A novel ligand for the NKG2D receptor activates NK cells and macrophages and induces tumor immunity

Andreas Diefenbach, Jennifer K. Hsia, Ming-Yu B. Hsiung and David H. Raulet

Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, USA

NK cells are involved in the immune response against viral and microbial infections and tumors. In contrast to B and T cells, NK cells employ various modes of immune recognition. An important mode of immune recognition employed by NK cells is "induced self recognition" exemplified by the NKG2D receptor–ligand system. The NKG2D immunoreceptor, expressed by NK cells, and by activated $CD8^+$ T cells and macrophages, recognizes one of several cell surface ligands that are distantly related to MHC class I molecules (*i.e.* H60 and Rae1 proteins in mice, and MHC class I chain-related proteins and UL-16-binding proteins in humans). These ligands are not expressed abundantly by most normal cells but are up-regulated on cells exposed to various forms of cellular insults. Here we report the cloning of another ligand for NKG2D; transcripts of this ligand are found in a wide variety of tissues and in various tumor cells. Cross-linking of NKG2D with the novel ligand potently activated NK cells and macrophages. Tumor cells ectopically expressing the molecule were efficiently rejected by naive mice, and induced strong protective immunity to the parental, ligand-negative tumor cells.

Received 28/10/02
Accepted 6/12/02

Key words: NK cell / NKG2D / Innate immunity / Tumor immunity / Macrophage

1 Introduction

NK cells are bone marrow (BM)-derived lymphocytes that are involved in the immune recognition of infected cells and tumors [1–3]. In contrast to B and T cells, NK cell receptor genes do not undergo recombination to create a highly diverse repertoire of specificities. Instead, NK cells employ other modes of immune recognition. One of these recognition modes is "missing-self recognition" [4]. To that end, NK cells are equipped with various families of inhibitory receptors (KIR proteins in humans, Ly49 proteins in mice and CD94–NKG2A proteins in mice and humans) that bind to class I MHC molecules. Binding of these receptors to their ligands results in profound inhibition of the NK cell [5]. Developing NK cells are "educated" by poorly understood mechanisms such that the population of fully mature NK cells is inhibited by cells expressing a full complement of self MHC molecules, and yet is capable of attacking cells that have extinguished one or more self MHC molecules [5]. During cellular transformation or viral infection MHC class I molecules are often down-regulated, releasing the NK cell from the inhibitory signal and shifting the balance towards activation [5].

NK cells also express a variety of stimulatory receptors. Until recently the significance of these receptors was unclear mainly because the specificity of these receptors was unknown. One of these receptors is NKG2D. This is a homodimeric C-type lectin-like protein that is expressed by all NK cells, subsets of NKT cells and subsets of $\gamma\delta$ T cells [6–8]. After stimulation, mouse NKG2D is also expressed by virtually all $CD8^+$ T cells and macrophages [7, 8]. NKG2D is a direct stimulatory receptor in the innate immune system (NK cells and macrophages) and a costimulatory receptor in the adaptive immune system ($CD8^+$ T cells) by virtue of regulated association with different adaptor proteins (*i.e.* DAP10 and KARAP/DAP12 in the innate immune response but only DAP10 in the adaptive immune response) [9].

Several ligands for the NKG2D receptor have been identified. In the mouse these ligands are the H60 minor histocompatibility antigen and the Rae1 family of proteins [7, 10]. H60 is a transmembrane protein whereas the Rae1 proteins are linked to the plasma membrane with a

[I 23635]

Abbreviations: aa: Amino acid **BM:** Bone marrow **GPI:** Glycosyl-phosphatidyl inositol **MFI:** Mean fluorescence intensity **MICA:** MHC class I chain-related protein A **MULT1:** Murine ULBP-like transcript 1 **ORF:** Open reading frame **PI-PLC:** GPI-specific phospholipase C **ULBP:** UL-16-binding protein

0014-2980/03/0202-381$17.50+.50/0

glycosyl-phosphatidyl inositol (GPI) anchor. Despite relatively divergent protein sequences (25.2% identity and 39.1% similarity), both protein families are composed of α_1 and α_2 ectodomains with similarities to the corresponding domains of class I MHC molecules [7, 11, 12]. In contrast to class I MHC molecules, H60 and Rae1 proteins lack an α_3 domain.

Interestingly, all known ligands for mouse NKG2D map close to the telomeric region of mouse chromosome 10 [7, 12, 13]. Significantly, the syntenic region on human chromosome 6 contains a similar group of proteins variously called UL-16-binding proteins (ULBP) [14], ALCAN [15] or RAET1 [16], suggesting that these proteins may be the human orthologs of the mouse ligands for NKG2D. Similar to the Rae1 proteins these GPI-linked proteins also display two ectodomains with significant similarities to class I MHC molecules. In addition, human NKG2D recognizes two closely related non-classical MHC class Ib molecules — MHC class I chain-related protein A (MICA) and MICB — both of which map close to the HLA-B locus [6]. No mouse homologs have been found for these proteins.

Human and mouse ligands for NKG2D are expressed poorly or not at all by most normal cells but are up-regulated in stressed cells (MIC proteins) [17], tumor cells (Rae1, H60, ULBP, MIC proteins) [7, 14, 15, 18, 19] and cells infected with viruses [20] or bacteria [21] (MIC proteins). This led us to propose that the up-regulation of NKG2D ligands occurs commonly in distressed cells and serves to alert the immune system to potential sites of cellular insult [2].

The fact that the NKG2D immunoreceptor binds to a wide variety of ligands that are only distantly related is unprecedented and the biological significance of each of these ligands has not yet been elucidated. In addition, the affinity of the various NKG2D ligands differs widely (with dissociation constants between 20–30 nM for H60 and Rae1ε, and 300–800 nM for Rae1α, β, γ, δ) [22, 23]. However, to date there is no evidence that the differing affinities of NKG2D ligands have significant functional consequences.

In this study we describe the cloning and functional characterization of a novel ligand of the mouse NKG2D receptor. The novel protein is encoded by a single gene in the telomeric region of mouse chromosome 10. In contrast to the Rae1 and H60 ligands for NKG2D, mRNA for this protein is expressed in a wide variety of tissues. In addition, mRNA is also expressed by various mouse tumor cell lines, some of which co-express other NKG2D ligands. Ectopic expression of the novel ligand for the NKG2D immunoreceptor in target cells very efficiently activates NK cells and macrophages and induces potent antitumor immunity. During the preparation of this manuscript Carayannopoulos and colleagues reported the cloning of the same gene, which they named murine ULBP-like transcript 1 (MULT1), and we will therefore use the same designation here [24].

2 Results

2.1 Cloning of a novel mouse protein related to NKG2D ligands

A database search for mouse nucleotide sequences similar to the human ULBP, using the "tblastn" algorithm, revealed a cDNA sequence with significant similarity to ULBP1 (Fig. 1). The cDNA clone (NCBI accession number AK020784) was derived and sequenced from a neonatal C57BL/6 (B6) thymus cDNA library by the RIKEN consortium [25]. The cDNA sequence contained a full open reading frame (ORF) of 1006 bp and encoded a predicted protein with a molecular weight of 37.1 kDa. Analysis of the amino acid (aa) sequence using various protein-prediction algorithms suggested that the protein is a type I transmembrane protein with an N-terminal signal sequence (25 aa) and two class I MHC like α domains (89 aa and 91 aa, respectively) (Fig. 1A). In contrast to the ULBP and the Rae1 family proteins that lack a transmembrane domain and are attached to the plasma membrane via a GPI anchor, the novel protein displays a transmembrane domain (17 aa) and a cytoplasmic domain of 109 aa (Fig. 1A). The protein is predicted to be a glycoprotein, as the two ectodomains contain four potential N-glycosylation sites (Fig. 1A, bold) and 1 potential O-glycosylation site (Fig. 1A, arrowhead).

Sequence alignments revealed that the protein is distantly related to known NKG2D ligands, all of which are even more distant relatives of MHC class I proteins (Fig. 1B–D). The sequence identity with known ligands ranged from 16.7% for MICA to 29.9% for human ULBP1. The greater similarity to ULBP1 (and other ULBP family members) was reflected in several short blocks of conserved residues (Fig. 1B, bold lines) that were less conserved in Rae1 and H60 sequences. Nevertheless, the new protein was not dramatically closer in sequence to ULBP1 (or ULBP3, data not shown) than to Rae1 or H60 (Fig. 1B–D). However, given the recent report by the Yokoyama group [24] we will use the name MULT1 throughout our manuscript.

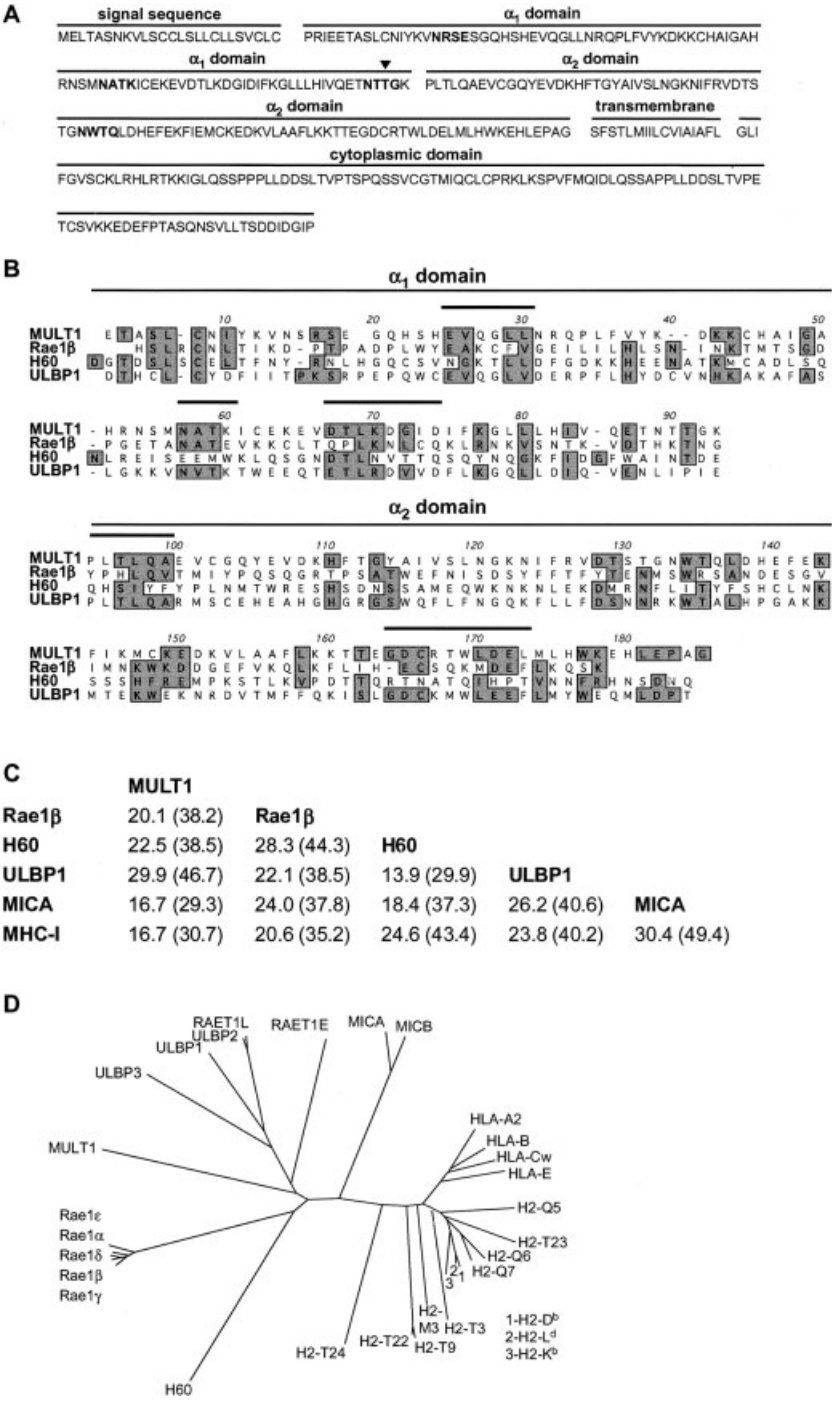

Fig. 1. Amino acid similarity of MULT1 and other MHC class I-like molecules. (A) MULT1 is a type I transmembrane protein. The domain structure of MULT1 is shown. Potential N-glycosylation sites are printed in bold and the single potential O-glycosylation site is marked by an arrowhead. (B) Comparison of the aa sequences of the ectodomains of MULT1, Rae1β, H60 and ULBP1 using the ClustalW alignment algorithm [34]. Shaded boxes show similar or identical amino acid residues. The bold lines indicate patches of conserved aa residues between MULT1 and ULBP1. (C) Sequence relatedness of MULT1 and other MHC class I-like proteins. The overall percentage of amino acid identity is shown (with similarity shown in parentheses) as determined by alignment using the ClustalW algorithm. (D) The tree dendogram illustrates the relationships between the mouse and human NKG2D ligands and classical and non-classical class I MHC molecules. The dendrogram was generated with Phylip 3.572.

2.2 MULT1 is a novel ligand for NKG2D

To determine whether MULT1 is a ligand for the NKG2D receptor, we stably transduced the RMA T cell lymphoma to express high levels of MULT1. RMA cells do not endogenously express mRNA for any of the known mouse ligands for NKG2D and do not stain with tetramers of the NKG2D extracellular domain that bind all known ligands (Fig. 2A) ([7, 19] and see below). As previously reported, RMA cells transfected with Rae1β or H60 stained brightly with NKG2D tetramers (Fig. 2A) [19]. Strikingly, MULT1-transfected RMA cells also bound NKG2D tetramers, indicating that MULT1 is a ligand for the NKG2D receptor (Fig. 2A).

Treatment of RMA cells expressing MULT1 or H60 with GPI-specific phospholipase C (PI-PLC), which cleaves GPI-linked proteins from their phospholipid anchors, did not decrease the mean fluorescence intensity (MFI) of staining with NKG2D tetramers, suggesting that MULT1 and H60 are not GPI-linked proteins (Fig. 2A). In contrast, addition of PI-PLC to RMA cells expressing Rae1β resulted in a dramatic decrease of the MFI (Fig. 2A). Thus, soluble NKG2D protein binds to cell surface MULT1 as well as to Rae1β and H60.

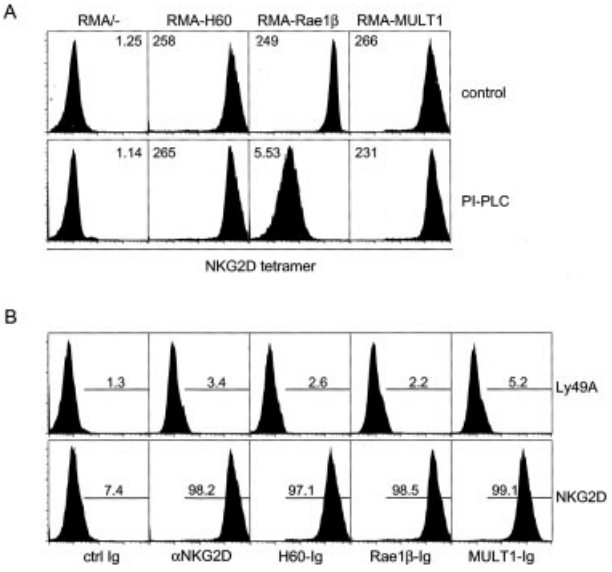

Fig. 2. MULT1 is a novel ligand for the NKG2D receptor. (A) MULT1 binds to the NKG2D receptor. The T cell lymphoma RMA was transduced with replication deficient retroviruses encoding the indicated proteins or with "empty" virus (RMA/–). The transduced cells were stained with NKG2D tetramer after 1 h of treatment with 1 U/ml PI-PLC (lower row) or PBS buffer ("control"; upper row). The numbers indicate the MFI of the tetramer staining. Data are representative of three experiments. (B) Soluble MULT1 binds to the NKG2D receptor. CHO cells that had been stably transfected with Ly49A or NKG2D were stained with a mAb to NKG2D (αNKG2D) or with the indicated Ig-fusion proteins. Data are representative of two experiments.

To address whether soluble MULT1 binds to cell surface NKG2D, we generated a soluble version of the MULT1 ectodomains attached to the Fc part of mouse IgG_1 (MULT1–Ig) as well as H60–Ig and Rae1β–Ig fusion proteins. As a control we used a fusion protein consisting of the ectodomains of the mouse NKp46 receptor [26] and the Fc portion of IgG_1. None of the Ig-fusion proteins bound to CHO cells stably transfected with the inhibitory C-type lectin-like Ly49A protein (Fig. 2B, upper panels). Significantly, MULT1–Ig strongly stained cell lines transfected with NKG2D, as did Rae1β–Ig and H60–Ig (Fig. 2B, lower panels). The NKp46–Ig fusion protein did not bind to NKG2D. These data confirmed that MULT1 is a novel ligand for the NKG2D immunoreceptor.

2.3 MULT1 mRNA is detected in various normal tissues and in some tumor cell lines

In contrast to the other mouse ligands for NKG2D, MULT1 mRNA was detected in a wide variety of tissues, including thymus and spleen, lymph nodes, and to a lesser extent liver, heart and lung (Fig. 3A and data not shown). The transcripts were detected by Northern blotting, suggesting that the mRNA levels were appreciable (Fig. 3A and data not shown). MULT1 transcripts were not detected by this method in total RNA of kidney or brain (Fig. 3A).

Interestingly, we showed previously that single-cell suspensions of thymocytes and splenocytes from B6 mice do not stain detectably with NKG2D tetramers [7]. Comparable analysis of lymph node, liver and kidney cells also did not reveal cell surface expression of NKG2D ligands (data not shown). These findings raise the possibility that MULT1 may be regulated post-transcriptionally. This is reminiscent of reported discrepancies between the expression of ULBP mRNA and ULBP protein in various tumor cell lines [14]. In contrast, there are no indications in the literature thus far that Rae1 and H60 proteins are regulated post-transcriptionally (Fig. 3B; and A. Diefenbach and D. H. Raulet, unpublished observations). Notably, one of the transcripts detected by Northern blotting was considerably larger in size (ca. 5.5 kb) than the expected size of the full length MULT1 mRNA (1.5 kb), suggesting the existence of a large precursor mRNA species.

We could also detect MULT1 mRNA expression in multiple tumor cell lines (Fig. 3B). In some cases (the

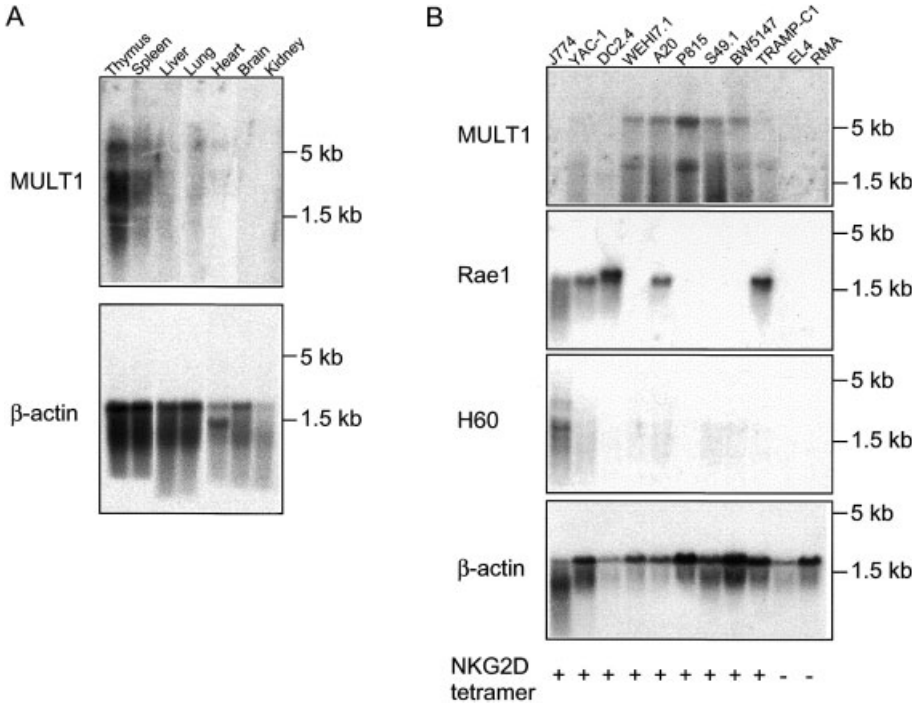

Fig. 3. Expression of MULT1 mRNA in tissues and tumor cell lines. Total RNA (20 μg) of the indicated tissues (A) or tumor cell lines (B) was separated via agarose gel electrophoresis and immobilized on a charged nylon membrane. The membrane was hybridized with a [^{32}P]-labeled full-length MULT1 probe. In the case of the tumor cell lines (B) the blot was consecutively rehybridized with full-length Rae1β or H60 probes. After the final exposure the membrane was stripped and rehybridized with a β-actin probe to control for loading of all lanes. In the case of the tumor cell lines (B) the result of the staining with the NKG2D tetramer is indicated under the Northern-blot panel. Data are representative of two (A) or three (B) experiments.

WEHI7.1, S49.1 and BW5147 T cell lymphomas, and the P815 mastocytoma) MULT1 is the only known NKG2D ligand expressed in the cells, suggesting that MULT1 is responsible for tetramer staining. Other cell lines coexpressed MULT1 and Rae1 (the A20 B cell lymphoma and TRAMP-C1 prostate carcinoma) (Fig. 3B). In this case, both ligands may contribute to tetramer staining.

2.4 MULT1 functionally activates NK cells and macrophages

RMA cells transfected with the three mouse NKG2D ligands (MULT1, Rae1β or H60) and control-transfected RMA cells were used as target cells in a [^{51}Cr] release cytotoxicity assay. Control-transfected RMA cells were not lysed by freshly isolated NK cells from syngeneic B6.Rag1$^{-/-}$ mice treated with poly (I:C) (Fig. 4A), nor by IL-2-stimulated NK cells from normal B6 mice (data not shown). Strikingly, RMA cells transduced with MULT1 were highly susceptible to NK mediated lysis (Fig. 4A). This effect could be almost entirely blocked by inclusion of a blocking antibody to NKG2D, confirming that recognition of MULT1 on target cells depends on the interaction with the NKG2D immunoreceptor (Fig. 4A). Nearly identical results were obtained with RMA cells transduced with Rae1β or H60.

RMA cells expressing MULT1, Rae1β or H60 also strongly induced IFN-γ production from IL-2-expanded NK cells (Fig. 4B, left) as well as from freshly isolated NK cells from poly(I:C)-treated mice (Fig. 4B, right). NK cells incubated with RMA cells transduced with an empty vector did not produce significant levels of IFN-γ. Thus, MULT1 functionally activates NK cells to produce IFN-γ and induces NK cell cytotoxicity.

We had previously shown that BM-derived macrophages express NKG2D after stimulation with either LPS, IFN-α/β or IFN-γ [7, 8]. BM-derived macrophages were treated for 24 h with IFN-α/β to induce expression of NKG2D and then cocultured for another 48 h with either control-transduced RMA cells or RMA transductants expressing MULT1, Rae1β or H60. Macrophages stimulated with MULT1 transductants produced abundant nitric oxide, similar to the amount stimulated by Rae1- or H60-

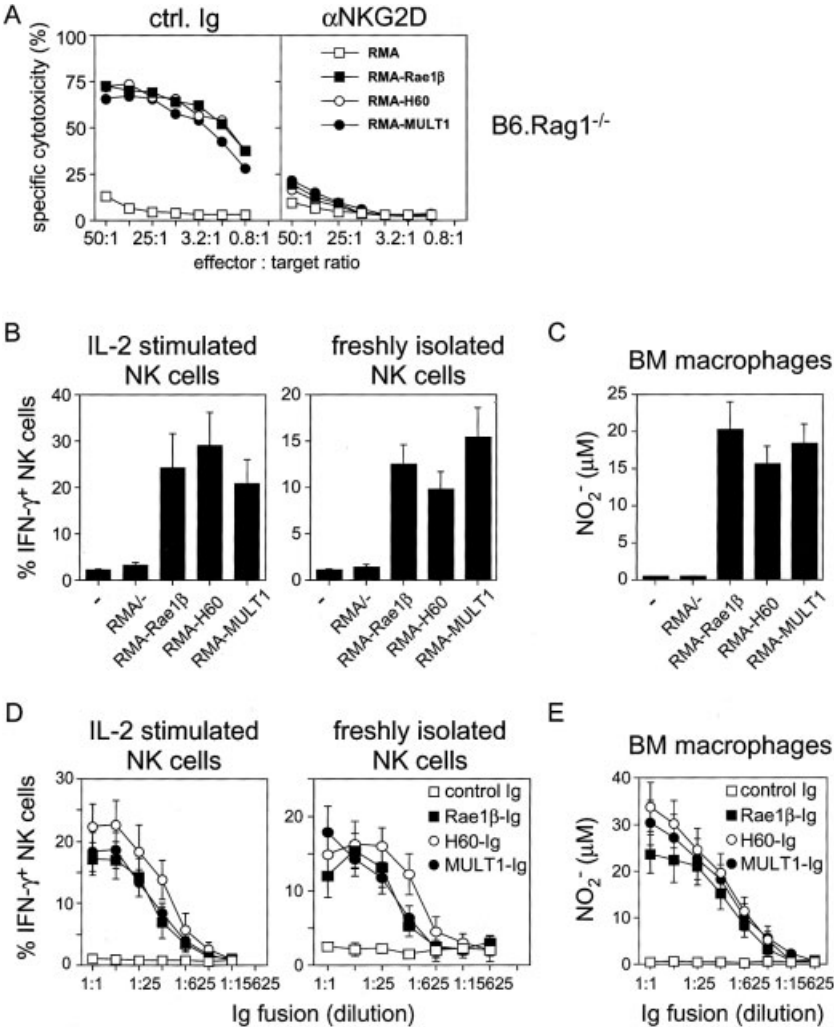

Fig. 4. MULT1 functionally activates NK cells and macrophages. (A) Ectopic expression of MULT1 induces NK cell cytotoxicity. Freshly isolated NK cells from poly(I:C)-treated B6.Rag1$^{-/-}$ mice were used as effector cells against the indicated target cells in a standard 4 h [^{51}Cr] release assay. The anti-NKG2D mAb MI-6 (αNKG2D) was used to block interactions. Data are representative of three experiments. (B, D) Stimulation of NK cells with MULT1-transfected cells and MULT1–Ig fusion protein. IL-2-stimulated NK cells and freshly isolated NK cells [from poly(I:C)-treated mice] were stimulated for 6 h with the indicated RMA transductants (B) or with the indicated plate-bound Ig-fusion proteins (D). Accumulation of IFN-γ in NK cells was determined by intracellular cytokine staining and electronic gating on NK1.1^{+}CD3^{-} cells. Data are representative of two experiments. (C, E) IFN-α/β-stimulated NKG2D^{+} BM-derived macrophages from B6 mice were incubated with the indicated RMA cell transductants (C) or with the indicated plate-bound Ig-fusion proteins (E). Nitrite accumulation in the culture supernatants was determined with the Griess assay. Data are representative of two experiments.

transductants (Fig. 4C). Control-transfected RMA cells failed to induce significant nitric oxide production.

Activation of NK cells or macrophages by transfected tumor cell lines (Fig. 4A–C) may well involve the interaction and contribution of multiple receptor–ligand systems. To address whether cell activation occurs as a result of engagement of NKG2D alone by MULT1 or the other ligands, we stimulated NK cells or activated-macrophages with plate-bound ligand–Ig fusion proteins. Strikingly, both freshly isolated NK cells from poly(I:C)-injected mice and IL-2-activated NK cells readily produced IFN-γ in response to MULT1–Ig, Rae1β–Ig or H60–Ig, but did not respond when stimulated with a control Ig-fusion protein (Fig. 4D). Similarly, IFN-α/β-stimulated BM macrophages produced nitric oxide in response to MULT1–Ig, Rae1β–Ig or H60–Ig but did not respond to a control Ig-fusion protein (Fig. 4E). Thus,

MULT1, like the other ligands, was able to functionally activate NK cells and BM-derived macrophages even in the absence of other receptor–ligand interactions.

2.5 Tumor cells expressing MULT1 are rejected in syngeneic mice and induce a potent memory response

We investigated whether RMA tumor cells ectopically expressing MULT1 would be eliminated *in vivo*, as we had observed for tumor cells expressing the Rae1β and H60 ligands for the NKG2D receptor [19]. Mice that were injected subcutaneously with 10^5 control-transfected RMA tumor cells developed progressive tumors that uniformly led to the death of the animals after 4–6 weeks post inoculation (Fig. 5). In contrast, tumor cells expressing high levels of MULT1, like those expressing Rae1β or H60, were efficiently rejected by syngeneic B6 mice (Fig. 5, left-hand panel). The mice remained tumor free for more than 12 weeks (Fig. 5, left-hand panel).

Next, we analyzed whether the rejection of MULT1-expressing tumor cells would induce a memory immune response against the parental (untransfected) tumor cell lines, as we had previously observed for Rae1β- or H60-expressing RMA tumor cells [19, 27]. Significantly, mice that had rejected RMA cells expressing MULT1 were completely immune to a challenge of 10^5 untransduced RMA cells, indicating that the initial exposure to MULT1-expressing cells induced a strong immunity to RMA tumor antigens (Fig. 5, right-hand panel). Similar results were obtained with RMA cells expressing Rae1β or H60.

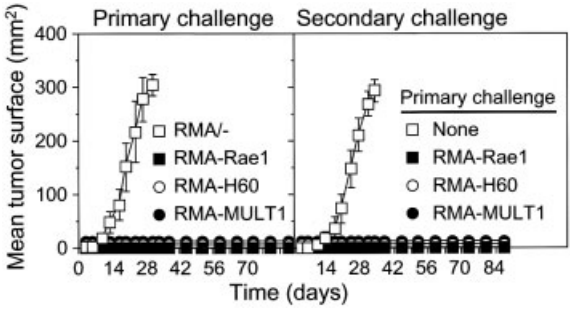

Fig. 5. MULT1-expressing RMA tumor cells are rejected in syngeneic mice and induce tumor immunity. B6 mice were inoculated subcutaneously with 1×10^5 RMA transductants (left-hand panel). Tumor growth was monitored. Mice that had rejected a primary challenge with the indicated RMA transductants were rechallenged with 1×10^5 wild-type RMA cells (right-hand panel). As a control, normal B6 mice inoculated with RMA cells are shown ("None" in the right-hand panel). Data are representative of two experiments.

As a control, naïve B6 mice challenged with the same tumor cells developed progressively growing tumors and had to be killed (Fig. 5, right-hand panel). These data indicate that the MULT1 ligand of the NKG2D receptor exerts a potent barrier to tumor cell growth and promotes long-term immunity to tumor antigens.

3 Discussion

We have identified another ligand for the mouse NKG2D receptor. The protein is distantly related to the mouse Rae1 proteins, the H60 minor histocompatibility antigen and the human ULBP, all of which are ligands for the NKG2D immunoreceptor. In contrast to the Rae1 family and H60, MULT1 mRNA seems to be expressed in a wide variety of tissues. The fact that these transcripts could be detected using a relatively insensitive assay — Northern blotting — suggests that the expression levels were appreciable. Interestingly, however, these tissues did not stain detectably with the NKG2D tetramer. The fact that cell lines transduced with MULT1 stained extremely brightly with tetramers indicates that the assay should be sufficiently sensitive to detect even weak ligand expression. MULT1 may, therefore, be regulated post-transcriptionally. Interestingly, we detected heterogeneity in MULT1 transcripts, including a large transcript of about 5.5 kb. This suggests the existence of a large precursor mRNA that may need to be processed before translation. Further studies will be necessary to determine the stage at which cell surface expression is prevented and the underlying mechanism. Reported discrepancies between ULBP mRNA and protein expression raise the possibility that other ligands may also be regulated post-transcriptionally [14].

Another interesting finding was that some tumor cell lines co-express mRNA for different NKG2D ligands, whereas others expressed only one ligand. There may be several interesting explanations for combinatorial ligand expression. One possibility is that the different ligands are all initially up-regulated by a common signal. If immunogenicity depends on the dose of NKG2D ligands, immunoselection could, in principle, lead to tumor variants that lose one or the other ligand. Alternatively, the different ligands may be regulated differently and have distinguishable biological activities, for example in inducing different types of cellular responses. This could occur if the NKG2D receptor can respond differently to different ligands (but see below), or if other receptors bind to one or the other of the ligands. Production of antibodies specific for the different ligands should help in testing these and other hypotheses.

NK cells and macrophages were efficiently activated by MULT1 in cell transductants or by plate-bound MULT1–Ig fusion protein. These experiments significantly extend the results obtained by Carayannopoulos and colleagues [24] by using a system that exclusively tests the interaction between the NKG2D receptor and its respective ligands. However, we did not observe significant quantitative differences between MULT1, Rae1β and H60 in the potential to activate these cell types. So far there is no indication that the various ligands for NKG2D fulfill qualitatively distinct functions in activating NK cells or macrophages. This is particularly surprising as the affinity of MULT1 for NKG2D is several-fold higher than that of H60 and up to nearly 100-fold higher than that of Rae1 [22–24]. Additional studies will be necessary to gauge the functional significance of the differing affinities of the NKG2D receptor for its various ligands.

MULT1, as well as Rae1β and H60 [19], when ectopically expressed by tumor cells, induced a very potent antitumor response *in vivo* that led to strong rejection of the transduced tumor cells in syngeneic B6 mice. Strikingly, the MULT1-transduced tumor cells also primed the mice, rendering them immune to the tumor antigens of the parental tumor cell. We had previously shown that Rae1β and H60 transductants also induce protective immunity to untransduced tumor cells, and that immunity was mediated by tumor-antigen-specific $CD8^+$ T cells [19, 27]. Thus, although induction of tumor immunity was not observed in one study using RMA cells transduced with Rae1γ, we and others [27, 28] have found that tumor cells expressing three different NKG2D ligands (MULT1, Rae1β or H60) induce protective immunity, and that this occurs in multiple tumor cell lines in the case of Rae1β and H60 transductants (RMA, B16-BL6 and EL4). The finding that MULT1, like Rae1 family members, is expressed by multiple tumor cell lines suggests that these molecules may contribute to immune surveillance of tumors developing *in situ* [29].

It remains unresolved why so many different ligands have evolved for a single immunoreceptor. One possibility is that the different ligands are regulated by different types of cellular distress signals, such as activation of different oncogenes, inactivation of different tumor repressors, and/or events associated with different forms of infection. Alternatively, the existence of multiple ligands for the same receptor could represent a "fail-safe" mechanism that reduces the probability of cells escaping surveillance by the NKG2D system. Viral proteins have been reported to specifically interfere with expression of specific NKG2D ligands [14, 30], and this may constitute selection pressure for expression of additional ligands.

4 Materials and methods

4.1 Cloning of MULT1

The MULT1 transcript was detected in the NCBI database (AK020784) as the most significant hit in a database search for nucleotide sequences related to ULBP1, using the "tblastn" algorithm [31]. The sequence was derived by the RIKEN consortium from a B6 neonatal thymus cDNA library [25]. The sequence contained a complete ORF of 1006 bp. Using RT-PCR from neonatal thymus RNA we created a PCR insert of the full ORF. After verifying the insert sequence, the ORF was subcloned into a retroviral expression vector [32]. The 5'-primer was 5'-ctcgagccaccATGGAGCTGACTGCCAGTAACAAGGTC-3' and the 3'-primer was 5'-gcggccgcTCATGGGATCCCATCAATATCGTC-3' (restriction sites in small letters, 5' consensus Kozak sequence underlined).

The primary structure of MULT1 was determined using algorithms provided by the PredictProtein server (http://cubic.bioc.columbia.edu/pp/) [33]. For prediction of GPI-linkage we used an algorithm provided by the Swiss Institute for Bioinformatics (accessible via http://www.expasy.ch/). Comparison of the MULT1 ectodomains with the other NKG2D ligands was performed using the ClustalW alignment algorithm [34]. The dendogram was created with Phylip 3.572 [35].

4.2 Northern blotting

Total cellular RNA was prepared from the indicated tissues and tumor cell lines using TRIZOL reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Northern blots were performed as described elsewhere [36]. Blots were hybridized with the indicated [^{32}P]-α-CTP-labeled full-length cDNA probes. The blots were washed at 65°C in 0.5× SSC/1% SDS. After autoradiography, the blot was stripped and re-hybridized with a ^{32}P-labeled β-actin probe as a loading control.

4.3 Retroviral transductants and tetramer staining

The T cell lymphoma RMA was transduced with replication deficient retroviruses encoding MULT1, as described previously [19]. The retrovirus vector did not encode a selectable marker or other foreign protein. The generation of the RMA-H60 and RMA-Rae1β transductants was previously reported [19]. NKG2D tetramer staining and the removal of GPI-anchored proteins by PI-PLC (Sigma, St. Louis, MO) were described in detail elsewhere [7].

4.4 Generation of Ig-fusion proteins

For generation of Ig-fusion proteins of the mouse NKG2D ligands, PCR inserts were created using 5'-primers match-

ing the translation start of the mouse NKG2D ligands (5'-MULT1–Ig, 5'-ggtaccccaccATGGAGCTGACTGCCAGTA-ACAAGGTC-3'; 5'-Rae1β–Ig, 5'-ggtaccccaccATGGCCAA-GGCAGCAGTGACCAAGCGC-3', 5'-H60–Ig, 5'-ggtac-cccaccATGGCAAAGGGAGCCACCAGCAAG-3') and 3'-primers truncating the protein at the end of the α_2 domain (3'-MULT1–Ig, 5'-ggatccTCTTTCCAGTGCAACATGAGCT-CATC-3'; 3'-Rae1β-Ig, 5'-ggatccTTAGAAGTAGAGTGGCT-GGG-3'; 3'-H60–Ig, 5'-ggatccTGGTTGTCAGAATTAT-GTCGGAAG-3'; restriction sites in small letters, 5' consensus Kozak sequence underlined). The PCR inserts were ligated in frame to the Pro227 residue of the hinge region of the CH2–CH3 domain of mouse IgG_1 which had been inserted in the pcDNA4 vector (kindly provided by W. C. Sha, University of California Berkeley). For purification and detection, a C-terminal 6×His tag and a c-myc tag were added in frame to the Fc portion of IgG_1. 293T cells were stably transfected with the Ig-fusion constructs and tissue culture supernatants containing the Ig-fusion proteins were collected. The Ig-fusion proteins were purified using a Ni^{2+} resin (ProBond Resin; Invitrogen) following the manufacturer's instructions. For flow cytometry analysis, cells were incubated for 30 min with the purified Ig-fusion proteins at a 1:50 dilution. After washing, the cells were incubated with a biotinylated mAb specific for the c-myc epitiope tag (9E10; Covance, Richmond, CA) followed by streptavidin–-phycoerythrin (Molecular Probes, Eugene, OR). The generation of CHO cell lines expressing Ly49A and NKG2D was described elsewhere [8, 37].

4.5 *Ex-vivo*-derived cell populations

Splenocyte suspensions from B6 mice or B6.Rag1$^{-/-}$ mice were incubated for 3 days in medium containing 1 μg/ml recombinant hIL-2 (Chiron, Emeryville, CA). NK cells from B6 mice were purified by using an autoMACS (Miltenyi Biotec, Auburn, CA) to negatively select cells that stained with FITC-conjugated mAb specific for the TCRβ chain (H57–597; Ebioscience, San Diego, CA), CD8α (CT-CD8α), CD4 (CT-CD4), (both from Caltag, Burlingame, CA), or CD19 (1D3; BD Pharmingen, San Diego, CA), followed by anti-FITC microbeads. The resulting cell populations were >90% NK1.1$^+$NKG2D$^+$CD3$^-$ NK cells. Freshly isolated NK cells from mice injected with 200 μg poly(I:C) (Sigma) 18 h earlier were enriched by depleting B cells, T cells and granulocytes as described elsewhere [9]. In the case of freshly isolated NK cells from B6 mice, the resulting cell population was >50% NK1.1$^+$CD3$^-$ NK cells. Freshly isolated NK cells from B6.Rag1$^{-/-}$ were enriched by depleting macrophages via adherence for 90 min to plastic surfaces and collection of the non-adherent cells. The resulting cell population was >90% NK1.1$^+$CD3$^-$ NK cells. BM macrophages were differentiated as described elsewhere [38].

4.6 Cytotoxicity assays

RMA (T cell lymphoma) target cell lines that had been retrovirally transduced to express high levels of the NKG2D ligands were labeled with [^{51}Cr]. Lysis was determined using a standard 4 h [^{51}Cr] release assay. Spontaneous lysis never exceeded 10%. The procedure to block interaction of the NKG2D receptor with its ligands on the target cells has been described elsewhere [8]. Data represent the mean ± SD of triplicate measurements.

4.7 Intracellular cytokine staining

For determination of IFN-γ secretion, the indicated cell populations were seeded into 96-well plates and stimulated for 6 h with the indicated target cell populations. Induction of IFN-γ production by stimulation with serial dilutions of plate bound Ig-fusion proteins was performed in microtiter plates that had been coated overnight with the indicated dilutions of Ig-fusion proteins. IFN-γ production in response to target cells was determined by intracellular cytokine staining [39]. Data represent the mean ± SD of triplicate measurements.

4.8 Macrophage stimulation

BM-derived macrophages that had been activated with 500 U/ml IFN-α/β (a kind gift from I. Gresser, France) to upregulate expression of NKG2D were transferred to plates that had been coated with the indicated dilutions of Ig-fusion proteins. In some experiments macrophages were co-cultured at a 1:1 ratio with RMA cells expressing NKG2D ligands or control RMA cells. After 48 h, nitrite accumulation was determined from triplicates with the Griess reagent as described previously [40]. Data represent the mean ± SD of triplicate measurements.

4.9 Tumor challenge

All tumor rejection experiments employed five mice per group. B6 mice were purchased from Jackson Laboratories. All mice were used between 8 and 18 weeks of age. 1×10^5 tumor cells were injected subcutaneously in 100 μl of PBS in the right flank. Tumor development was monitored by measuring the tumor size twice weekly with a metric caliper. For the rechallenge experiments, groups of five mice that had completely rejected the initial tumor (12 weeks after initial tumor challenge) were injected in the opposite flank with the respective untransduced RMA tumor cells (*i.e.* lacking NKG2D ligands).

Acknowledgements: This research was supported by grants of the National Institute of Health (to D. H. R.). A. D. is a Physician Postdoctoral Fellow of the Howard Hughes Medical Institute.

References

1 **Trinchieri, G.,** Biology of natural killer cells. *Adv. Immunol.* 1989. **47:** 187–376.

2 **Diefenbach, A. and Raulet, D. H.,** Strategies for target cell recognition by natural killer cells. *Immunol. Rev.* 2001. **181:** 170–184.

3 **Moretta, L., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M., Biassoni, R. and Moretta, A.,** Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol.* 2001. **19:** 197–223.

4 **Ljunggren, H. G. and Karre, K.,** In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* 1990. **11:** 237–244.

5 **Raulet, D. H., Vance, R. E. and McMahon, C. W.,** Regulation of the natural killer cell receptor repertoire. *Annu. Rev. Immunol.* 2001. **19:** 291–330.

6 **Bauer, S., Groh, V., Wu, J., Steinle, A., Phillips, J. H., Lanier, L. L. and Spies, T.,** Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* 1999. **285:** 727–729.

7 **Diefenbach, A., Jamieson, A. M., Liu, S. D., Shastri, N. and Raulet, D. H.,** Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat. Immunol.* 2000. **1:** 119–126.

8 **Jamieson, A. M., Diefenbach, A., McMahon, C. W., Xiong, N., Carlyle, J. R. and Raulet, D. H.,** The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* 2002. **17:** 19–29.

9 **Diefenbach, A., Tomasello, E., Lucas, M., Jamieson, A. M., Hsia, J. K., Vivier, E. and Raulet, D. H.,** Selective associations with signaling molecules determines stimulatory versus costimulatory activity of NKG2D. *Nat. Immunol.* 2002. **3:** 1142–1149.

10 **Cerwenka, A., Bakker, A. B. H., McClanahan, T., Wagner, J., Wu, J., Phillips, J. H. and Lanier, L. L.,** Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* 2000. **12:** 721–727.

11 **Zou, Z., Nomura, M., Takihara, Y., Yasunaga, T. and Shimada, K.,** Isolation and characterization of retinoic acid-inducible cDNA clones in F9 cells: a novel cDNA family encodes cell surface proteins sharing partial homology with MHC class I molecules. *J. Biochem.* 1996. **119:** 319–328.

12 **Malarkannan, S., Shih, P. P., Eden, P. A., Horng, T., Zuberi, A. R., Christianson, G., Roopenian, D. and Shastri, N.,** The molecular and functional characterization of a dominant minor H antigen, H60. *J. Immunol.* 1998. **161:** 3501–3509.

13 **Nomura, M., Zou, Z., Joh, T., Takihara, Y., Matsuda, Y. and Shimada, K.,** Genomic structures and characterization of Rae1 family members encoding GPI-anchored cell surface proteins and expressed predominantly in embryonic mouse brain. *J. Biochem.* 1996. **120:** 987–995.

14 **Cosman, D., Müllberg, J., Sutherland, C. L., Chin, W., Armitage, R., Fanslow, W., Kubin, M. and Chalupny, N. J.,** ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* 2001. **14:** 123–133.

15 **Onda, H., Ohkubo, S., Shintani, Y. et al.** A novel secreted tumor antigen with a glycosylphosphatidylinositol-anchored structure ubiquitously expressed in human cancers. *Biochem. Biophys. Res. Commun.* 2001. **285:** 235–243.

16 **Radosavljevic, M., Cuillerier, B., Wilson, M. J., Clement, O., Wicker, S., Gilfillan, S., Beck, S., Trowsdale, J. and Bahram, S.,** A cluster of ten novel MHC class I related genes on human chromosome 6q24.2-q25.3. *Genomics* 2002. **79:** 114–123.

17 **Groh, V., Steinle, A., Bauer, S. and Spies, T.,** Recognition of stress-induced MHC molecules by intestinal epithelial $\gamma\delta$ T cells. *Science* 1998. **279:** 1737–1740.

18 **Groh, V., Rhinehart, R., Secrist, H., Bauer, S., Grabstein, K. H. and Spies, T.,** Broad tumor-associated expression and recognition by tumor-derived $\gamma\delta$ T cells of MICA and MICB. *Proc. Natl. Acad. Sci. USA* 1999. **96:** 6879–6884.

19 **Diefenbach, A., Jensen, E. R., Jamieson, A. M. and Raulet, D. H.,** Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 2001. **413:** 165–171.

20 **Groh, V., Rhinehart, R., Randolph-Habecker, J., Topp, M. S., Riddell, S. R. and Spies, T.,** Costimulation of CD8 $\alpha\beta$ T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat. Immunol.* 2001. **2:** 255–260.

21 **Tieng, V., Le Bouguenec, C., du Merle, L., Bertheau, P., Desreumaux, P., Janin, A., Charron, D. and Toubert, A.,** Binding of *Escherichia coli* adhesin AfaE to CD55 triggers cell-surface expression of the MHC class I-related molecule MICA. *Proc. Natl. Acad. Sci. USA* 2002. **99:** 2977–2982.

22 **O'Callaghan, C. A., Cerwenka, A., Willcox, B. E., Lanier, L. L. and Bjorkman, P. J.,** Molecular competition for NKG2D: H60 and RAE1 compete unequally for NKG2D with dominance of H60. *Immunity* 2001. **15:** 201–211.

23 **Carayannopoulos, L. N., Naidenko, O. V., Kinder, J., Ho, E. L., Fremont, D. H. and Yokoyama, W. M.,** Ligands for murine NKG2D display heterogeneous binding behavior. *Eur. J. Immunol.* 2002. **32:** 597–605.

24 **Carayannopoulos, L. N., Naidenko, O. V., Fremont, D. H. and Yokoyama, W. M.,** Cutting edge: murine UL16-binding protein-like transcript 1: a newly described transcript encoding a high-affinity ligand for murine NKG2D. *J. Immunol.* 2002. **169:** 4079–4083.

25 **Kawai, J., Shinagawa, A., Shibata, K. et al.,** Functional annotation of a full-length mouse cDNA collection. *Nature* 2001. **409:** 685–690.

26 **Biassoni, R., Pessino, A., Bottino, C., Pende, D., Moretta, L. and Moretta, A.,** The murine homologue of the human NKp46, a triggering receptor involved in the induction of natural cytotoxicity. *Eur. J. Immunol.* 1999. **29:** 1014–1020.

27 **Hayakawa, Y., Kelly, J. M., Westwood, J., Darcy, P. K., Diefenbach, A., Raulet, D. H. and Smyth, M. J.,** Tumor rejection mediated by NKG2D receptor-ligand interaction is strictly dependent on perforin. *J. Immunol.* 2002. **169:** 5377–5381.

28 **Gilfillan, S., Ho, E. L., Cella, M., Yokoyama, W. M. and Colona, M.,** NKG2D recruits two distinct adapters to trigger natural killer cell activation and costimulation. *Nat. Immunol.* 2002. **3:** 1150–1155.

29 **Diefenbach, A. and Raulet, D. H.,** The innate immune response to tumors and its role in the induction of T cell immunity. *Immunol. Rev.* 2002. **188:** 19–21.

30 **Krmpotic, A., Busch, D. H., Bubic, I., Gebhardt, F., Hengel, H., Hasan, M., Scalzo, A. A., Koszinowski, U. H. and Jonjic, S.,** MCMV glycoprotein gp40 confers virus resistance to CD8+ T cells and NK cells *in vivo*. *Nat Immunol.* 2002. **3:** 529–535.

31 **Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J.,** Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997. **25:** 3389–3402.

32 **Pear, W. S., Nolan, G. P., Scott, M. L. and Baltimore, D.,** Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* 1993. **90:** 8392–8396.

33 **Rost, B.,** PHD: predicting one-dimensional protein structure by profile-based neural networks. *Methods Enzymol.* 1996. **266:** 525–539.

34 **Thompson, J. D., Higgins, D. G. and Gibson, T. J.,** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 1994. **22:** 4673–4680.

35 **Vance, R. E., Tanamachi, D. M., Hanke, T. and Raulet, D. H.,** Cloning of a mouse homolog of CD94 extends the family of C-type lectins on murine natural killer cells. *Eur. J. Immunol.* 1997. **27:** 3236–3241.

36 **Maniatis, T., Fritsch, E. F. and Sambrook, J. (Eds.)** *Molecular cloning: a laboratory manual*, Cold Spring Harbor Laboratory Press*, Cold Spring Harbor* 1989.

37 **Tanamachi, D. M., Hanke, T., Takizawa, H., Jamieson, A. M. and Raulet, D. H.,** Expression of natural killer cell receptor alleles at different Ly49 loci occurs independently and is regulated by major histocompatibility complex class I molecules. *J. Exp. Med.* 2001. **193:** 307–315.

38 **Portnoy, D. A., Jacks, P. S. and Hinrichs, D. J.,** Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. *J. Exp. Med.* 1988. **167:** 1459–1471.

39 **Murali-Krishna, K., Altman, J. D., Suresh, M., Sourdive, D., Zajac, A., Miller, J., Slansky, J. and Ahmed, R.,** Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 1998. **8:** 177–187.

40 **Diefenbach, A., Schindler, H., Donhauser, N., Lorenz, E., Laskay, T., MacMicking, J., Röllinghoff, M., Gresser, I. and Bogdan, C.,** Type 1 interferon (IFNα/β) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. *Immunity* 1998. **8:** 77–87.

Correspondence: David H. Raulet, Department of Molecular and Cell Biology and Cancer Research Laboratory, 485 Life Sciences Addition, University of California at Berkeley, Berkeley, CA 94720–3200, USA
Fax: +1-510-642-1443
e-mail: raulet@uclink4.berkeley.edu