NKG2D recruits two distinct adapters to trigger NK cell activation and costimulation

Susan Gilfillan[1], Emily L. Ho[2], Marina Cella[1], Wayne M. Yokoyama[2] and Marco Colonna[1]

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NKG2D is a receptor on natural killer (NK) cells and cytotoxic T lymphocytes that binds major histocompatibility complex (MHC) class I–like ligands expressed primarily on virally infected and neoplastic cells. *In vitro* studies indicate that NKG2D provides costimulation through an associated adapter, DAP10, which recruits phosphatidylinositol-3 kinase. Here we show that in DAP10-deficient mice, CD8$^+$ T cells lack NKG2D expression and are incapable of mounting tumor-specific responses. However, DAP10-deficient NK cells express a functional NKG2D receptor due to the association of NKG2D with another adapter molecule, DAP12 (also known as KARAP), which recruits protein tyrosine kinases. Thus, NKG2D is a versatile receptor that, depending on the availability of adapter partners, mediates costimulation in T cells and/or activation in NK cells.

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NKG2D is a C-type lectin-like receptor encoded within the natural killer (NK) gene complex that is constitutively expressed on all NK cells and up-regulated on activated CD8$^+$ T cells, γδ T cells and macrophages. Among the numerous activating receptors expressed primarily on NK cells[1], NKG2D is distinct in that it recognizes a number of disparate major histocompatibility complex (MHC) class I–related molecules; these include MICA and MICB in humans[2], the murine minor histocompatibility antigen H-60[3,4] and retinoic acid–inducible gene products encoded by the murine genes *Raet1a*, *Raet1b*, *Raet1c* and *Raet1d* [3,4] and their putative human counterparts, the ULBP genes[5,6]. NKG2D ligands do, however, share some characteristics: many are not constitutively expressed in adult tissues, but are inducible and expressed in tumor cells. MICA and MICB are frequently expressed in epithelial tumors and can be induced by stress[7,8]. *Raet1a*, *Raet1b*, *Raet1c* and *Raet1d* (which encode Rae-1α, Rae-1β, Rae-1γ and Rae-1δ, respectively) are expressed early during ontogeny but not in adult tissues[3,4]. ULBP expression is low in various tissues but higher in some tumors[5]. Expression of MIC and ULBP on human tumor cells is sufficient to overcome the inhibitory effects of MHC class I expression on NK cell killing[2,5]. Similarly, overexpression of Rae-1 and H-60 on murine tumor cells leads to NKG2D-mediated rejection of the tumors by NK, CD8$^+$ T and γδ T cells[9–11]. In addition, NKG2D augments the αβ TCR responses of CD28$^-$CD8$^+$ T cells to targets that have up-regulated MIC due to cytomegalovirus (CMV) infection[12]. Together, these observations indicate that NKG2D provides first-line surveillance against stressed or "abnormal" cells that have been induced to express one of its ligands.

Lacking intrinsic cytoplasmic signaling motifs, NKG2D is thought to depend solely on the transmembrane adapter DAP10 for cell-surface expression and function[13]. The majority of transmembrane adapters—including CD3γ, CD3δ, CD3ε, CD3ζ, FcRγ and DAP12 (also known as KARAP)—trigger intracellular signaling pathways that are dependent on the protein tyrosine kinases Syk and/or ZAP-70 *via* immunoreceptor tyrosine-based activation motifs (ITAMs), defined by the consensus sequence YxxL$_{6-8x}$YxxL/I, in their cytoplasmic tails[14–17]. In contrast, DAP10 contains an YxxM motif that, when phosphorylated, binds the p85 subunit of phosphatidylinositol-3 kinase (PI3K)[13,18,19] and Grb2[18]. Engagement of NKG2D-DAP10 is expected to induce costimulatory signals analogous to those transmitted by CD28, which shares the DAP10 YxxM motif[20,21]. Given the distinct nature of the DAP10 adapter and its only known ligand NKG2D, we decided to further elucidate their functions by generating DAP10-deficient mice.

Results

Generation of DAP10-deficient mice

The DAP10 targeting construct was designed to replace exon 1 and intron 1 of the gene encoding DAP10 with the MC1-*neo*^{r} gene flanked by *loxP* sites (**Fig. 1a**). MC1-*neo*^{r} was then deleted by breeding mice heterozygous for the MC1-*neo*^{r} insertion (DAP10$^{n/+}$ mice) to mice expressing a *Cre* transgene[22]. DAP10$^{-/+}$ mice were crossed to produce mice homozygous for the deletion (**Fig. 1b**); these mice expressed virtually no full-length mRNA that was detectable by northern blot analysis of spleen RNA (**Fig. 1c**). Immunoblot analysis confirmed that no DAP10 protein was present in either activated NK or CD8$^+$ T cells from the DAP10$^{-/-}$ animals (**Fig 1d**). Because the gene encoding DAP12 (*Hcst*) is located only 307 bp from the 3′ end of the gene encoding DAP10 (*Tyrobp*), we ensured that our targeting had not caused an unanticipated effect on DAP12 expression (**Fig. 1c,d**).

DAP10$^{-/-}$ mice were born at the expected frequency, bred well and were phenotypically normal. Histological analysis of heart, lung, liver, kidney, stomach, intestine, brain, thymus and skin from mice aged

[1]*Department of Pathology and Immunology, Washington University School of Medicine, 660 S. Euclid, St. Louis, MO 63110, USA.* [2]*Howard Hughes Medical Institute, Rheumatology Division, Department of Medicine, Barnes-Jewish Hospital and Washington University School of Medicine, 660 S. Euclid, St. Louis, MO 63110, USA. Correspondence should be addressed to M. C. (mcolonna@pathology.wustl.edu).*

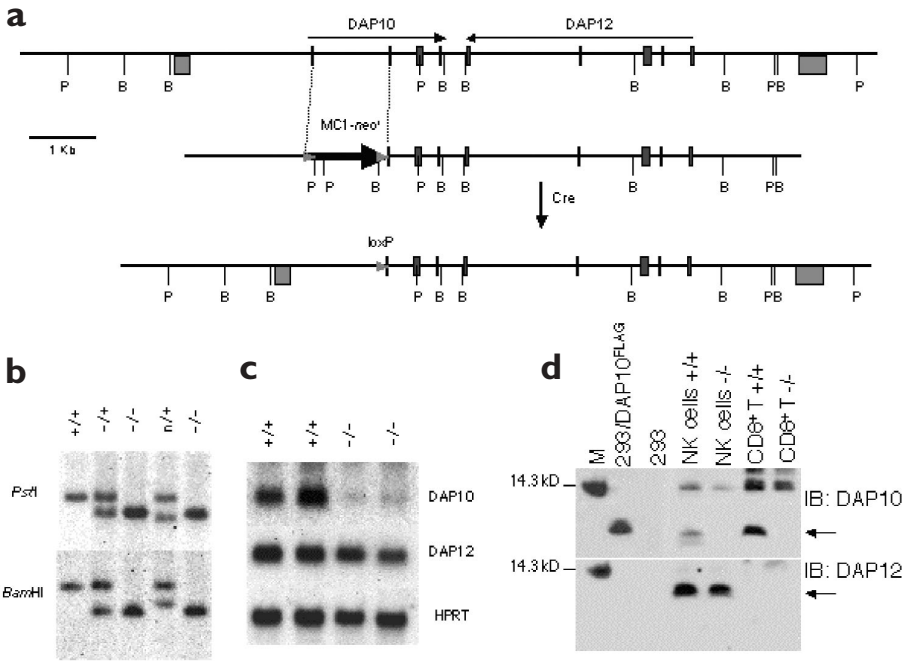

Figure 1. Generation of DAP10$^{-/-}$ mice. (**a**) Structure of the endogenous allele, targeting construct and mutant allele after excision of MC1-*neo*^{r} by Cre. Exons of the genes encoding DAP10 and DAP12 are shown with arrows indicating transcriptional orientation. The 5′ and 3′ external probes used for screening ES clones are designated by gray boxes. Only restriction enzyme sites relevant to screening are shown. P, *Pst*I; B, *Bam*HI. (**b**) Southern blot analysis of tail DNA isolated from various mice, which was digested with *Pst*I and *Bam*HI and hybridized with the DAP10 5′ external probe. (**c**) Northern blot analysis of total spleen RNA isolated from DAP10$^{+/+}$ and DAP10$^{-/-}$ littermates; the blot was sequentially hybridized with DAP10, DAP12 and HPRT probes. (**d**) Immunoblot analysis of lysates from activated DAP10$^{+/+}$ and DAP10$^{-/-}$ NK and CD8^{+} T cells. Lysates were prepared from cells and immunoblotted with DAP10 or DAP12 antisera. Lysates from 293 cells transiently transfected with a Flag-DAP10 cDNA (293/DAP10^{FLAG}) served as a positive control for the DAP10 antiserum. Arrows indicate the positions of the DAP10 and DAP12 bands. M, molecular weight marker.

6 weeks revealed no major abnormalities. Phenotyping of the immune system by flow cytometry demonstrated that the major lymphoid populations (αβ, CD4^{+} and CD8^{+} T cells, γδ T cells and B cells) were present at the expected frequency and numbers in the thymus, lymph nodes (LNs), spleen and bone marrow from DAP10$^{-/-}$ mice. NK cells were also present in normal numbers and frequency in the bone marrow, spleen, liver and peripheral blood—as were NK1.1^{+} T cells—in the DAP10$^{-/-}$ mice (data not shown). Thus, DAP10 is not critical for normal development nor is required for the development of a phenotypically normal immune system, including the NK cell population.

Expression of NKG2D in WT and DAP10$^{-/-}$ mice

Because DAP10 is only known to associate with NKG2D and is required for NKG2D cell-surface expression in transfectants[13], we assessed the expression of this receptor in mutant mice using monoclonal antibodies (mAbs) specific for murine NKG2D[23]. NKG2D was expressed on all NK1.1^{+}CD3^{-} NK cells and on ~50% of NK1.1^{+}CD3^{lo} NKT cells in littermate controls (**Fig. 2a**). In contrast, no NKG2D could be detected on NKT cells from the DAP10$^{-/-}$ mice (**Fig. 2**). However, low NKG2D expression was consistently observed on NK1.1^{+}CD3^{-} NK cells during analyses of spleen (**Fig. 2a**), bone marrow (**Fig. 2b**), peripheral blood, liver and thymus (data not shown) from the DAP10$^{-/-}$ mice. Expression of receptors recognized by an antibody specific for NKG2A, NKG2E and NKG2C (NKG2A/E/C) as well as the DAP12-associated receptors Ly49D and Ly49H was normal (**Fig. 2b** and data not shown). Thus, NKG2D expression is low but can still be detected on NK cells, but not T cells, freshly isolated from DAP10$^{-/-}$ mice.

Expression of NKG2D on activated NK and T cells

This discrepancy in NKG2D expression was magnified on NK and T cells activated *in vitro*. DAP10$^{-/-}$ and DAP10$^{+/+}$ spleen and LN cells

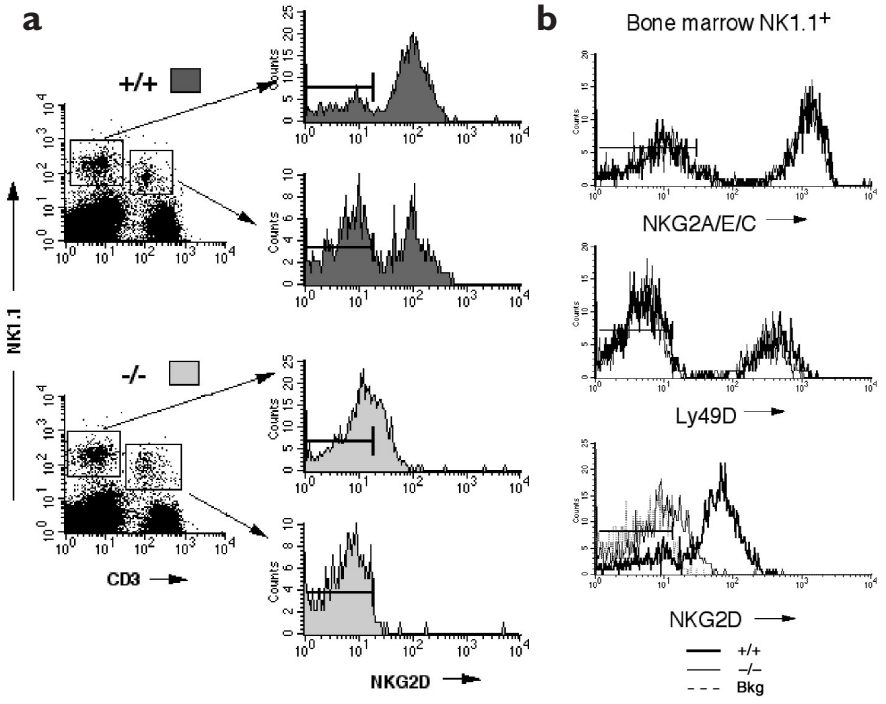

Figure 2. Expression of NKG2D in DAP10$^{-/-}$ and wild-type mice. (**a**) Three-color staining of spleen cells with NK1.1, CD3 and NKG2D mAbs for detection of NKG2D expression on NKT cells (CD3^{lo}NK1.1^{+}) and NK cells (CD3^{-}NK1.1^{+}) from DAP10$^{-/-}$ and DAP10$^{+/+}$ mice. (**b**) NKG2D, NKG2A/E/C and Ly49D expression on NK1.1^{+} bone marrow cells derived from DAP10$^{+/+}$ and DAP10$^{-/-}$ mice. Cells stained with a second step reagent (allophycocyanin-streptavidin) only are indicated in the histograms as background (bkg).

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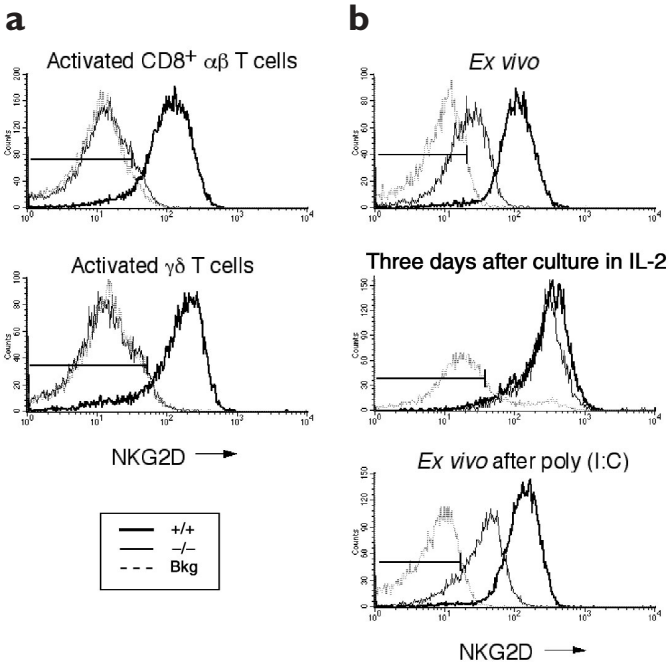

Figure 3. Expression of NKG2D on activated T and NK cells from DAP10$^{-/-}$ and control mice. (**a**) NKG2D expression on activated CD8$^+$ αβ and γδ T cells from DAP10$^{+/+}$ and DAP10$^{-/-}$ mice. LN cells were cultured on either TCRβ or γδ TCR mAb–coated plates with IL-2 for 2–3 days, then expanded with IL-2 for 5–8 days. NKG2D expression on gated CD8$^+$ T cells and γδ T cells is shown. (**b**) NKG2D expression on resting and activated DX5$^+$ spleen cells from DAP10$^{-/-}$ and control mice. Purified NK cells were activated *in vitro* with IL-2 or by intraperitoneal injection of poly(I•C) *in vivo*. Cells stained with only the second step reagent (allophycocyanin-streptavidin) are shown.

were incubated in interleukin 2 (IL-2)–containing medium on plates coated with either γδ or αβ TCR mAbs. The majority of γδ and CD8$^+$

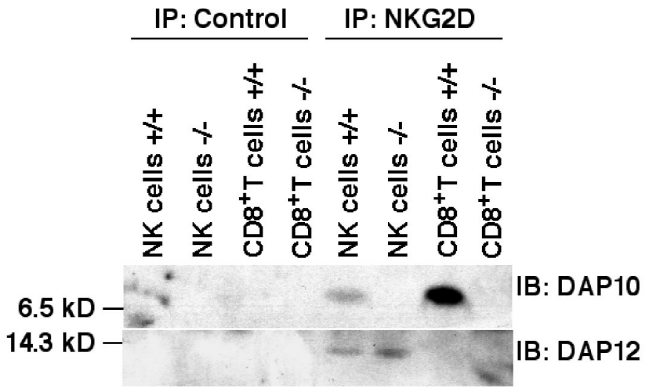

Figure 4. Association of NKG2D with DAP10 and DAP12 in NK cells. Digitonin lysates from the indicated cells were immunoprecipitated with either a control or NKG2D mAbs as designated and immunoblotted with either DAP10 antisera (upper panel) or DAP12 antisera (lower panel).

T cells from DAP10$^{+/+}$ mice expanded in these cultures expressed high amounts of NKG2D, whereas similar populations expanded from DAP10$^{-/-}$ mice expressed no NKG2D on the cell surface (**Fig. 3**). In contrast, wild-type expression of NKG2D was observed on DAP10$^{-/-}$ NK cells 3 days after culture in IL-2. NKG2D on NK cells was also up-regulated after intraperitoneal injection of poly(I•C) (**Fig. 3**). Thus, NKG2D was not expressed on resting T cells in the DAP10$^{-/-}$ mice and was not induced upon T cell activation. NKG2D was, however, expressed on the surface of resting NK cells and was further up-regulated by *in vitro* and *in vivo* activation.

Role of DAP12 in NKG2D expression

One explanation for the unexpected expression of NKG2D on DAP10$^{-/-}$ NK cells is the association with another adapter. DAP12 is a plausible candidate because it is expressed in NK cells but not activated CD8$^+$ T cells (**Fig. 1**). To address this possibility, we performed

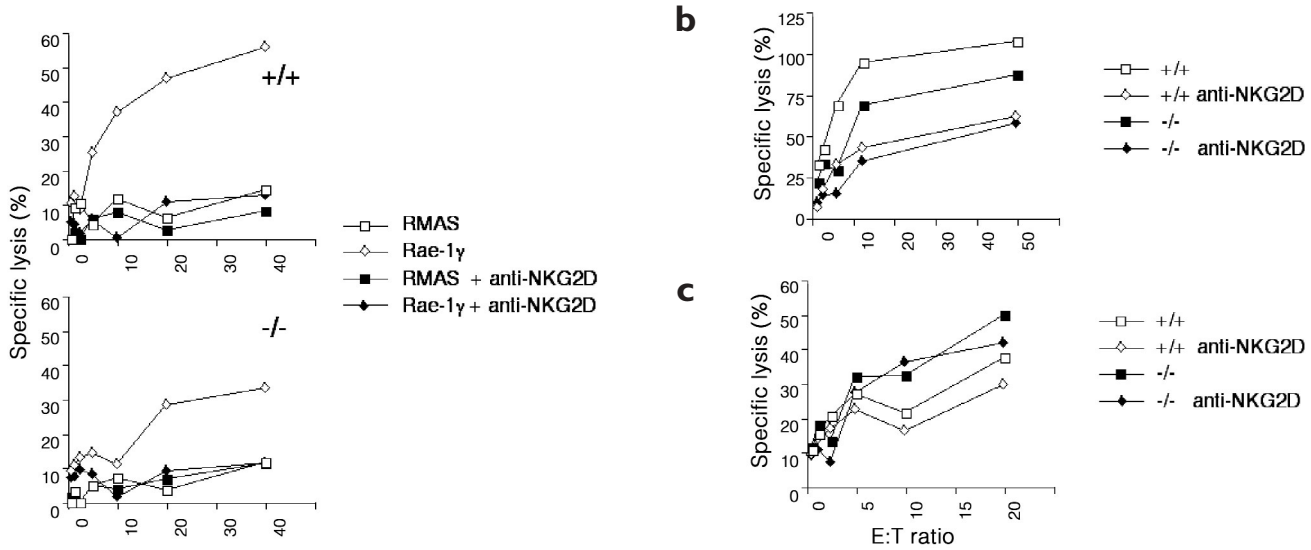

Figure 5. NKG2D-mediated cytotoxic activity of resting and IL-2–cultured NK cells from DAP10$^{+/+}$ and DAP10$^{-/-}$ mice. (**a**) After 5 days expansion in IL-2, DX5$^+$ purified NK cells from DAP10$^{+/+}$ (upper panel) and DAP10$^{-/-}$ mice (lower panel) were analyzed in a 4-h ^{51}Cr-release assay against RMAS or RMAS.Rae1γ target cells in the presence or absence of NKG2D mAb. (**b**) Freshly DX5$^+$ purified NK cells from both wild-type and DAP10$^{-/-}$ mice were analyzed in a 4-h ^{51}Cr-release assay against YAC cells in the presence or absence of NKG2D mAb. (**c**) Five days after expansion in IL-2, DX5$^+$ purified DAP10$^{+/+}$ and DAP10$^{-/-}$ NK cells were analyzed in a 4-h ^{51}Cr-release assay against the CHO target cell line in the presence or absence of NKG2D mAb.

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Table 1. Tumor growth in DAP10$^{-/-}$ mice

Tumor cells	Injected cells	DAP10^{+} mice with tumors/mice injected	DAP10$^{-/-}$ mice with tumors/mice injected
RMAS	5×10^5	6/7	4/6
	1×10^6	8/8	5/5
	2×10^6	5/5	4/4
		19/20[a]	13/15[a]
		(95%)	(87%)
RMAS.Rae-1γ	2.5×10^5	0/7	1/8
	1×10^6	0/14	0/12
	2.5×10^6	0/4	0/5
	3×10^6	0/6	2/7
		0/31[a]	3/32[a]
		(0%)	(9%)

Mice were injected subcutaneously in the flank with RMAS and RMAS.Rae-1γ cells and tumor growth was monitored. Only tumors with a diameter >12 mm within 3 weeks of injection that showed no sign of rejection were considered positive. The numbers of mice used for each experiment are shown as well as total numbers for each group; the percentages of mice with tumors are shown only for the total numbers in each group. DAP10^{+} controls included DAP10$^{+/+}$, DAP$^{n/+}$ and DAP10$^{-/+}$ mice. [a]Total for the experiment.

immunoblot analysis on NKG2D immunoprecipitates. NKG2D associated with DAP12 in NK cells from both wild-type and DAP10$^{-/-}$ mice (**Fig. 4**). As expected, NKG2D coimmunoprecipitated with DAP10 in NK cells and CD8^{+} T cells from wild-type but not DAP10$^{-/-}$ mice. Therefore, the association of NKG2D with DAP12 could explain the unexpected expression of NKG2D on NK cells in the DAP10$^{-/-}$ mice. However, because DAP12 is not expressed in the majority of CD8^{+} T cells, NKG2D apparently has no suitable adapter partner and hence is not expressed on the cell surface of DAP10$^{-/-}$ T cells.

Functional association of NKG2D with DAP12

To functionally evaluate the intracellular promiscuity of NKG2D, we examined DAP10$^{-/-}$ NK cell–mediated cytoxicity. Target cells used included RMAS, a TAP2-deficient cell line that expresses low amounts of MHC class I but no NKG2D ligands[4,24]; an RMAS cell line stably transfected with Rae-1γ (RMAS.Rae-1γ); YAC, a prototypic NK cell target expressing NKG2D ligands[4]; and Chinese hamster ovary (CHO) cells which are killed in a Ly49D-DAP12–dependent manner[25]. Purified NK cells were expanded in IL-2 for 5 days and challenged with all target cells. Both DAP10$^{-/-}$ and DAP10$^{+/+}$ NK cells killed RMAS–Rae-1γ transfectants more proficiently than they killed the parental RMAS cells (**Fig. 5a**). However, DAP10$^{-/-}$ killing of RMAS.Rae-1γ cells was consistently less efficient than that of DAP10$^{+/+}$ NK cells. An NKG2D mAb blocked both DAP10$^{+/+}$ and DAP10$^{-/-}$ NK cell killing of RMAS.Rae-1γ target cells (**Fig. 5a**), demonstrating that the Rae-1γ–dependent lysis was mediated by NKG2D in both wild-type and DAP10$^{-/-}$ NK cells.

This was confirmed by a series of experiments that used YAC cells as targets. Freshly isolated NK cells from both DAP10$^{+/+}$ and DAP10$^{-/-}$ mice lysed YAC cells, although the DAP10$^{-/-}$ NK cells were slightly less efficient (**Fig. 5b**). However, the NKG2D mAb blocked killing by both DAP10$^{+/+}$ and DAP10$^{-/-}$ NK cells. Thus, DAP10$^{-/-}$ primary and IL-2–activated NK cells retained some NKG2D-mediated activity, which was most likely due to association with the DAP12 adapter. NK cell function was not globally affected by the abrogation of DAP10: DAP10$^{+/+}$ and DAP10$^{-/-}$ NK cells killed CHO cells equally well and, as expected, the NKG2D mAb had no effect (**Fig. 5c**). Thus, DAP10$^{-/-}$ NK cells killed NKG2D ligand–negative cells in a Ly49-DAP12–mediated manner as effectively as their wild-type counterparts.

Rejection of tumors in DAP10$^{-/-}$ mice

Tumors induced by small inoculi of RMAS cells are primarily rejected by NK cells, as RMAS expresses low amounts of MHC class I[24]. However, large inoculi overcome NK surveillance unless they have high cell surface expression of stimulatory ligands[9,10,26–28]. To further test DAP10$^{-/-}$ NK cell function *in vivo*, we challenged DAP10$^{-/-}$ and control littermates with either RMAS or RMAS.Rae-1γ cells and monitored tumor growth. After subcutaneous (s.c.) injection of 5×10^6 or more RMAS cells, almost all DAP10$^{-/-}$ and control mice developed tumors (**Table 1**). Similarly, 5/6 DAP10$^{-/-}$ and 6/6 control littermates developed tumors after being challenged with a large inoculum (3×10^6 cells) of RMAS.Rae-1γ cells expressing intermediate amounts of Rae-1γ (data not shown). In contrast, only 3/32 DAP10$^{-/-}$ and 0/31 littermate controls developed exponentially growing tumors after s.c. injection of RMAS transfectants expressing high amounts of Rae-1γ (**Table 1**). Thus, expression of Rae-1γ on RMAS promoted tumor rejection in both wild-type and DAP10$^{-/-}$ mice, indicating that NK cells can mount an NKG2D-mediated antitumor response in the absence of DAP10. This was consistent with the cytotoxicity data and provided additional evidence that DAP12 can substitute for DAP10 *in vivo*.

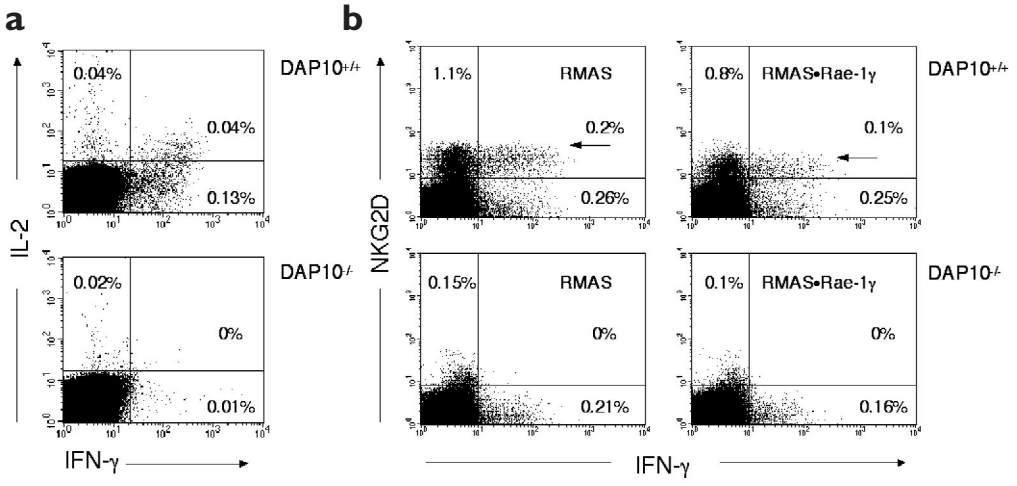

Figure 6. Tumor-specific CTL responses in DAP10$^{+/+}$ and DAP10$^{-/-}$ mice. DAP10$^{+/+}$ and DAP10$^{-/-}$ mice were injected subcutaneously with 2×10^6 RMAS.Rae-1γ cells. After 6–8 weeks, mice were challenged intravenously with 3×10^5 RMAS.Rae-1γ cells and killed after 5 additional days. CD8^{+} T cells were purified from spleens and incubated with (**a**) RMAS.Rae-1γ or (**b**) RMAS.Rae-1γ and RMAS cells. After 6 h, production of (**a**) IFN-γ and IL-2 or (**b**) expression of NKG2D and secretion of IFN-γ were determined. The percentages of positive cells are indicated in the relevant quadrants. In **b**, NKG2D expression on DAP10$^{+/+}$ CD8^{+} T cells incubated with RMAs and RMAS.Rae-1γ cells is indicated by arrows.

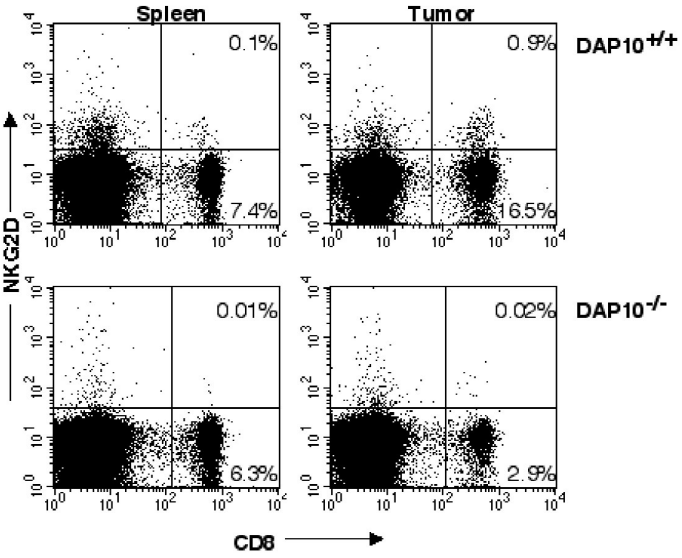

Figure 7. Comparison of NKG2D$^+$ CD8$^+$ T cell frequency in spleens and RMAS.Rae-1γ tumors. Mice were injected subcutaneously with RMAS transfectants expressing intermediate levels of Rae-1γ and tumors were analyzed when they reached 20 mm in diameter. The frequencies of NKG2D$^+$ CD8$^+$ T cells in both the spleen and the tumors were analyzed in DAP10$^{+/+}$ and DAP10$^{-/-}$ mice. Data from two representative mice are shown. The percentages of CD8$^+$NKG2D$^-$ and CD8$^+$NKG2D$^+$ cells are indicated in the relevant quadrants.

The role of NKG2D in generating a CD8$^+$ T cell response

NKG2D has been implicated in generating an antitumor γδ T cell[11] and CD8$^+$ T cell[9,12,29] response. Although RMAS expresses low amounts of MHC class I, CD8$^+$ T cells specific for RMAS are elicited by "vaccination" with an RMAS transfectant expressing high amounts of CD80[26,27]. Additionally, vaccination with an RMAS line expressing CD70, a ligand for the costimulatory molecule CD27, elicits memory CD8$^+$ T cells that specifically recognize the parental TAP-sufficient MHC class I^{hi} RMA tumor cell line[28]. We assessed the antitumor CD8$^+$ T cell responses generated by RMAS.Rae-1γ cells in DAP10$^{+/+}$ and DAP10$^{-/-}$ mice that remained tumor-free 6–8 weeks after being challenged with RMAS.Rae-1γ cells. Mice were boosted with an intravenous injection of RMAS.Rae-1γ cells 3–5 days before analysis. CD8$^+$ T cells were purified and incubated with RMAS.Rae-1γ cells *in vitro*, and interferon-γ (IFN-γ) and IL-2 secretion were measured by intracellular staining. A population of IFN-γ-secreting CD8$^+$ T cells was detected in wild-type but not in DAP10$^{-/-}$ mice (**Fig. 6a**).

Further analysis of NKG2D expression revealed that most of the IFN-γ–producing T cells expressed NKG2D. Production of IFN-γ was also evident after incubation of DAP10$^{+/+}$CD8$^+$ T cells with the parental RMAS line, showing that the CD8$^+$ T cell response was tumor-specific and not solely dependent on Rae-1γ expression. No IFN-γ was observed with an unrelated cell line or with CD8$^+$ T cells from naïve mice, demonstrating the specificity of the CD8$^+$ T cell response (data not shown). This indicated that CD8$^+$ T cells specific for TAP-2–deficient RMAS cells were generated in wild-type but not DAP10$^{-/-}$ mice that had been "vaccinated" with RMAS.Rae-1γ cells.

In addition, NKG2D was down-regulated on CD8$^+$ T cells after incubation with RMAS.Rae-1γ but not RMAS tumor cells (**Fig. 6b**). This, again, reflected NKG2D engagement of a ligand, which was probably important for the priming or generation of tumor-specific CD8$^+$ T cells.

We also analyzed the lymphocytes infiltrating the tumors that arose in DAP10$^{+/+}$ and DAP10$^{-/-}$ mice after injection of RMAS cells expressing intermediate amounts of Rae-1γ. Consistent with the induction of an antitumor CD8$^+$ T cell response in DAP10$^{+/+}$ mice, we found that the proportion of NKG2D$^+$CD8$^+$ T cells within the tumor was much higher than that observed in the spleens of the same animals. This suggested that NKG2D$^+$CD8$^+$ T cells were preferentially recruited to or, more likely, expanded within the tumors (**Fig. 7**). Together, these results indicated that DAP10$^{-/-}$ mice failed to generate a CD8$^+$ T cell memory response to tumors expressing NKG2D ligands.

Discussion

Our analysis of DAP10$^{-/-}$ mice shows that NKG2D not only uses conformational plasticity to recognize a large number of ligands but also has the capacity to associate with two adapter proteins, each linked to a distinct intracellular signaling pathway. However, the association with different signaling chains is cell type– and activation state–dependent. In T cells, NKG2D associates only with DAP10 and therefore is limited to the YxxM "costimulatory" pathway. As a result, DAP10$^{-/-}$ mice show impaired development of tumor-specific CD8$^+$ T cell responses that could be due to the absence of NKG2D-DAP10 signaling during either antigen-specific T cell priming or effector activation. In contrast, in NK cells—which express both DAP10 and DAP12—NKG2D can activate ZAP-70 or Syk protein tyrosine kinases through the ITAM of DAP12 and the p85 subunit of PI3K and Grb2 through the YxxM motif of DAP10. Consequently, NK cells can reject tumors expressing NKG2D ligands even in the absence of DAP10.

The relative contributions of DAP12 and DAP10 pathways to NKG2D function in wild-type NK cells are likely to change with their state of activation. We have observed that NKG2D expression and NKG2D-mediated lysis in culture are optimal early after IL-2 activation of NK cells, whereas they decline at later time points of culture (data not shown). In an accompanying study published in this issue, Raulet and colleagues show that NKG2D includes two isoforms generated by alternative splicing of the same gene[30]. One isoform is associated only with DAP10, whereas the other is associated with both DAP10 and DAP12, but both apparently retain similar ligand specificity. Consistent with our study, the DAP12-associated isoform of NKG2D increases upon IL-2 and poly(I•C)-induced NK cell activation and slowly decreases over time in culture. These observations may also explain the apparent discrepancy between our data and a published study that demonstrated the association of DAP10, but not DAP12, with NKG2D in a B cell transfection system and in coimmunoprecipitation experiments with a human NK cell line[19]. The reported NKG2D-DAP10 specificity probably describes the human NKG2D equivalent of the mouse DAP10-specific isoform; whether a "promiscuous" isoform of NKG2D is expressed in humans remains to be tested.

These NKG2D signaling options may reflect the disparate nature of NK and T cells. Individual T cells require the clonotypically expressed T cell receptor for specific recognition of a peptide–MHC class I complex and rely on a number of costimulatory molecules, such as NKG2D-DAP10, to modify the strength and duration of the signal. In this situation, an additional receptor that directly activates the cell, such as NKG2D-DAP12, would jeopardize T cell specificity. In contrast, individual NK cells simultaneously express a broad variety of activating receptors that are dominated by inhibitory receptors, with the functional outcome determined by signal integration[31]. In this context, NKG2D-DAP10–mediated "costimulation" may augment stimulation induced by other NK cell–activating receptors recognizing different

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ligands[23,32]. Alternatively, the same MHC class I–like ligand can trigger both activation and costimulation through the NKG2D-DAP12 and NKG2D-DAP10 complexes, permitting innate host defense early in an immune response. Therefore, NKG2D has adapted to appropriately serve both cell types in surveillance for abnormal and stressed cells.

Methods

Generation of DAP$^{-/-}$ mice. A targeting construct in which exon 1 and intron 1 of DAP10 were replaced by MCI-*neo*^{r} flanked by *loxP* sites was made by amplifying a 1.9-kb fragment 5′ of DAP10 exon 1 and a 6.5-kb 3′ fragment containing exon 2 from phage DNA with the oligo pairs 5′-TAGTAGGCGGCCGCAGCCGCAGAGCTAACTTAGAC TCTGA-3′, 5′-TAGTAGGCGGCCGCGCTGGGCTGGGACCTGAAGGGATCTGG-3′ (*Not*I sites are underlined) and 5′-TAGTAGACTAGTAGTGGCTGCAAGTCAGACAT CGGCA-3′, 5′-TAGTAGACTAGTCTGGACATGCTAACACCACCCAAGAGAGTAG-3′ (*Spe*I sites are underlined). The PCR fragments were digested with *Not*I and *Spe*I, respectively, and cloned into pmmNeoFlox-8 (a gift of R. Torres, Denver, CO), which contains MCI-*neo*^{r} flanked by *loxP* sites. The construct was electroporated into E14.1 embryonic stem (ES) cells[33] and G418-resistant clones were screened by Southern blot analysis with multiple enzyme digests and external probes located 5′ and 3′ of the targeting vector as well as a *neo*^{r} internal probe. One correctly targeted clone was injected into C57BL6 blastocysts and two chimeras were obtained. One of these transmitted the targeted allele when bred with C57BL/6 mice. The MCI-*neo*^{r} insertion was deleted by crossing DAP10$^{w/+}$ mice with B6N14 mice expressing a Cre transgene under the CMV promoter[22]. Mice were genotyped by either Southern blot analysis or PCR analysis of tail DNA with the oligos DAP105′, 5′-GGTCCTCTTGCCACTCCA-3′; Neo 3′, 5′-TTGCC GAATATCATGGTGGAAAAT-3′; DAP10 leader, 5′-GAAGCAGGAACAGGAGGTAG-3′; and DAP10 exon 2, 5′-GATGTCTGACTTGCAGCCAC-3′. These probes distinguished between all three genotypes: the endogenous (+) band was 263 bp, the neo insertion (n) was 365 bp and targeted with neo-deleted (–) was 302 bp. Southern blotting of genomic DNA isolated from ES clones or tail tissues and RNA blot analysis of spleen RNA from DAP10$^{-/-}$ and DAP10$^{+/+}$ mice was done as described[33]. DAP10, DAP12 and HPRT probes were amplified from either LN or liver cDNA with the following primers: DAP10, 5′-TACCTCCTGTTCCTGCTTCTG-3′ and 5′-GCCTCTGCCAGGCATGTTG AT-3′; DAP12, 5′-GAGCCCTCCTGGTGCCTTCTG-3′ and 5′-TGGTCTCTGACCCTG AAGCTC-3′; and HPRT, 5′-GCTGGTGAAAAGGACCTCT-3′, 5′-CACAGGACTAGA ACACCTGC-3′.

Immunoblot analysis. Cell lysates of day 5 IL-2–cultured NK cells (2×10^5 cells) and day 4 anti-CD3–stimulated CD8$^+$ T cells (1×10^6) from DAP10$^{+/+}$ and DAP10$^{-/-}$ mice were separated by SDS-PAGE and immunoblotted with DAP10- and DAP12-specific rabbit antisera raised against CPAQEDGRVYINMPGRG (DAP10) and CESPYQELQGQRPEVYSD (DAP12) peptides. Negative and positive controls included lysates from 293 cells untransfected or transiently transfected with a cDNA encoding NH_2-terminal Flag-DAP10 fusion protein, respectively.

Flow cytometric analysis. The mAbs phycoerythrin (PE)–anti-γδ (GL3), PE–anti-CD4 (H129.19), fluorescein isothiocyanate (FITC)–anti-CD8α (53-6.7), FITC–anti-CD8β (53-5.8), PE–anti-NK1.1 (PK136), FITC–anti-CD3ε (145-2C11), FITC–anti-Ly49D (4E5) and biotin–anti-NKG2A/C/E (20D5) were from PharMingen (San Diego, CA). Allophycocyanin-streptavidin was from Molecular Probes (Eugene, OR). mAbs A10 and C7 to NKG2D were purified and biotinylated[23]. Thymus, LN, spleen, bone marrow and liver suspensions were prepared and stained essentially as described[33]. Cells were analyzed on a FACScalibur with Cell Quest software (Becton Dickinson, San Jose, CA).

Immunoprecipitations. NKG2D was immunoprecipitated from digitonin lysates of 10^7 wild-type and DAP10$^{-/-}$ NK cells 4–6 days after activation in IL-2 with the C7 and A10 NKG2D mAbs[23]. In addition, we immunoprecipitated NKG2D from 4×10^7 activated CD8$^+$ T cells from wild-type and DAP10$^{-/-}$ mice activated on day 4 with plate-bound CD3 mAb. Immunoprecipitates were analyzed by immunoblotting with DAP12 and DAP10 antisera.

Cytotoxicity assays. NK cells were purified with DX5 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) as described[3]. Purified DX5$^+$ cells were >90% NK1.1$^+$CD3$^-$ NK cells were cultured for different time periods in 1000 U/ml of recombinant human IL-2 (Roche, Basel, Switzerland) and 5% of heat-inactivated human serum. Cytotoxic activity of freshly purified NK cells and NK cells cultured in IL-2 was tested against target cells in standard 4-h ^{51}Cr-release assays. Rae-1γ was expressed in RMAS cells as an NH_2-terminal Flag-peptide fusion protein by cloning Rae-1γ cDNA into pFLAG-CMV1 (Sigma, St. Louis, MO). RMAS cells were cotransfected with pFLAG-CMV1–Rae-1γ and a *neo*^{r} gene and G418-resistant clones were sorted for high expression of Flag–Rae-1γ by cell sorting with the Flag mAb M2 (Sigma).

Priming of tumor specific T cells and determination of intracellular IFN-γ. DAP10$^{+/+}$ and DAP10$^{-/-}$ mice were challenged with 2×10^6 RMAS.Rae-1γ cells. After 6–8 weeks, mice were challenged intravenously with 3×10^5 RMAS.Rae-1-γ cells and, after 5 additional days, CD8$^+$ T cells were purified from spleens with CD8 microbeads (Miltenyi Biotech). Purified cells were incubated at a 1:2 ratio with RMAS or RMAS/Rae-1γ cells for 6 h. In the last 4 h, 2 μg/ml of monensin (Sigma) was added. Cells were then fixed, permeabilized with the Cytofix-Cytoperm kit (PharMingen) and stained.

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Competing interests statement

The authors declare that they have no competing financial interests.

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