fluxing Ca<sup>2+</sup> (Figures 5B and 5C). All three types of responses were induced by control anti-CD3 or anti-TCR $\beta$  mAbs (Figures 5A–5C). In addition, cross-linking of the NKG2D receptor alone failed to stimulate proliferation of CFSE-labeled CD8<sup>+</sup> T cells (Figure 5D). The same T cells responded well to anti-CD3 mAb (Figure 5D). Although MI-6 mAb was not sufficient to stimulate CD8<sup>+</sup> T cell proliferation, it did augment the response of CD8<sup>+</sup> T cells to limiting doses of anti-TCR mAb. The enhancement in proliferation was most marked for cells undergoing three or more cell divisions. Anti-CD28 mAb tested in parallel behaved nearly identically (Figure 5D). Thus, whereas NKG2D cross-linking with MI-6 mAb provides a sufficient signal for activation of NK cells and macrophages, it provides instead an enhancing or costimulatory signal for activation of NKG2D<sup>+</sup> CD8<sup>+</sup> T cells.

#### The NKG2D Receptor Mediates Natural Killing of Tumor Cells

To test the efficacy of MI-6 mAb in functionally blocking the NKG2D-ligand interaction, we employed two tumor cell lines that do not express NKG2D ligands and compared them to retroviral transductants that express high



**Figure 5. NKG2D Cross-Linking Costimulates, but Does Not Stimulate, CD8<sup>+</sup> T Cells**  
 Representative experiments of at least two are shown. Each analysis was performed in other CD8<sup>+</sup> T cell lines with similar results.  
 (A) MI-6 antibody fails to redirect cytotoxicity of FcR<sup>+</sup> Daudi cells by a CD8<sup>+</sup> T cell line specific for the LCMV-derived peptide GP33, unlike control anti-CD3 mAb.  
 (B and C) Activated NKG2D<sup>+</sup> CD8<sup>+</sup> T cells (a line specific for LCMV) do not produce IFN- $\gamma$  (B) or flux calcium (C) in response to NKG2D cross-linking, but do respond to anti-TCR $\beta$  mAb. Nearly 40% of NK1.1<sup>+</sup> LAK cells responded in a parallel test (data not shown). A representative experiment of three performed is shown.  
 (D) NKG2D cross-linking costimulates T cell proliferation. CFSE-labeled, activated NKG2D<sup>+</sup> CD8<sup>+</sup> T cells (a line specific for LCMV) were stimulated for 4 days with plate-bound anti-TCR $\beta$  in combination with a constant dose (9  $\mu$ g/ml) of plate bound MI-6 mAb (top) or anti-CD28 mAb (bottom). MI-6 mAb enhanced CD8<sup>+</sup> T cell proliferation to a comparable extent as did anti-CD28 mAb. Costimulation is most pronounced in the case of cells undergoing three or more divisions (right panels).



**Figure 6. NKG2D Ligand-Induced NK Cell Activation Is Blocked by MI-6 mAb**  
 (A) MI-6 mAb (50  $\mu$ g/ml) blocks NKG2D-dependent killing by LAK cells of target cells transduced to express H60 or Rae1 ligands. RMA cells (top) were not killed unless the cells were transduced with NKG2D ligands, and MI-6 mAb blocked the induced level of killing completely. B16-BL6 cells (bottom) lacking NKG2D ligands were sensitive to NK cells, but H60 or Rae1 transduction enhanced their sensitivity. The ligand-dependent component was blocked by MI-6 mAb. Control Ig was added to the control assays. Standard deviations are shown, but in most cases are too small to visualize in the figure. A representative analysis of six is shown.  
 (B and C) MI-6 mAb (50  $\mu$ g/ml) blocked IFN- $\gamma$  secretion by LAK cells (B) or freshly isolated NK cells (C) induced by H60 or Rae1-transduced cells. Control Ig was added to the control assays. IFN- $\gamma$  production was visualized by intracellular staining after 5 hr of stimulation. Representative analyses of three (LAK cells) or two (NK cells) are shown. N.D., not done.

levels of the ligands. As reported previously (Diefenbach et al., 2001), RMA cells are resistant to NK cells, but RMA cells transduced with Rae1 or H60 are rendered

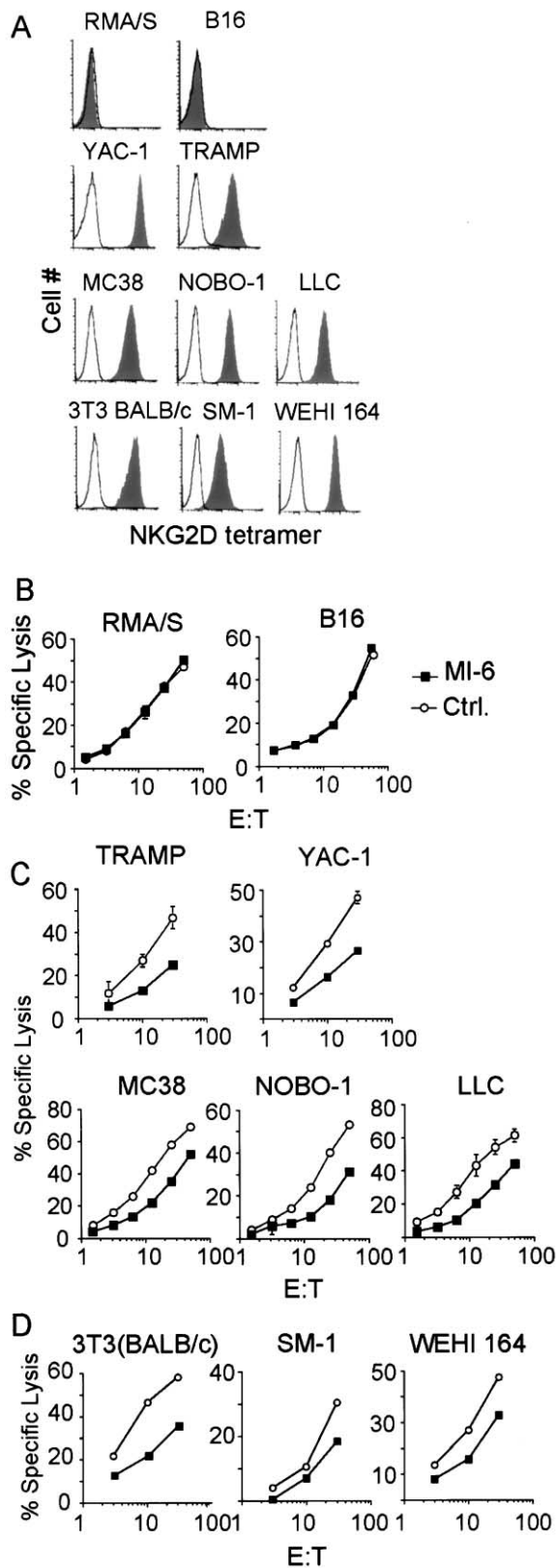


Figure 7. NKG2D Participates in Natural Killing of Target Cells that Naturally Express NKG2D Ligands

(A) NKG2D ligand expression was visualized by staining with NKG2D tetramers (shaded) versus control irrelevant tetramers (open). The

sensitive to NK cells (Figure 6A). MI-6 F(ab')<sub>2</sub> but not control F(ab')<sub>2</sub> blocked the ligand-dependent killing to background levels. B16-BL6 melanoma cells also lack NKG2D ligands, yet these cells are killed by NK cells, suggesting the involvement of other stimulatory receptor interactions (Figure 6A). Transduction of B16-BL6 with Rae1 or H60 significantly enhanced killing by NK cells, and MI-6 F(ab')<sub>2</sub> blocked the enhanced level of killing (Figure 6A). MI-6 F(ab')<sub>2</sub> also specifically blocked IFN- $\gamma$  production by LAK cells and NK cells that had been stimulated with ligand-transduced RMA or B16 tumor cells (Figures 6B and 6C). Thus, MI-6 mAb is highly efficacious in specifically blocking NKG2D-ligand interactions without interfering with other natural killing recognition systems.

Notably, untransduced B16 cells failed to stimulate significant IFN- $\gamma$  production by NK cells compared to the response with no stimulator cells, despite the fact that these cells were quite sensitive to NK killing (compare Figures 6A and 6B). This may reflect a requirement for more intense stimulation for cytokine production as opposed to killing, a phenomenon that has been previously documented with CD8<sup>+</sup> T cells (Valitutti et al., 1996).

A majority of tumor cell lines that have been previously examined express NKG2D ligands (Diefenbach et al., 2000). To detect NKG2D ligand expression, NK-sensitive tumor cell lines of B6 and BALB/c origin were stained with a previously described soluble tetrameric NKG2D reagent (Diefenbach et al., 2000). Two of the cell lines (B16 and RMA/S) failed to stain, whereas eight of the cell lines stained well with NKG2D tetramers but not with control tetramers (Figure 7A). Each cell line was tested as a target cell for NK cells in the presence of a predetermined excess dose of MI-6 mAb or control antibody. The NK cells were from B6 mice for the B6 tumor cells or from BALB/c mice for the BALB/c tumor cells. In the case of the cell lines that lack NKG2D ligands, the killing curves with MI-6 mAb and control antibody were superimposable, indicating that NKG2D binding by the antibody does not impart any inhibitory effect in the absence of ligands (Figure 7B). In contrast, partial inhibition of NK killing by MI-6 mAb was evident with all eight cell lines that expressed NKG2D ligands (Figures 7C and 7D). Addition of more antibody did not lead to greater inhibition (data not shown). The results indicate that for most tumor target cells, recognition leading to natural killing is mediated to a significant

B16 and RMA/S tumor cell lines were among the few tumor lines tested that did not express ligands for NKG2D.

(B) MI-6 mAb (50  $\mu$ g/ml) did not influence killing by LAK cells of susceptible cell lines that do not express NKG2D ligands.

(C and D) MI-6 mAb (50  $\mu$ g/ml) partially blocks lysis by LAK cells of B6-derived (C) or BALB/c-derived (D) tumor target cells that naturally express NKG2D ligands. The antibody was in excess, as this dose was sufficient to completely block lysis of transduced target cells expressing higher levels of NKG2D ligands (Figure 6). Furthermore, addition of higher antibody doses did not increase the level of inhibition (data not shown). Standard deviations are shown, but in most cases are too small to visualize in the figure. A representative analysis of three is shown.

degree by NKG2D, but that other receptor-ligand systems also play an important role. These other recognition systems presumably also account for the NK-sensitivity of certain NKG2D-ligand-negative tumor target cell lines, such as B16 and RMA/S (Figure 7).

## Discussion

Stimulatory NK cell receptors specific for MHC molecules have been characterized previously, but much evidence suggests that natural killing of most tumor target cells is mediated by non-MHC-specific receptors (reviewed in Moretta et al., 2001). Our results provide direct evidence that NKG2D and its ligands represent one such recognition system employed by NK cells to attack tumor cell lines. Excluding the MHC-specific receptors, NKG2D is the only receptor involved in natural killing for which ligands have been identified. The results also indicate, however, that additional receptor-ligand systems participate in natural killing. Studies of human NK cells have implicated several other stimulatory receptors in natural killing, including NKp46, NKp30, and NKp44 (Moretta et al., 2001; Pende et al., 2001). A functional homolog of NKp46 has been identified in mice. Evidence suggests that NKR-P1 receptors also play a role in natural killing of some tumor cells (Ryan et al., 1995). The involvement of multiple receptor-ligand systems in natural killing is reminiscent of other recognition systems of the innate immune system, such as the Toll-like receptor system, in which the various isoforms recognize different molecular patterns associated with pathogens. In the case of NK cells, however, NKG2D (and possibly the other receptors as well) recognize self-ligands that are induced by transformation, infection, and/or cellular stress.

Another significant finding of our study is that cross-linking of NKG2D with immobilized mAb is a sufficient signal to trigger NK cells and macrophages, demonstrating that NKG2D is a directly stimulatory receptor in these cell types. This in turn suggests that upregulation of NKG2D ligands by a tumor cell or other cell is likely to be sufficient for targeting of the cell by NK cells and activated macrophages. The results are not readily compatible with the notion that NKG2D delivers signals only through its interaction with a costimulatory adaptor molecule. Previous studies have shown that other costimulatory receptors containing the YxxM motif, such as CD28, fail to stimulate either  $Ca^{2+}$  mobilization or effector functions (Tsuchihashi et al., 2000). It remains possible that DAP10 is not solely involved in costimulation and can deliver directly activating signals in certain cellular contexts. Alternatively, NKG2D may interact with other signaling molecules in addition to DAP10.

In contrast to our results with mouse NK cells, it was reported that human NK cells are not directly activated by immobilized anti-NKG2D antibody (Wu et al., 2000). It is therefore possible that NKG2D signals differently in human versus mouse NK cells. Alternatively, the distinct results may reflect experimental differences, such as the use of an NK cell line in the human studies or the use of different concentrations of anti-NKG2D antibody.

Interestingly, NKG2D cross-linking was not directly stimulatory in the case of preactivated mouse  $CD8^+$

T cells, which express abundant cell surface NKG2D. Cross-linking of NKG2D did enhance proliferation of  $CD8^+$  T cells induced by T cell receptor cross-linking, in line with evidence that human NKG2D provides primarily a costimulatory signal for T cells (Groh et al., 2001). Thus, our results suggest that NKG2D signals differently in distinct immune cell types. It will be of interest to determine the molecular basis for these differences in signaling.

Results with the MI-6 mAb confirmed our earlier findings that activated mouse macrophages express NKG2D (Diefenbach et al., 2000). Furthermore, we showed that receptor expression is induced not only by LPS, but also by  $IFN-\alpha/\beta$  or  $IFN-\gamma$ , but not by  $TNF\alpha$ . Notably, all of these NKG2D-upregulating agents are involved as mediators or effector molecules in the innate immune system, suggesting that NKG2D upregulation by macrophages is likely to be an accompaniment of innate immune responses. The additional finding that NKG2D engagement is sufficient to trigger certain macrophage effector functions supports the possibility that the receptor plays a role in macrophage responses *in vivo*. The full spectrum of macrophage functions induced or regulated by NKG2D engagement remains to be investigated.

NKG2D is also expressed by subsets of  $NK1.1^+$  T cells and  $\gamma\delta$  T cells. Interestingly, all  $NK1.1^+$  T cells express CD44, and expression of NKG2D by  $\gamma\delta$  T cells is limited to the  $CD44^+$  subset, suggesting the NKG2D expression by T cells is related to previous activation or acquisition of a memory phenotype. On the other hand, not all  $NK1.1^+$  T cells or  $CD44^+$   $\gamma\delta$  T cells express NKG2D. Most striking was the finding that intestinal epithelial  $\gamma\delta$  T cells, despite expressing CD44 and being chronically activated *in vivo*, do not express NKG2D. In contrast,  $TCR\gamma\delta^+$  DECJs in the skin uniformly express NKG2D. It is possible that some  $\gamma\delta$  subtypes vary in their capacity to express NKG2D, just as is seen for  $CD8^+$  versus conventional  $CD4^+$   $\alpha\beta$  T cells. Alternatively, factors in addition to previous activation may control NKG2D expression by  $\gamma\delta$  T cells. Further studies will be necessary to determine whether NKG2D expression is determined by the activation state, developmental state, or other determinants. In this vein, however, it was of interest that memory  $CD8^+$   $\alpha\beta$  T cells maintained high levels of NKG2D even months after priming. An intriguing possibility is that NKG2D plays a role in the rapid and efficacious recall response of memory  $CD8^+$  T cells and possibly of other types of memory T cells.

## Experimental Procedures

### Mice

C57Bl/6 (B6) and BALB/c mice were obtained from Jackson Laboratory. OT-1 transgenic mice (Hogquist et al., 1994) on a C57Bl/6 background were provided by James Allison.  $Fc\gamma RIII^{-/-}$  mice (Ujike et al., 1999), which had been backcrossed once to C57Bl/6 mice, were a generous gift of Jeffrey Ravetch (Rockefeller University).

### The NKG2D Monoclonal Antibody

Lewis Rats were injected four times at 2 week intervals with CHO cells expressing a fusion protein consisting of the extracellular portion of NKG2D and the transmembrane and cytoplasmic portions of Ly49A. Three days after the last immunization, splenocytes were fused to the P3X8653 fusion partner as described elsewhere (Coli-



gan et al., 1991). Hybridoma supernatants were screened for antibody that stained NKG2D-transfected CHO cells but not untransfected CHO cells. A positive hybridoma, MI-6, was expanded and subcloned four times. The MI-6 mAb is an IgG2a/λ, as determined with a mAb-based rat Ig isotyping kit (Pharmingen, San Diego, CA). MI-6 mAb stained NKG2D-transfected Chinese hamster ovary (CHO) cells, but did not stain CHO cells transfected with NKG2A/CD94, NKG2C/CD94 (see Supplemental Figure S1 at <http://www.immunity.com/cgi/content/full/17/1/19/DC1>), or NKG2E/CD94 (data not shown), in line with the fact that these other NKG2 family members exhibit less than 40% sequence identity with NKG2D, despite their similar names. In addition, MI-6 mAb failed to react with 14 other NK cell receptors tested, including Ly49A-I, NKRP-1A-D, and CD69 (data not shown).

MI-6 hybridoma supernatant was collected from an INTEGRA CELLLine CL1000 (Integra Biosciences, Switzerland) cell cultivation system. The mAb was purified by precipitation with 45% saturated ammonium sulfate followed by gel filtration chromatography with a Superdex 200 column on an FPLC system. MI-6 mAb was biotinylated using EZ-Link NHS-LC-Biotin (Pierce, Rockford, IL).

F(ab)<sub>2</sub> fragments were prepared using immobilized pepsin (Pierce) according to the manufacturer's instructions. No intact antibody was detected by SDS-PAGE after pepsin digestion. After purification of F(ab)<sub>2</sub> fragments by size exclusion chromatography, no Fc fragments or intact antibody were detected by SDS-PAGE, nor did an anti-rat Fc reagent stain cells incubated with the F(ab)<sub>2</sub> reagent. Polyclonal rat IgG F(ab)<sub>2</sub> (Jackson Immunoresearch, Westgrove, PA) was used as a control.

#### Cell Preparations

Splenic NK cells from untreated or poly(I:C)-treated B6 mice were enriched by depleting B cells with anti-CD24 (mAb J11d) antibody and Low-tox H complement (Cedarlane Laboratories, Ontario, Canada), followed by centrifugation in Lympholyte-Mammalian M (Cedarlane Laboratories). Lymphokine activated killer (LAK) cells were prepared by incubating B6 splenocytes for 4 days in medium containing 1 μg/ml IL-2. In the calcium flux and redirected lysis experiments, LAK cells were 97% NK1.1<sup>+</sup> following depletion of CD8<sup>+</sup>, CD4<sup>+</sup>, TCR αβ<sup>+</sup>, and CD19<sup>+</sup> cells using the AutoMACS (Miltenyi Biotec, Auburn, CA) according to manufacturer's instructions.

Fetal NK cells were enriched from fetal day 15 thymocytes by treatment with anti-CD24 and complement (Carlyle and Zúñiga-Pflücker, 1998). Dendritic epidermal cells (Sullivan et al., 1985) and intestinal IELs (Chu et al., 1999) were prepared as described. Bone marrow macrophages were differentiated in L cell culture supernatant for 5 days before use (Portnoy et al., 1988).

#### NKG2D Upregulation

For analysis of NKG2D upregulation in T cells, enriched splenic T cells were stimulated in 96-well plates that had been coated overnight at 4°C with 5–10 μg/ml anti-TCR antibody (H57-597) (Kubo et al., 1989). Alternatively, unfractionated splenocytes from OT-1 TCR transgenic mice were incubated with irradiated C57Bl/6 splenocytes that had been pulsed with the cognate peptide (SIINFEKL). For analysis of NKG2D upregulation in vivo on T cells, mice were infected with 2 × 10<sup>5</sup> PFU of LCMV (Armstrong CA1371 strain) for primary responses or 2 × 10<sup>6</sup> PFU for secondary responses (which occurred at least 1 month after clearance of the primary infection). To examine NKG2D upregulation in macrophages, the cells (see Cell Preparations) were stimulated for 24–72 hr with 20 ng/ml LPS (Sigma-Aldrich, St. Louis, MO), 10 ng/ml IFN-γ (R&D Systems, Minneapolis, MN), 1000 U/ml IFN-α/β, or 1 ng/ml TNFα (R&D Systems).

#### mAbs, Tetramers, and Staining Protocols

We employed PK136-FITC (anti-NK1.1), anti-CD3 TC, anti-γδ-FITC, anti-CD44-TC, anti-Vα2-FITC, anti-IFN-γ-FITC (Pharmingen), anti-HA-FITC (CoVance, Berkeley, CA), anti-CD8 and anti-CD4 (Caltag, Burlingame, CA), anti-TCRβ (H57-597), anti-CD28 (37N.51) (a gift of James Allison), anti-rat IgG-FITC (Jackson Immunoresearch). Cells were incubated in 96-well plates for 30 min with MI-6-biotin, followed by washing and incubation with streptavidin PE (Molecular Probes, Eugene, OR) in combination with relevant antibodies as indicated in the figures. Macrophages were stained with 1 μg/ml MI-6 F(ab)<sub>2</sub>

or control F(ab)<sub>2</sub> followed by biotinylated donkey anti-rat F(ab)<sub>2</sub> fragment specific F(ab)<sub>2</sub> fragments (Jackson Immunoresearch), which were detected with streptavidin PE (Molecular Probes). In some experiments, the MI-6 mAb was preincubated with a 10-fold molar excess of recombinant NKG2D protein.

For analysis of LCMV-specific T cells, splenocytes were stained in four steps: (1) unconjugated MI-6 mAb (40 μg/ml) or isotype control mAb; (2) donkey F(ab)<sub>2</sub> anti-rat IgG-FITC (Jackson Immunoresearch); (3) normal rat serum to block free Ab sites; (4) anti-CD8α-tricolor (CalTag) and class I tetramers multimerized with PE-streptavidin (Molecular Probes). Staining with NKG2D tetramers was as previously described (Diefenbach et al., 2000). Tetrameric complexes of D<sup>b</sup> bound with LCMV-derived peptides GP<sub>33-41</sub> and NP<sub>396-404</sub>, were prepared as described previously (Murali-Krishna et al., 1998). After staining, cells were fixed with 1% formaldehyde. All flow cytometry was performed on EPICS XL-MCL machines (Coulter, Hialeah, FL), and data were analyzed with FlowJo software (Tree Star, Stanford University).

#### Cellular Stimulation by Anti-NKG2D mAb

For redirected lysis assays, effector cells were incubated with 100 μg/ml antibody for 45 min. <sup>51</sup>Cr-labeled Daudi cells were then added to the wells for 4 hr. To assay calcium mobilization, cells were labeled with 3 μM INDO-1AM and pluronic detergent (Molecular Probes) according to manufacturer's instructions. Effector cells were incubated with primary antibody on ice for 30 min, washed, and run for 5 min at 37°C on a Coulter Elite-ESP (Miami, FL). Cross-linking F(ab)<sub>2</sub> anti-rat IgG antibody was added after 40 s of data collection. Induction of IFN-γ production was performed in round bottom high-protein binding plates (Costar, Fischer Scientific, Pittsburgh, PA) that had been coated overnight at 4°C with the indicated concentrations of intact mAb or F(ab)<sub>2</sub> fragments, as indicated, in 20 μl PBS, followed by four rinses with 200 μl PBS. The IFN-γ release assay was done using Cytotfix/Cytoperm intracellular staining kits (Pharmingen). For analysis of NK cells, 1 × 10<sup>5</sup> LAK cells, or splenocytes isolated from poly(I:C)-injected mice that had been enriched for NK cells by treatment with anti-HSA and complement, were incubated in each well for 5 hr in the presence of Golgi Plug before transfer to V-bottom plates and staining for surface NK1.1, CD3, and intracellular IFN-γ. Results represent percent of gated NK1.1<sup>+</sup> CD3<sup>+</sup> cells.

For analysis of T cell activation by anti-NKG2D, we established a CTL line specific for the modified LCMV peptide GP33M (KAVYNFATM) in the context of D<sup>b</sup>. Splenocytes from mice infected 8 days before with LCMV were cultured with irradiated B6 splenocytes pulsed with 1 μM GP33M peptide and restimulated weekly. The medium was supplemented with 5% T-STIM (BD, Franklin Lakes, NJ) beginning at the third stimulation. Seven days after the last restimulation, 1 × 10<sup>5</sup> cells were incubated in triplicate in wells that had been coated at 4°C overnight with MI-6 mAb, anti-CD28 mAb, or control Ig, alone or in combination with anti-TCRβ mAb. Two days later, Golgi Plug was added for 5 hr before staining and analysis of intracellular IFN-γ as described for NK cells. For proliferation studies, the cells were preincubated for 10 min in 0.5 μM CFSE, quenched with FCS, and washed three times in RPMI media with 10% FCS. After 4 days with plate-bound antibody, cells were counterstained with propidium iodide and analyzed by flow cytometry. Separate analysis indicated that all the cells were CD8<sup>+</sup> T cells. For analysis of macrophage activation, the cells (see Cell Preparations) were preincubated for 48 hr with or without 1000 U/ml IFN-α/β, washed, and incubated in triplicate at 1 × 10<sup>5</sup> per well in 96-well plates that had been coated with MI-6 or control F(ab)<sub>2</sub>. After 48 hr, nitrite accumulation was determined with the Griess reagent as described (Diefenbach et al., 2000), and secreted TNFα was determined in a bioassay using the TNF-sensitive cell line WEHI 164/cl.13 (Espevik and Nilsen-Meyer, 1986).

#### Northern Blots

BM macrophages were left unstimulated or were stimulated for 48 hr with either 20 ng/ml LPS (Sigma-Aldrich), 10 ng/ml IFN-γ (R&D Systems), 1000 U/ml IFN-α/β, or 1 ng/ml TNFα (R&D Systems). RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Northern blots were per-

formed as described elsewhere (Maniatis et al., 1989). Blots were hybridized with a [ $\alpha$ - $^{32}$ P]CTP-labeled full-length NKG2D cDNA probe. The blots were washed at 65°C in 0.5× SSC/1% SDS. After autoradiography, the blot was stripped and rehybridized with a  $^{32}$ P-labeled  $\beta$ -actin probe.

#### Inhibition of Target Cell Recognition by MI-6 mAb

LAK cells, titrated in triplicate in 96-well plates, were preincubated for 30 min with 50  $\mu$ g/ml MI-6 mAb, rat IgG, or medium alone before addition of  $^{51}$ Cr-labeled target cells ( $1 \times 10^4$ ) for 4 hr (Liao et al., 1991). Comparable results were obtained with intact mAb and F(ab')<sub>2</sub> fragments. In Figure 7, RMA, B16, RMA/S, MC38, LLC, and NOBO-1 were blocked with F(ab')<sub>2</sub> fragments, while TRAMP, YAC-1, 3T3 (BALB/c), SM-1, and WEHI-164 were blocked with intact mAb. Addition of twice as much antibody gave identical results, indicating that the reagent was in excess.

For analysis of IFN- $\gamma$  production, LAK cells or enriched splenic NK cells were preincubated with F(ab')<sub>2</sub> fragments of MI-6 as described above and then incubated with target cells at a 1:1 ratio for 5 hr in the presence of Golgi Plug before staining for intracellular IFN- $\gamma$ , NK1.1, and CD3 as described above.

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