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

Andreas Diefenbach[1], Elena Tomasello[2], Mathias Lucas[2], Amanda M. Jamieson[1], Jennifer K. Hsia[1], Eric Vivier[2] and David H. Raulet[1]

Published online 11 November 2002; doi:10.1038/ni858

Optimal lymphocyte activation requires the simultaneous engagement of stimulatory and costimulatory receptors. Stimulatory immunoreceptors are usually composed of a ligand-binding transmembrane protein and noncovalently associated signal-transducing subunits. Here, we report that alternative splicing leads to two distinct NKG2D polypeptides that associate differentially with the DAP10 and KARAP (also known as DAP12) signaling subunits. We found that differential expression of these isoforms and of signaling proteins determined whether NKG2D functioned as a costimulatory receptor in the adaptive immune system ($CD8^+$ T cells) or as both a primary recognition structure and a costimulatory receptor in the innate immune system (natural killer cells and macrophages). This strategy suggests a rationale for the multisubunit structure of stimulatory immunoreceptors.

Antigen receptors and other stimulatory receptor complexes expressed by lymphoid and myeloid cells typically transduce intracellular signals by associating with immunoreceptor tyrosine-based activation motif (ITAM)–bearing signaling polypeptides, such as CD3ζ, immunoglobulin α (Igα, also known as CD79a) Igβ (CD79b) and FcRγ. Killer cell–activating receptor–associated polypeptide (KARAP[1], also known as DAP12[2]) is another ITAM-containing signaling adaptor molecule that is associated with various receptors expressed by natural killer (NK) and myeloid cells[3,4]. The immunoreceptors associate with the signaling adaptors *via* interactions between charged or polar amino acid residues in the transmembrane domains. Receptor engagement induces tyrosine phosphorylation of the ITAMs by Src family protein tyrosine kinases (PTKs), recruitment and activation of Syk–ZAP-70 family PTKs and downstream signaling events. Full activation often depends on engagement of a second (or costimulatory) receptor, such as CD28, inducible costimulator (ICOS) or CD19, which provides another degree of control over the immune response. Costimulatory receptors lack ITAMs, but display an intracytoplasmic YxxM motif, the phosphorylation of which leads to recruitment and activation of phosphatidylinositol-3 kinase (PI3K) family lipid kinases. NKG2D is an activating cell surface receptor that reportedly associates selectively with DAP10, an YxxM-bearing adaptor protein[5–7]. In the mouse, NKG2D is expressed by NK cells, activated macrophages, activated $CD8^+$ T cells, γδ T cells and $NK1.1^+$ T cells[8,9]. Cell surface ligands for NKG2D include the retinoic acid early transcript 1 (Rae-1) and H-60 protein families in mice[8,10] and the MIC[11] and ULBP families[12] in humans. Some of these ligands are up-regulated on transformed, infected or distressed cells[13,14]. Ligand expression by a tumor cell leads to potent activation of NK cells and $CD8^+$ T cells and induction of tumor immunity[15,16]. Consistent with the signaling properties of DAP10, NKG2D engagement provides a costimulatory rather than primary stimulatory signal to activated $CD8^+$ T cells[9,15,17]. Evidence suggests that NKG2D provides a direct stimulatory signal rather than a costimulatory signal in NK cells and activated macrophages[9]. Because receptors that deliver stimulatory signals in some cells and costimulatory signals in others have not been demonstrated, we investigated here the basis of the differential signaling properties of NKG2D in distinct cell types.

Results

Regulated expression of NKG2D splice variants

A comparison of two murine NKG2D cDNA sequences[18,19] revealed differences in both the 5′ and 3′ ends (**Fig. 1a**). Alignment with genomic sequences indicated that the two cDNAs had been generated by alternative splicing from a single gene encoding NKG2D (*D6H12S2489E*). The two open-reading frames (ORFs) were identical except for the 5′ end: one sequence—NKG2D long (NKG2D-L)—contained an additional upstream AUG codon compared to the other sequence—NKG2D short (NKG2D-S). This resulted in a predicted 13–amino acid extension at the NH_2 terminus of the protein (that is, in the cytoplasmic domain of these type II transmembrane proteins). Analysis of mRNA by semiquantitative polymerase chain reaction (PCR) demonstrated that the two isoforms were expressed differentially in distinct cell types and were regulated by cellular activation. Freshly isolated "naïve" NK cells contained large amounts of

[1]*Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, CA 94720-3200, USA.* [2]*Centre d'Immunologie de Marseille-Luminy, INSERM-CNRS-Univ. Méditerranée, 13288 Marseille cedex 09, France. Correspondence should be addressed to D. H. R. (raulet@uclink4.berkeley.edu) or E.V. (vivier@ciml.univ-mrs.fr).*

NKG2D-L mRNA, but NKG2D-S mRNA was almost undetectable (**Fig. 1b**). NKG2D-S mRNA was up-regulated in NK cells after activation for 18 h *in vivo* with poly(I•C) or activation for 2–4 days *in vitro* with interleukin 2 (IL-2). However, the NKG2D-S isoform was down-regulated after 9 days of culture in IL-2 (data not shown). Unstimulated CD8⁺ T cells and macrophages did not express either isoform (**Fig. 1b**). After T cell receptor (TCR) stimulation, CD8⁺ T cells substantially up-regulated both isoforms. Macrophages activated with bacterial lipopolysaccharide (LPS) up-regulated the NKG2D-S isoform but only modestly up-regulated NKG2D-L.

Adaptor associations of NKG2D splice variants

We analyzed next the potential of the two NKG2D splice variants to associate with signaling adaptor proteins expressed by NK cells. DAP10 and KARAP each supported surface expression of NKG2D-S, as determined by flow cytometric analysis of 293T cells that were transiently expressing a hemagglutinin (HA)-tagged version of the NKG2D-S isoform with different Flag-tagged adaptor proteins (**Fig. 2a**). In contrast, FcRγ had a smaller effect and CD3ζ had no effect. Associations of NKG2D-S with DAP10 or KARAP were confirmed by coimmunoprecipitating the adaptor proteins with NKG2D, or *vice versa*, from digitonin lysates of the transfected cells (**Fig. 2b**). In contrast to NKG2D-S, NKG2D-L was highly selective in its associations, as only DAP10 and not the other adaptors rescued cell surface expression (**Fig. 2**).

NKG2D was also associated with both DAP10 and KARAP in NK cells that had been stimulated for 4 days with IL-2, as shown by coimmunoprecipitation with polyclonal antibodies specific for a sequence in NKG2D that is shared by both isoforms (**Fig. 3a**). Converse immunoprecipitation experiments with antibodies specific for adaptor proteins confirmed that both DAP10 and KARAP associate with NKG2D in normal NK cells. No association of NKG2D with FcRγ or CD3ζ was detected in normal NK cells, which suggested that the association with FcRγ observed in transient transfections may have been due to protein overexpression (**Fig. 3a**). Comparable analysis revealed that NKG2D also associated with both KARAP and DAP10 in bone marrow–derived LPS-activated macrophages (**Fig. 3a**), which was consistent with selective expression of the promiscuous NKG2D-S isoform in these cells. In contrast, NKG2D was associated only with DAP10 in activated CD8⁺ T cells (**Fig. 3a**). Although these cells expressed the promiscuous NKG2D-S isoform, they did not express KARAP, as shown by reverse-transcribed–PCR (RT-PCR) (**Fig. 1b**) and direct immunoprecipitation (**Fig. 3a**). Thus, the immunoprecipitation experiments indicated that NKG2D associates with both KARAP and DAP10 in activated NK cells and activated macrophages, but only with DAP10 in CD8⁺ T cells.

Immunoprecipitation of KARAP consistently coimmunoprecipitated trace amounts of DAP10 and *vice versa* from lysates of NK cells and macrophages (**Fig. 3a**). This observation raised the possibility that KARAP associates with DAP10-NKG2D complexes indirectly by forming covalent heterodimers with DAP10. However, DAP10 and KARAP failed to coimmunoprecipitate from NK cell lysates prepared in the dissociating detergents Triton X-100 and NP-40, which argued against covalent dimerization of DAP10 and KARAP (**Fig. 3b**).

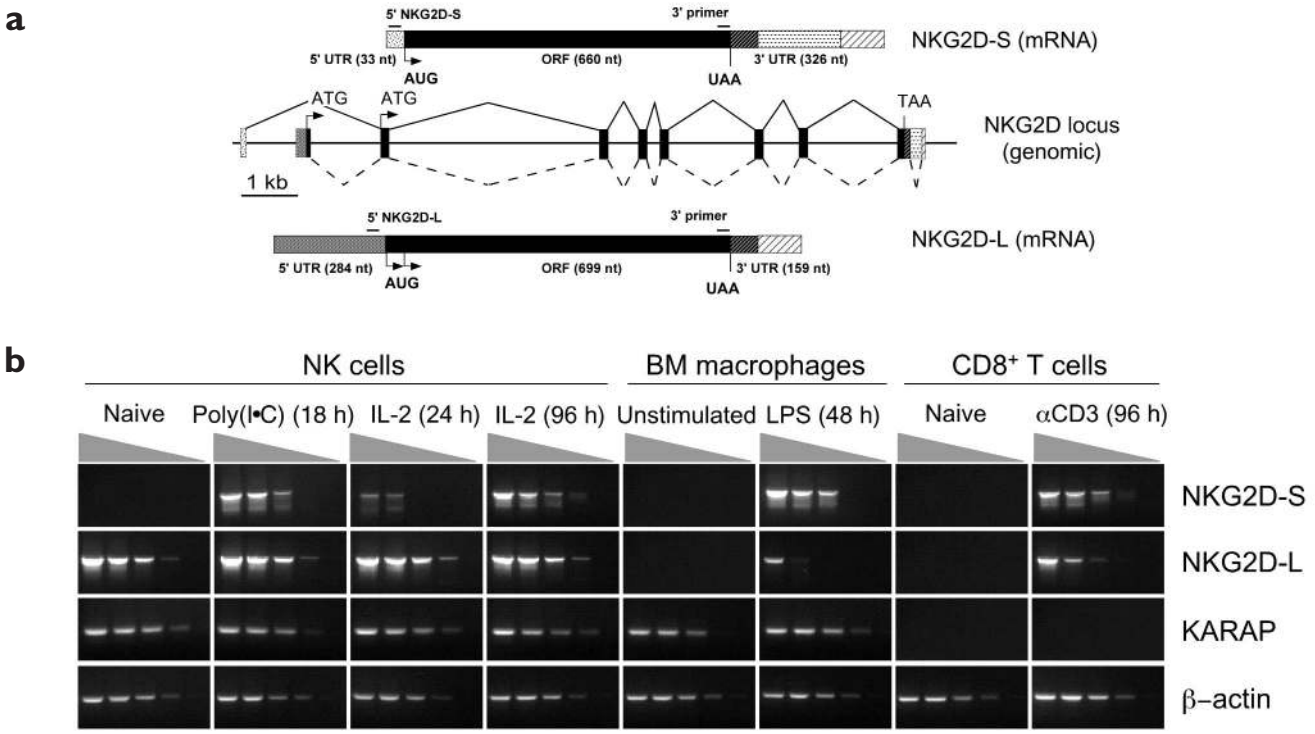

Figure 1. Expression of NKG2D splice variants in NK cells, CD8⁺ T cells and macrophages. (**a**) Exon-intron organization of the gene encoding NKG2D and the splices that generated the two alternatively spliced NKG2D mRNA species. The start (AUG) and the stop (UAA) codons as well as the positions of the primers used for detection of the different splice variants are indicated. (**b**) Semiquantitative RT-PCR analysis to detect the expression of NKG2D splice variants, KARAP and β-actin in highly purified NK cells, bone marrow macrophages and CD8⁺ T cells. Total RNA was prepared from cells and reverse transcribed. β-actin was quantified from each sample by quantitative-competitive PCR (data not shown). The cDNAs were normalized according to the calculated β-actin amounts. Serial tenfold dilutions of normalized cDNAs were used for amplifying the indicated mRNA species. A representative experiment is shown (n = 2).

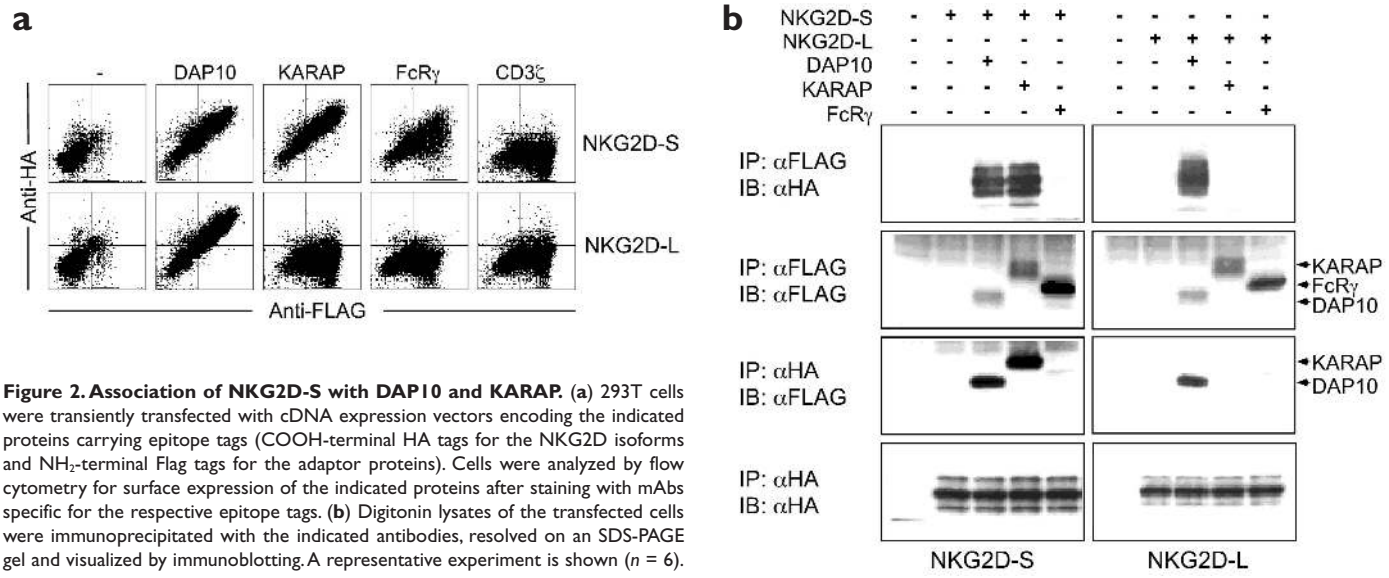

Figure 2. Association of NKG2D-S with DAP10 and KARAP. (**a**) 293T cells were transiently transfected with cDNA expression vectors encoding the indicated proteins carrying epitope tags (COOH-terminal HA tags for the NKG2D isoforms and NH_2-terminal Flag tags for the adaptor proteins). Cells were analyzed by flow cytometry for surface expression of the indicated proteins after staining with mAbs specific for the respective epitope tags. (**b**) Digitonin lysates of the transfected cells were immunoprecipitated with the indicated antibodies, resolved on an SDS-PAGE gel and visualized by immunoblotting. A representative experiment is shown (n = 6).

NKG2D function in KARAP-mutant mice

Consistent with the associations demonstrated by immunoprecipitation, cross-linking of NKG2D on NK cells and bone marrow–derived macrophages with a monoclonal antibody (mAb) to NKG2D resulted in tyrosine phosphorylation of both DAP10 and KARAP (**Fig. 4a**). Therefore, the interaction of NKG2D with both adaptors is likely to lead to signaling and to be of functional significance. To directly investigate the role played by KARAP in NKG2D function, we used the gene-targeted mouse strain KΔ75/KΔ75 in which the ITAM in the cytoplasmic domain of KARAP was inactivated[3]. The mutant KARAP adaptor protein is devoid of stimulatory function, but maintains the capacity to interact with stimulatory receptors and support their cell surface expression[3] (**Web Fig. 1** online). As expected, the mutation did not affect the expression of NKG2D by $CD8^+$ T cells, which do not express KARAP. Unexpectedly, the cell surface expression—mean fluorescence intensity (MFI)—of NKG2D was enhanced on NK cells or macrophages that expressed one or more mutant KARAP alleles. This suggested that wild-type KARAP normally limits NKG2D expression on the cell surface (**Web Fig. 1** online). Because it was possible that the mutant KARAP molecule dominantly interferes with the function of DAP10 in the same receptor complex, we examined tyrosine phosphorylation of DAP10 in NK cells from the mutant mice after NKG2D cross-linking (**Fig. 4a**). Comparable DAP10 tyrosine phosphorylation was observed in NK cells from homozygous mutant (KΔ75/KΔ75), wild-type and heterozygous mice, which ruled out a major dominant-negative effect of the mutant KARAP molecule on DAP10 function. In addition, the mutant molecule did not exert significant dominant-negative effects on KARAP function because heterozygous NK cells responded as well as wild-type NK cells to stimulation through Ly49D, a KARAP-dependent receptor (**Fig. 4b**)[3].

To directly assess the role of KARAP in NKG2D function, we analyzed the response of poly(I•C)-activated NK cells derived from KARAP-mutants and wild-type littermates to B16-BL6 (B16) melanoma cells or B16 transductants expressing Rae-1β or H-60

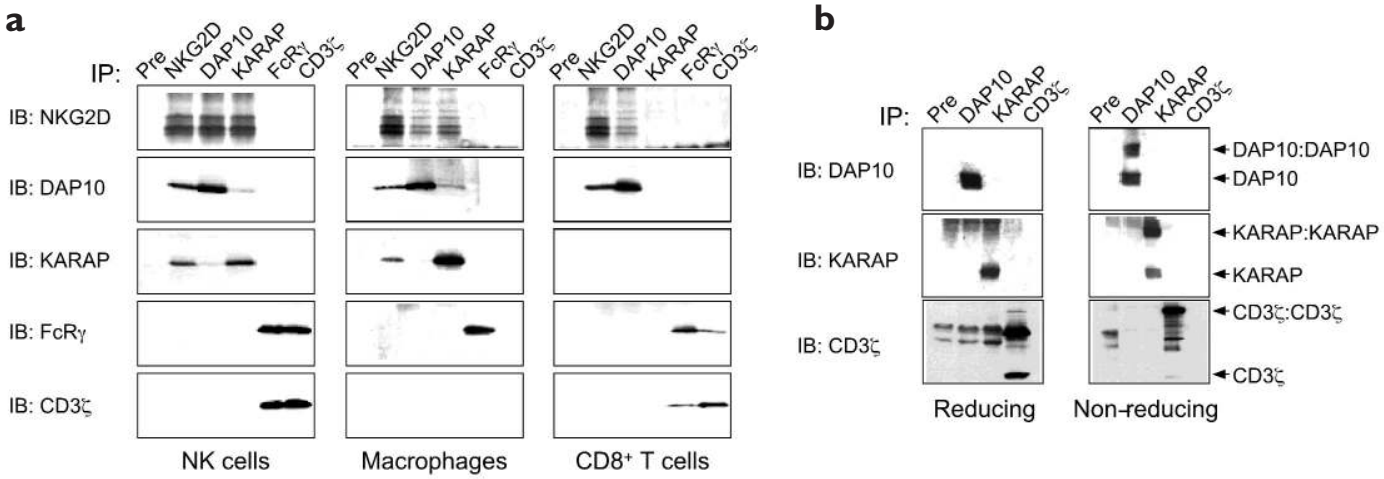

Figure 3. Association of NKG2D with DAP10 and KARAP in NK cells and macrophages. (**a**) Digitonin lysates from the indicated cell populations (purity >95%) were immunoprecipitated with the indicated antibodies, separated in a SDS-PAGE gel and proteins were detected by immunoblotting. A representative experiment is shown (n = 5). (**b**) TritonX-100–NP-40 lysates from NK cells (>95% purity) were immunoprecipitated with the indicated antibodies, resolved on an SDS-PAGE gel under reducing or nonreducing conditions and visualized by immunoblotting. A representative experiment is shown (n = 2). Pre, preimmune serum.

(referred to hereafter as B16.Rae-1β and B16.H-60 cells, respectively), which are two ligands for NKG2D[8]. Interferon-γ (IFN-γ) production (**Web Fig. 2** online) and cytolytic activity (**Fig. 4c**) were assessed. The mutant NK cells were highly impaired in both functional assays. A mAb specific for NKG2D blocked the response of wild-type NK cells to B16.Rae-1β or B16.H-60 cells, which confirmed that the Rae-1–H-60 response depends on NKG2D (**Fig. 4c**). An almost identical trend in cytotoxicity was observed with transductants of a second tumor cell line, the lymphoma line RMA, which are referred to as RMA.Rae-1β or RMA.H-60 cells (**Web Fig. 2** online). In addition, the recognition of Rae-1β– or H-60–transduced target cell lines by day 3 IL-2–stimulated NK cells was severely impaired (data not shown). The mutant NK cells did not show general defects in the capacity to produce IFN-γ, as they responded normally to a nonspecific stimulatory signal, ionomycin combined with 4-α-phorbol 12-myristate 13-acetate (PMA) (**Fig. 4d**). In addition, the *in vivo* elimination of B16.Rae-1β, B16.H-60, RMA.Rae-1β or RMA.H-60 cells was substantially impaired in KARAP loss-of-function mutant compared to wild-type mice (data not shown). These experiments showed that KARAP plays a pivotal role in the response of NK cells to Rae-1β– or H-60–transduced target cells.

It remained possible that NKG2D provides only a costimulatory signal to NK cells *via* DAP10 and that another NK receptor with an unknown ligand provides the essential KARAP-mediated stimulatory signal. To directly investigate the role of KARAP in NKG2D signaling, NK cells from poly(I•C)-treated mice (**Web Fig. 2** online) or day 3 IL-2–stimulated NK cells (**Fig. 4b**) were activated with F(ab′)$_2$ fragments of an NKG2D mAb affixed to plastic wells, and the induced accumulation of intracellular IFN-γ was measured. In agreement with published data, cross-linking of NKG2D with antibody was sufficient to activate wild-type NK cells[9]. In contrast, acti-

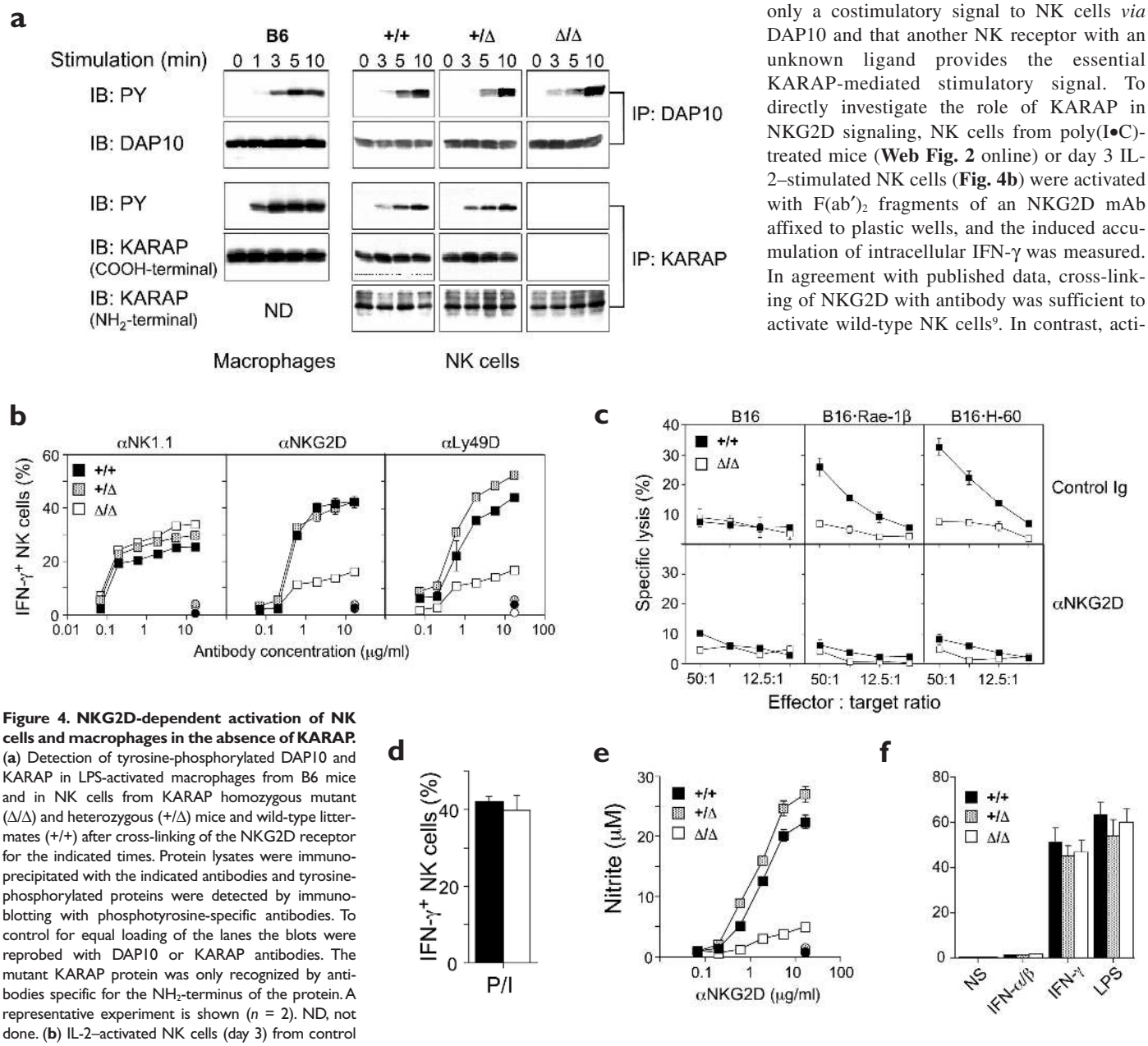

Figure 4. NKG2D-dependent activation of NK cells and macrophages in the absence of KARAP. (**a**) Detection of tyrosine-phosphorylated DAP10 and KARAP in LPS-activated macrophages from B6 mice and in NK cells from KARAP homozygous mutant (Δ/Δ) and heterozygous (+/Δ) mice and wild-type littermates (+/+) after cross-linking of the NKG2D receptor for the indicated times. Protein lysates were immunoprecipitated with the indicated antibodies and tyrosine-phosphorylated proteins were detected by immunoblotting with phosphotyrosine-specific antibodies. To control for equal loading of the lanes the blots were reprobed with DAP10 or KARAP antibodies. The mutant KARAP protein was only recognized by antibodies specific for the NH$_2$-terminus of the protein. A representative experiment is shown (n = 2). ND, not done. (**b**) IL-2–activated NK cells (day 3) from control and mutant mice were stimulated with the indicated plate-bound antibodies (squares) or control antibodies (circles) and accumulation of IFN-γ was evaluated by intracellular cytokine staining. A representative experiment is shown (n = 4). (**c**) The cytotoxicity of NK cells from poly(I•C)-treated KARAP-mutant mice (open squares) or wild-type littermates (closed squares) in response to B16.Rae-1β and B16.H-60 cells was assessed. Effector cells were incubated with a control antibody or a mAb (MI-6) to NKG2D. A representative experiment is shown (n = 3). (**d**) Freshly isolated NK cells from poly(I•C)-treated KARAP-mutant mice (open bars) or wild-type littermates (filled bars) were stimulated with PMA and ionomycin (P/I) and accumulation of IFN-γ was evaluated by intracellular cytokine staining. (**e,f**) IFN-α/β stimulated BM-derived macrophages from control and mutant mice were stimulated on plates coated with the indicated antibodies (circles) (**e**) or incubated with the indicated cytokines (**f**). Nitrite accumulation in the culture supernatants was determined with the Griess assay. A representative experiment is shown (n = 2).

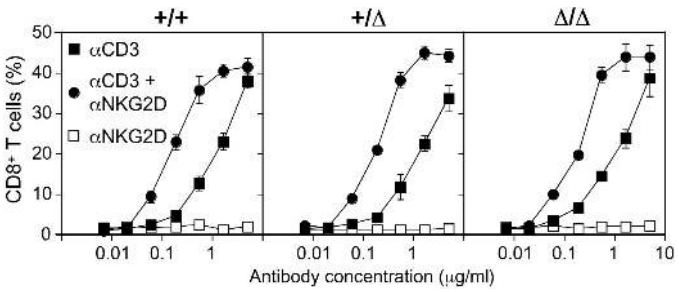

Figure 5. NKG2D-DAP10–dependent costimulation of CD8⁺ T cells. Activated CD8⁺ T cells (>97% purity) from homozygous KARAP-mutant (Δ/Δ) and heterozygotes (+/Δ) mice or wild-type littermates (+/+) were labeled with CFSE and then stimulated with the indicated plate-bound antibodies. Proliferation is shown as the percentage of CD8⁺ T cells undergoing three or more divisions, as determined by flow cytometry after 4 days. A representative experiment is shown (n = 2).

vation of KARAP-mutant NK cells by NKG2D mAb was markedly reduced, as was activation with a mAb specific for a known KARAP-dependent receptor, Ly49D (**Fig. 4b** and **Web Fig. 2** online)[3]. Stimulation with a mAb specific for NKR-P1C, which is not KARAP-dependent, was unaffected by the mutation. NK cells from KARAP heterozygous mice showed normal responses to stimulation with plate-bound Ly49D and NKG2D mAbs, which ruled out the possibility of a major dominant-negative effect of the mutant molecule (**Fig. 4b**).

A similar approach was used to investigate the activation of bone marrow macrophages, which were cultured with IFN-α/β to up-regulate NKG2D. Nitric oxide (NO) production induced by $F(ab')_2$ fragments of NKG2D mAbs was markedly impaired in macrophages from KARAP-mutant mice (**Fig. 4e**). The mutant macrophages were fully responsive to other NO-inducing signals, including IFN-γ and LPS (**Fig. 4f**). Thus, NKG2D signaling in macrophages, as in NK cells, was dependent on KARAP. In contrast, the costimulatory function of NKG2D was not impaired in KARAP-mutant CD8⁺ T cells, as shown by stimulating the cells simultaneously through NKG2D and the TCR (**Fig. 5**).

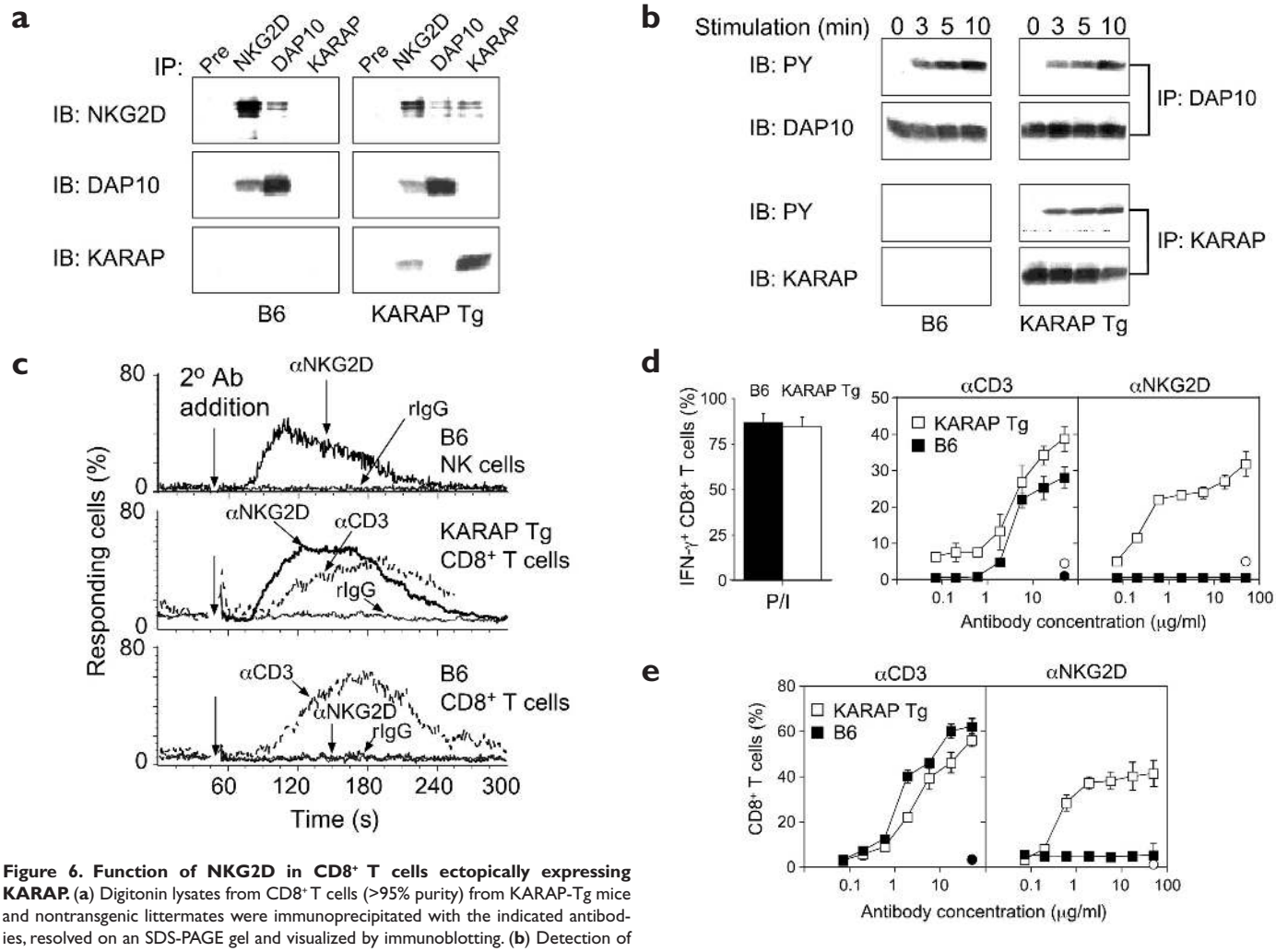

Figure 6. Function of NKG2D in CD8⁺ T cells ectopically expressing KARAP. (**a**) Digitonin lysates from CD8⁺ T cells (>95% purity) from KARAP-Tg mice and nontransgenic littermates were immunoprecipitated with the indicated antibodies, resolved on an SDS-PAGE gel and visualized by immunoblotting. (**b**) Detection of tyrosine-phosphorylated signaling adaptor proteins from CD8⁺ T cells from KARAP-Tg mice and littermate controls after cross-linking of the NKG2D receptor. Protein lysates were immunoprecipitated with the indicated antibodies and tyrosine-phosphorylated proteins were detected by immunoblotting with phosphotyrosine-specific antibodies. To control for equal loading of the lanes the blots were reprobed with DAP10 or KARAP antibodies, respectively. (**c**) Previously activated CD8⁺ T cells from KARAP-Tg mice and littermate controls were restimulated with the indicated mAbs and mobilization of intracellular Ca^{2+} was analyzed by flow cytometry. 2° Ab, secondary antibody. (**d,e**) Activated CD8⁺ T cells from KARAP-Tg mice and littermate controls were stimulated with PMA and ionomycin or with the indicated plate-bound antibodies (squares) or control antibodies (circles) and (**d**) IFN-γ production and (**e**) proliferation of CFSE-labeled CD8⁺ T cells were analyzed by flow cytometry. A representative experiment is shown (n = 2).

Ectopic expression of KARAP and NKG2D function

Activated CD8+ T cells express both NKG2D-S and NKG2D-L (**Fig. 1b**), but are unresponsive to stimulation with NKG2D mAb alone (**Fig. 5**)[9]. Therefore, we tested the hypothesis that the failure of CD8+ T cells to respond to NKG2D stimulation is due to the absence of KARAP in these cells. We examined CD8+ T cells from mice that ectopically expressed KARAP due to the presence of a KARAP transgene under the control of a major histocompatibility complex class I promoter[20], referred to as KARAP transgenic (Tg) mice. Surface expression of NKG2D was comparable on CD8+ T cells from KARAP-Tg mice and non-Tg littermates after 5 days of TCR stimulation (**Web Fig. 1** online). NKG2D in the transgenic CD8+ T cells was associated with both KARAP and DAP10, as demonstrated by coimmunoprecipitation (**Fig. 6a**). Cross-linking of NKG2D led to rapid tyrosine phosphorylation of KARAP in transgenic CD8+ T cells (**Fig. 6b**), whereas tyrosine phosphorylation of DAP10 was comparable between transgenic and control CD8+ T cells. Cross-linking of NKG2D on transgenic but not control CD8+ T cells resulted in substantial mobilization of intracellular Ca^{2+}, production of IFN-γ and proliferation, which showed that the transgene converted NKG2D into a directly stimulatory receptor (**Fig. 6c–e**). Thus, the introduction of KARAP into CD8+ T cells enabled the NKG2D receptor to provide a direct stimulatory signal that was comparable to a TCR-derived signal (**Fig. 6c–e**). Together with the data we obtained using KARAP-mutant mice, these results showed that KARAP can confer direct stimulatory functions to the NKG2D receptor.

Discussion

Our results demonstrate discrete biological activities for NKG2D in different cell types as a result of associations with different adaptor proteins. The association pattern is determined by the differential expression of KARAP *versus* DAP10 as well as the regulated availability in immune cells of the appropriate NKG2D splice variant. The adaptor association pattern enables NKG2D to provide functions most suitable to the cell type in which it occurs. Thus, in cells of the innate immune system (NK cells and macrophages), the receptor is predicted to provide both stimulatory and costimulatory activity and will therefore efficiently elicit a direct attack upon transformed or infected cells expressing NKG2D ligands[14,21]. In contrast, the same ligands provide most activated CD8+ T cells with only a costimulatory signal, which may aid in amplifying the cytolytic T lymphocyte response specific for tumor or pathogen-associated antigens. It remains possible that signaling molecules other than the adaptor proteins studied here can also regulate functional interconversions of the NKG2D receptor.

Our results are well supported by complementary data from Colonna and colleagues published in this issue[22]. Colonna's group investigated mice in which the gene encoding DAP10 was disrupted by gene targeting. They found that in DAP10-deficient mice, NKG2D function was abrogated in CD8+ T cells but only partially impaired in activated NK cells. This was consistent with our findings that NKG2D signals through KARAP (as well as DAP10) in NK cells. In addition, naïve NK cells from DAP10-deficient mice showed markedly reduced surface NKG2D[22], which was consistent with our finding that naïve NK cells express only the NKG2D-L isoform, which pairs with DAP10 and not KARAP.

CD8+ T cell clones acquire "promiscuous" or "natural" cytotoxicity when cultured *in vitro* under certain conditions[23,24]. The acquisition by T cells of these functional activities characteristic of the innate immune response could be due, at least in part, to the induction of KARAP in these cells. KARAP up-regulation may also occur in T cells under physiological conditions, as suggested by the identification of discrete KARAP+ T cell subsets in humans[25] (and unpublished observations). If so, such T cells could function like NK cells in eliminating transformed or infected cells that express NKG2D ligands, despite lacking TCR specificity for these target cells.

Interconversions of NKG2D functional isoforms may also regulate NK cell activity. Freshly isolated "naïve" NK cells, which generally respond poorly to NK-sensitive target cells[15,26], lack the NKG2D-S isoform necessary for associations with KARAP. NKG2D-S is induced in these cells by poly(I•C) *in vivo* or IL-2 *in vitro* in parallel with the up-regulation of NK effector functions (a process called NK cell "priming"). Hence, regulated RNA splicing that leads to production of NKG2D-S may constitute one component of NK cell priming and may represent an example of how one type of innate immune stimulus (double-stranded RNA) regulates the response to another type of innate immunity stimulus (NKG2D signaling). Consequently, although naïve NK cells do not initially express the directly stimulatory isoform of NKG2D, this isoform may be rapidly induced by innate stimuli so that the cells can respond directly to target cells expressing NKG2D ligands. The finding that NKG2D-S is down-regulated after prolonged culture in IL-2 may be a manifestation of mechanisms that moderate NK cell functions under specific conditions of activation.

Transmembrane charged amino acid residues are critical for the assembly of other receptor-adaptor complexes[27], including NKG2D-DAP10[7]. The differential associations of NKG2D-S and NKG2D-L with adaptor proteins is notable because the two isoforms share identical transmembrane and ectodomains and only differ by the presence of a 13-aa NH_2-terminal extension (MALIRDRKSHHSE) in NKG2D-L compared to NKG2D-S. This difference is maintained in the cytoplasmic domains of the mature protein because the NH_2-terminal sequences of type II transmembrane proteins like NKG2D are not removed after translation. Therefore, our results suggest that the cytoplasmic domain can be an important determinant of the specificity of receptor-adaptor interactions. One possibility is that the extended NH_2 terminus of NKG2D-L specifically obstructs interactions with the cytoplasmic domain of KARAP but not DAP10 as these complexes are being assembled in the endoplasmic reticulum[28].

Based on cotransfection experiments as well as coimmunoprecipitation experiments in the human NKL cell line[7], human NKG2D selectively associates with DAP10 and not with KARAP. In addition, stimulation of NKL cells with plate-bound NKG2D mAb alone does not lead to IFN-γ secretion, but potently costimulates cytokine secretion if cross-linked with an ITAM-containing receptor[7]. At present, a single product of the gene encoding human NKG2D has been reported, and its predicted sequence aligns with the mouse NKG2D-L isoform[29]. Therefore, these results would be compatible with the selective association of NKG2D-L with DAP10, which confers costimulatory, but not direct stimulatory, function. However, analysis of the genomic locus that contains the human NKG2D sequence suggests a similar splice acceptor-donor site that could lead to a splice variant similar to the mouse NKG2D-S isoform (unpublished observation). Further experiments with peripheral blood human NK cells that take into account the regulated expression of NKG2D-S will be required to determine whether human NK cells express a functional analog of NKG2D-S.

The genes encoding DAP10 and KARAP are directly adjacent to each other in the mouse and human genomes, separated by 131 and 307 bp, respectively[30]. This genomic clustering and their shared capacity to associate with a single receptor (NKG2D-S) is reminiscent of CD3ζ and FcRγ. Both the TCR in T cells and CD16 in NK cells can associate with FcRγ homodimers, CD3ζ homodimers or FcRγ-CD3ζ heterodimers[31–33].

It has been suggested that the signaling strength of TCRs can differ depending on the associated signaling molecules[34]. The biological relevance of combinatorial adaptor associations has not been clearly established in these multisubunit complexes. The differential associations of NKG2D with distinct adaptor proteins we report here efficiently couples different biological responses to a common receptor-ligand system and suggests an evolutionary rationale for the multisubunit structure of most stimulatory receptors in the immune system.

Methods

Genomic organization of the NKG2D locus and NKG2D splice variants. The genomic organization of the NKG2D locus was resolved by aligning the two published NKG2D cDNA sequences[18,19] to the public mouse genome database published by the Mouse Genome Sequencing Consortium (MGSC) with the BLAST 2 sequences alignment algorithm[35]. For the semiquantitative PCR experiments, cell-sorted NK cells (>98.3% $NK1.1^+CD3^-$ cells) were stimulated for 1, 4 and 9 days with 1 μg/ml of recombinant human IL-2 (hIL-2, Chiron, Emeryville, CA). Sorted $CD8^+$ T cells (>98.5% $CD8^+CD3^+$ T cells) were stimulated with 10 μg/ml of plate-bound CD3 mAb (500A2, a gift of J. P. Allison, UC Berkeley) for 96 h. Bone marrow–derived macrophages were prepared as described[36] and left unstimulated or stimulated for 48 h with 10 ng/ml of LPS (from *E. coli* O55:B5, Calbiochem, La Jolla, CA). β-actin mRNA concentrations in each sample were determined by quantitative-competitive RT-PCR[37]. After adjusting the concentration of each cDNA preparation accordingly, tenfold dilutions of the samples were subjected to PCR with various primers. Primer sequences that bind in the unique 5′ UTR of the two splice variants were 5′-TCC CTTCTCTGCTCAGAG-3′ (5′ NKG2D-S) and 5′-CAGGAAGCAGAGGCAGATTATCTC-3′ (5′ NKG2D-L). The common 3′ NKG2D primer was 5′-TTACACCGCCCTTTTCAT GCAGATG-3′. For detection of KARAP, we used 5′-CAGAGTGACACTTTCCCAAGAT GCG-3′ as a 5′ primer and 5′-TCATCTGTAATATTGCCTCTGTGTG-3′ as a 3′ primer. The β-actin primers were as described[37].

Transfections and vectors. cDNAs for the adaptor molecules were inserted into an expression vector (pME18S)[38] that contained the mouse CD8 leader segment followed by the Flag epitope (DYKDDDDK), which resulted in an extracellular, NH_2-terminal Flag tag for these type I transmembrane proteins. The cDNAs for the NKG2D-S and NKG2D-L were modified by the in-frame addition of a COOH-terminal HA epitope (YPYDVPDYA) and cloned into the same expression vector. After confirmation of the sequences, 293T cells were transiently transfected with expression vectors with Lipofectamine, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, the cells were either used to prepare digitonin lysates (see below) or stained with mAbs specific for the epitope tags (see below) and analyzed by flow cytometry.

Mice. C57BL/6/J mice were bred and housed under specific pathogen–free conditions in the UC Berkeley animal facilities in accordance with institutional guidelines. The gene-targeted mouse strain KΔ75/KΔ75, heterozygous mice and littermate controls that had been backcrossed to the C57BL/6 background for eight generations were as described[3]. KARAP-Tg mice were prepared as described[20]. Unless indicated, all the mice used in this study were 8–12 weeks old; they were age- and sex-matched.

***Ex vivo*–derived cell populations.** Splenocytes were incubated for 3 days in medium containing 1 μg/ml of recombinant hIL-2 to generate IL-2–stimulated NK cells for the biochemistry experiments and IFN-γ assays. The NK cells were purified with an autoMACS (Miltenyi Biotec, Auburn, CA) to negatively select cells that stained with fluorescein isothiocyanate (FITC)–conjugated mAbs specific for the TCRβ chain (H57-597, ebioscience, San Diego, CA), CD8α and CD4 (CT-CD8α and CT-CD4, respectively, both from Caltag, Burlingame, CA) and CD19 (1D3, BD Pharmingen, San Diego, CA), followed by anti-FITC microbeads. The resulting cell populations were >95% pure $NK1.1^+NKG2D^+CD3^-$ NK cells.

Freshly isolated NK cells from mice that had been injected with 200 μg of poly(I•C) (Sigma, St. Louis, MO) 18 h earlier were enriched by depleting B cells, T cells and granulocytes. Briefly, total splenocytes were depleted of B cells by incubating with goat anti–mouse IgG magnetic beads (Dynal, Oslo, Norway). Nonbinding cells were stained with mAbs (all rat anti-mouse) specific for CD4 (H129.19), CD8 (H59.101), CD3 (KT3) and Gr1 (RB6-8C5), followed by incubation with sheep anti–rat IgG beads (Dynal). The resulting cell population was 30–40% $NK1.1^+CD3^-$ NK cells.

Bone marrow macrophages were differentiated as described[36]. To up-regulate NKG2D expression, differentiated bone marrow macrophages (>98% $CD11b^+$) were stimulated with 10 ng/ml of LPS (Calbiochem) or with 500 U/ml of IFN-α/β (gift of I. Gresser, Centre National de la Recherche Scientifique, Villejuif, France).

For *in vitro* activation of T cells, splenocytes from mice were incubated on tissue culture plates coated with 10 μg/ml of CD3 mAb (500A2). Stimulated cell populations were collected after 5 days, $CD8^+$ T cells were stained with a FITC-conjugated mAb specific for CD8α and positively selected with anti-FITC microbeads and autoMACS. The resulting cell population was >97% $CD8^+CD3^+NKG2D^+$ T cells. Cells were then left to rest for 2 days in medium containing 10 ng/ml of IL-2 before use in the assays.

Generation of peptide antisera and antibodies for biochemistry. Antisera against mouse NKG2D, DAP10 and KARAP were obtained by immunizing rabbits three times with keyhole limpet hemocyanin (KLH)–conjugated peptides in adjuvant. The NKG2D peptide (KPAKWDTSQEQQKQ) is located near the NH_2 terminus of the molecule and is shared by both NKG2D isoforms (aa 10–24 of NKG2D-S and aa 23–37 of NKG2D-L). The DAP10 (PAQEDGRVYINMPGRG) peptide is located at the COOH terminus of the protein. Three KARAP-specific antisera were used; one was prepared in our laboratory as described above with a COOH-terminal peptide (PEVYSDLNTQRQYYR) for immunization and was used for analysis of cells from normal mice; antiserum C-20 (Santa Cruz Biotechnology, Santa Cruz, CA) specific for the COOH terminus and cross-reactive with mouse and human KARAP was used for analysis of KARAP-Tg mice; antiserum A-20 (Santa Cruz) specific for the NH_2 terminus was used for analysis of KARAP-mutant mice. The antibodies to FcRγ and CD3ζ were from Upstate Biotechnology (Lake Placid, NY) and Santa Cruz Biotechnology (6B10.2). For immunofluorescence analysis, immunoprecipitation and immunoblotting from cells transfected with epitope-tagged adaptor proteins, mAbs specific for the HA epitope (HA.11, Covance, Richmond, CA) or the Flag epitope (Flag-M2, Sigma) were used. Phosphotyrosine-containing proteins were detected with the PY99 mAb (Santa Cruz).

Stimulation for phosphotyrosine blots. Cells (5×10^7/ml) were incubated for 30 min on ice with 50 μg/ml of mAb MI-6[9] (specific for NKG2D), washed three times and treated at 37 °C with $F(ab')_2$ fragments of donkey anti–rat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) to cross-link NKG2D. At various times thereafter, ice-cold PBS containing 1 mM sodium orthovanadate was added, and protein lysates were prepared as described below.

Immunoprecipitation, SDS-PAGE and immunoblotting. For the coimmunoprecipitation experiments, cells were lysed in digitonin lysis buffer containing 1% Digitonin (Calbiochem), 0.12% Triton X-100, 150 mM NaCl, 20 mM triethanolamine, 1 mM EDTA at pH 8.0 (all from Sigma) and the protease inhibitors 1 mM PMSF and 5 μg/ml of pepstatin A, aprotinin, chymostatin and leupeptin (Roche Molecular Biochemicals, Indianapolis, IN). Immunoprecipitation was done by incubation with protein A/G agarose beads (Santa Cruz). Lysates from 20×10^6 cells were loaded per lane on 12.5% SDS-PAGE gels. After transfer to nitrocellulose membranes, proteins were immunoblotted with antibodies, followed by incubation with a horseradish peroxidase–coupled secondary antibody (Jackson Immunoresearch Laboratories) and visualization with a chemiluminescent substrate (Pierce, Richmond, IL). The Triton-X100–NP-40 lysis buffer was as described[39]. Preparation of lysates for the detection of tyrosine-phosphorylated proteins was done as described[39].

Cytotoxicity assays. The B16-BL6 (melanoma) and the RMA (T cell lymphoma) target cell lines that had been retrovirally transduced to express high amounts of the NKG2D ligands Rae-1β and H-60 were as described[15]. Lysis was determined with a standard 4-h ^{51}Cr-release assay[40]. Spontaneous lysis never exceeded 10%. The procedure to block interaction of the NKG2D receptor with its ligands on the target cells was as described[9]. Data represent the mean ± s.d. of triplicate measurements.

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 various target cell populations. Induction of IFN-γ production by stimulation with plate-bound antibodies was done in microtiter plates that had been coated overnight with intact mAb or $F(ab')_2$ fragments (anti-NKG2D, MI-6; anti-Ly49D, SED85; anti-NK1.1, PK136; and anti-CD3, 500A2)[9,41,42]. IFN-γ production in response to target cells and plate-bound antibodies was determined by intracellular cytokine staining[43]. Data represent the mean ± s.d. of triplicate measurements.

Macrophage stimulation. Bone marrow–derived macrophages that had been activated with IFN-α/β to up-regulate expression of NKG2D were transferred to plates that had been coated with mAbs. After 48 h, nitrite accumulation was determined from triplicates with the Griess reagent as described[37]. Data represent the mean ± s.d. of triplicate measurements.

T cell stimulation assays. Carboxyfluorescein diacetate succinimidyl diester (CFSE)–labeled $CD8^+$ T cells[9] were added to microtiter plates coated with mAbs. After 4 days of stimulation, cells were counterstained with propidium iodide and analyzed by flow cytometry. Data represent the mean ± s.d. of triplicate measurements.

To analyze calcium mobilization, cells were labeled with 3 μM Indo-1AM and pluronic detergent according to the manufacturer's instructions (Molecular Probes, Eugene, OR). 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). The cross-linking $F(ab')_2$ fragment of the secondary antibody donkey anti–rat IgG (Jackson Immunoresearch Laboratories) was added after 40 s of data collection.

Web addresses. The MGSC is at http://www.ensembl.org/Mus_musculus/.

Genbank accession numbers. The NKG2D-S[18] accession number is AF030313 and the NKG2D-L[19] accession number is AF054819.

Note: Supplementary information is available on the Nature Immunology *website.*

Acknowledgments

Supported by NIH grants (to D. H. R.) and by a Howard Hughes Medical Institute Physician Postdoctoral grant (to A. D.), by institutional grants from INSERM, CNRS and the Ministère de l'Enseignement Supérieur et de la Recherche (to E.V.), and specific grants from Ligue Nationale contre le Cancer (to M. L.) and 'Equipe labellisée La Ligue' (to E.V.).

Competing interests statement

The authors declare that they have no competing financial interests.

Received 17 August 2002; accepted 7 October 2002.

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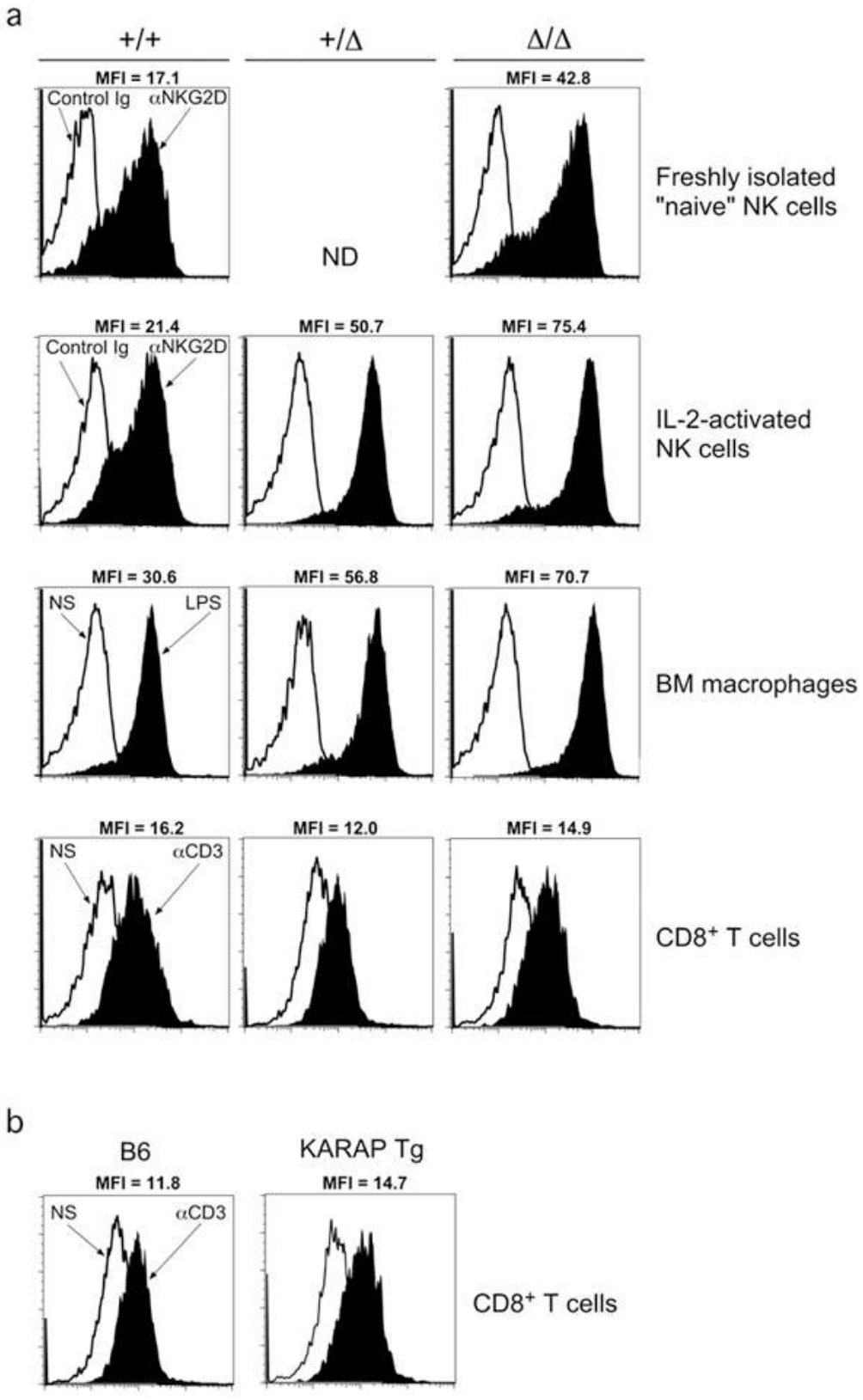
Web Fig. 1. Cell surface expression of NKG2D on cell populations from KARAP-mutant and KARAP-Tg mice. The indicated cell populations were analyzed by flow cytometry for cell surface expression of NKG2D by staining with a mAb. The histograms show electronic gating on the respective cell populations. The MFI of the gated positive cells is indicated above each histogram. (a) Analysis of NKG2D cell surface expression in homozygous KARAP-mutant mice (/), heterozygotes (+/) or wild-type littermates (+/+). (b) Analysis of CD8+ T cells from KARAP-Tg mice and nontransgenic littermates. ND, not done; NS, not stimulated.

Web Fig. 2. NKG2D-dependent NK cell activation in the absence of KARAP. (a) Freshly isolated NK cells from poly(IC)-treated KARAP-mutant mice (/, open bars) or wild-type littermates (+/+, solid bars) were stimulated with RMA lymphoma cells transduced or not with the NKG2D ligands Rae-1 or H-60 (left panel) or with the indicated plate-bound antibodies (right panel). Accumulation of IFN- was evaluated by intracellular cytokine staining. A representative experiment is shown ($n = 3$). (b) The cytotoxicity of freshly isolated NK cells from poly(IC)-treated KARAP-mutant mice (/, open squares) or wild-type littermates (+/+, closed squares) against RMA lymphoma cells transfected or not with Rae-1 or H-60 as indicated. The effector cells were incubated with a control antibody (upper panels) or a mAb to NKG2D (lower panels). A representative experiment is shown ($n = 4$).

Fig 1


a
+/+
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MFI = 42.8
Freshly isolated "naive" NK cells
ND
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Control Ig
αNKG2D
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MFI = 75.4
IL-2-activated NK cells
MFI = 30.6
NS
LPS
MFI = 56.8
MFI = 70.7
BM macrophages
MFI = 16.2
NS
αCD3
MFI = 12.0
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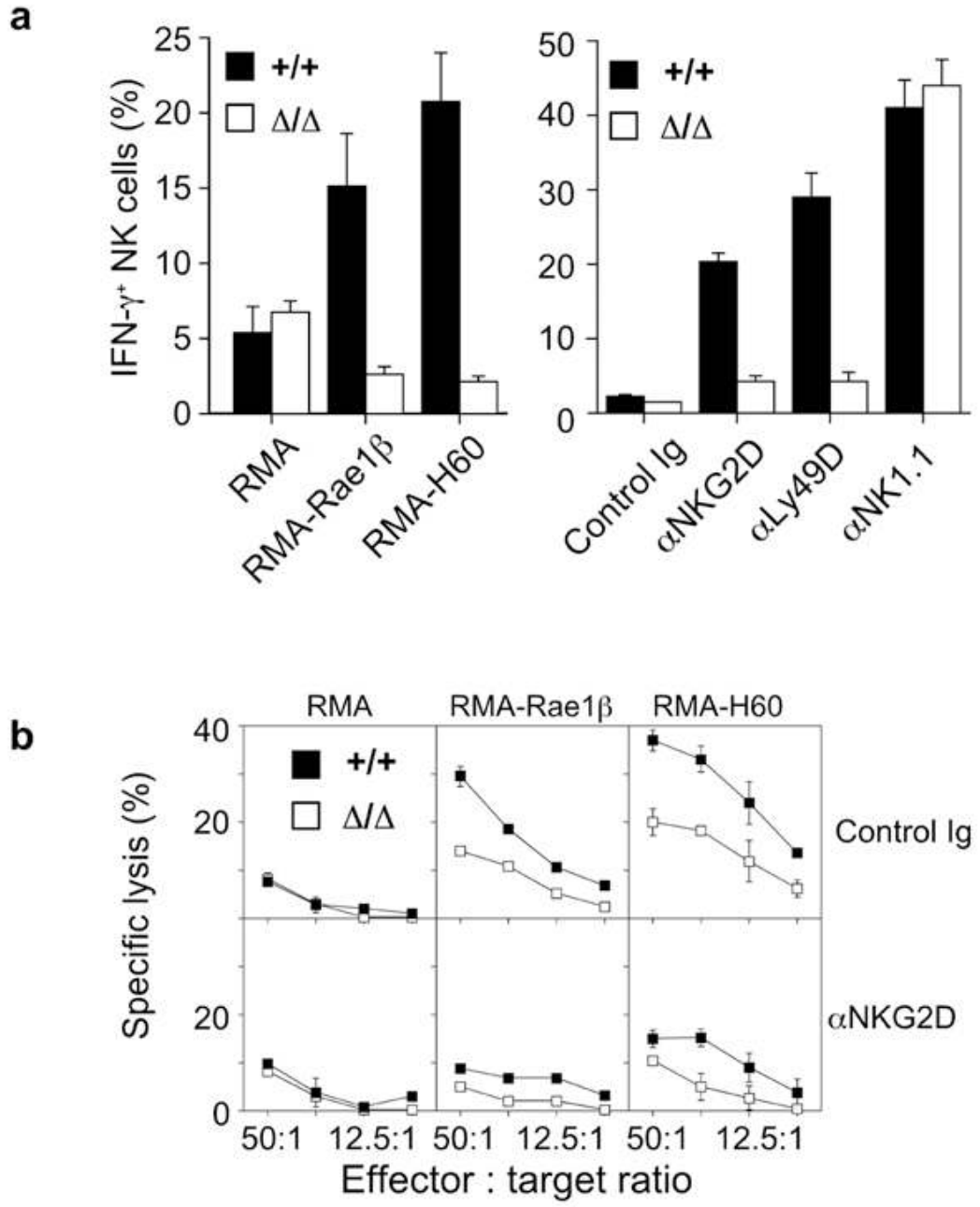
KARAP Tg
MFI = 11.8
NS
αCD3
MFI = 14.7
CD8+ T cells

Fig 2


a
IFN-γ+ NK cells (%)
+/+
Δ/Δ
RMA
RMA-Rae1β
RMA-H60
Control Ig
αNKG2D
αLy49D
αNK1.1
b
RMA
RMA-Rae1β
RMA-H60
Specific lysis (%)
Control Ig
αNKG2D
50:1
12.5:1
Effector : target ratio