Title
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Journal
Nature immunology, 6(9)

ISSN
1529-2908

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Publication Date
2005-09-01

Peer reviewed

A role for NKG2D in NK cell-mediated rejection of mouse bone marrow grafts

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ABSTRACT

Irradiation-resistant NK cells in a F1 recipient can reject parental bone marrow (BM), and host NK cells can also prevent engraftment of allogeneic BM. We show that repopulating BM cells in certain mouse strains express the RAE-1 proteins, which are ligands for the activating NKG2D NK cell receptor. Treatment with a neutralizing NKG2D antibody prevented rejection of parental BALB/c BM in (C57BL/6 x BALB/c) F1 recipients, and allowed engraftment of allogeneic BALB.B BM in C57BL/6 recipients. Additionally, BM from RAE-1ε transgenic C57BL/6 mice was rejected by syngeneic animals, but accepted with anti-NKG2D treatment. If other stem cells or tissues up-regulate expression of NKG2D ligands after transplantation, NKG2D may contribute to graft rejection in immunocompetent hosts.

Natural killer (NK) cells play a critical role in the elimination of virus-infected cells or transformed cells[1]. Although beneficial in host protection against infectious disease and cancer, irradiation-resistant mouse NK cells can reject bone marrow (BM) cell grafts[2-5]. This process whereby NK cells in F1 recipients reject parental BM grafts has been called F1 hybrid resistance[6,7]. Initially, the hypothesis proposed to explain hybrid resistance was the expression of hybrid histocompatibility (Hh) antigens on parental bone marrow cells that were not expressed in the F1 hybrid mice. Genetic mapping studies suggested that at least in some mouse strains the genes regulating the Hh antigens localized to the H-2S/D region[8]. More recently, the ability of NK cells to recognize and reject parental BM cells has been explained, in part, by the lack of inhibitory Ly49 receptors specific for parental H-2 proteins on a subset of NK cells in the F1 recipient[9-12]. Thus, a subset of NK cells in the F1 recipient lacking inhibitory receptors for the parental BM cells might eliminate these parental BM grafts. However, the NK cell receptors that initiate the attack against BM grafts have not been defined.

NKG2D is an activating receptor that is expressed on the cell surface of NK cells, activated $CD8^+$ T cells and $\gamma\delta TcR^+$ T cells[13]. In resting NK cells, NKG2D associates with the DAP10 adapter protein, and in activated mouse NK cells an NKG2D isoform generated by alternative splicing can also associate with the DAP12 adapter protein[14]. NKG2D binds to a family of ligands with structural homology to major histocompatibility complex (MHC) class I proteins (reviewed in [1,15]). In mice, the retinoic acid early inducible-1 (RAE-1) family of proteins, H60 and MULT1 function as high affinity ligands for NKG2D[16-18]. Although the genes encoding the RAE-1 proteins were first discovered by their expression in embryonic tissues[19,20], they are largely silent in normal, healthy tissues in adult mice, but are induced by viral infection or cellular transformation. Here, we have examined expression of the NKG2D ligands in BM cells repopulating irradiated mice and

have evaluated the role of NKG2D in hybrid resistance.

RESULTS

Expression of NKG2D ligands on mouse BM cells

In BALB/c mice, the *Raet1a, Raet1b* and *Raet1c* genes encode the RAE-1α, RAE-1β, and RAE-1γ proteins, respectively, whereas in C57BL/6 mice *Raet1d* and *Rae1e* encode the RAE-1δ and ε proteins, respectively[21]. Whether *Raet1d* and *Rae1e* in C57BL/6 mice represent distinct *Raet1* loci or are allelic variants of the *Raet1a, Raet1b* and *Raet1c* genes has not been determined because the genomic organization of the *Raet1* genetic complex has not been established in these mouse strains. BALB/c, but not C57BL/6, mice express functional H60 proteins[22]. To examine whether NKG2D ligands are expressed on BM cells, we analyzed BM cells isolated from BALB/c, C57BL/6, and (BALB/c x C57BL/6) F1 (CB6F1) mice. Cells were stained with a mouse NKG2D-IgG Fc fusion protein and analyzed by flow cytometry. Low expression of NKG2D ligands was detected on freshly isolated BALB/c BM cells, but not C57BL/6 BM cells (**Fig. 1a**). To determine which NKG2D ligands were expressed, we stained the BM cells with a pan RAE-1, H60 and MULT1 monoclonal antibody (mAb). RAE-1 and H60 were expressed at low abundance on freshly isolated BALB/c BM cells, whereas MULT1 was not detected (**Fig 1b**). By contrast, RAE-1 was not detected on freshly isolated splenocytes from BALB/c, C57BL/6 or CB6F1 mice (unpublished observation).

Prior studies have established that NK cells in F1 recipients are able to reject parental BM grafts[2-5]. Therefore, we examined whether the BALB/c BM cells that repopulate the spleen in an irradiated CB6F1recipient express NKG2D ligands. To prevent rejection of the transplanted BALB/c BM cells, the recipient CB6F1 mice were pre-treated with anti-NK1.1 to deplete the recipient's NK cells. As a control, a group of irradiated CB6F1 mice

were reconstituted with syngeneic CB6F1 BM cells. Seven days after grafting, we isolated the hematopoietic cells repopulating the spleens of the CB6F1mice and analyzed them for expression of NKG2D ligands. NKG2D ligands were detected on the repopulating hematopoietic cells isolated from the spleens of BALB/c BM -> CB6F1mice, but not on cells isolated from the spleens of CB6F1BM -> CB6F1mice (**Fig. 1c**). The BALB/c hematopoietic cells reconstituting the spleens of the irradiated CB6F1recipients predominantly expressed RAE-1, and not H60 or MULT1 (**Fig. 1d**).

To identify the population of hematopoietic cells that expressed RAE-1, we stained cells isolated from the spleens of CB6F1BM -> CB6F1 and BALB/c BM -> CB6F1 recipients with mAbs against hematopoietic lineage markers. At day 7 post-transplantation, RAE-1 was detected on the majority of cells isolated from the spleens of BALB/c BM -> CB6F1 recipients; however, RAE-1 was not detected on a substantial proportion of cells in CB6F1BM -> CB6F1 recipients. Essentially all RAE-1- positive cells isolated from the BALB/c BM -> CB6F1recipients expressed CD43 (**Fig. 1e**). RAE-1 was also present on most cells expressing the granulocyte-associated Ly-6C/G (Gr-1) protein and the myeloid cell-associated marker CD11b (Mac-1). Only a minor fraction of $B220^+$ (B cell-associated marker) cells and $Ter119^+$ (an erythrocyte-associated marker) cells expressed RAE-1, and RAE-1 was not detected on $CD3^+$ T cells (not shown). RAE-1 was detected on a small subset of cells expressing c-kit and Sca-1, although most RAE-1-positive cells did not have these markers (**Fig. 1f**). We evaluated the proliferation status of cells expressing RAE-1 in the BALB/c BM -> CB6F1 recipients by injecting bromodeoxyuridine (BrdU) into these mice 2 h and 12 h before harvesting the spleen cells on day 7 post-transplantation. RAE-1 was readily detected on a large fraction (but not all) of the proliferating progenitor cells in the spleen of the transplant recipients (**Fig. 1g**).

In initial experiments, CB6F1 mice were transplanted with whole BM isolated from

BALB/c donors. To address whether RAE-1 is expressed on the progeny of hematopoietic stem cells (HSC), donor BALB/c mice were treated with 5-fluorouracil (5-FU) before BM harvest to enrich for HSCs, and BM from 5-FU-treated donors was then transplanted into CB6F1 recipients that were pre-treated with NK1.1 mAb to deplete resident host NK cells. The BM cells harvested from the 5-FU-treated donors did not express RAE-1 (data not shown). When cells in the spleens of BALB/c 5-FU BM -> CB6F1recipients were analyzed on day 8 post-transplantation, essentially all RAE-1-positive cells expressed Ly-6C/G, CD11b and CD43 (**Fig. 1h**). A small population of RAE-1-positive cells expressed low amounts of c-kit and Sca-1, although a majority of the RAE-1-positive cells lacked both of these markers (not shown). These results indicated that the majority of proliferating BALB/c myeloid progenitor cells in the NK cell-depleted CB6F1recipients expresses RAE-1.

NKG2D is involved in hybrid resistance

Expression of RAE-1 on the proliferating progenitor cells in the spleens of CB6F1 mice reconstituted with BALB/c BM prompted us to examine whether NKG2D is involved in hybrid resistance. To this end, we transferred BALB/c BM cells into irradiated CB6F1mice pre-treated with a control antibody (cIg), a neutralizing, non-depleting NKG2D mAb (CX5)[23] (**Supplementary Fig.1**) or the NK cell-depleting NK1.1 mAb (PK136). Hematopoietic cell reconstitution of recipient mice was evaluated by injecting ^{125}I-UdR 12 h prior to harvesting spleens on day 7. cIg-treated mice rejected the BALB/c BM cells. Consistent with prior findings[3], depletion of NK cells in CB6F1 mice efficiently prevented rejection of the BALB/c BM cells – resulting in a substantial increase in incorporation of radiolabel in the spleens (**Fig. 2a**). The non-depleting, neutralizing NKG2D mAb also increased incorporation of ^{125}I-UdR, comparable to the effects of depleting NK cells.

The ability of NKG2D mAb treatment to prevent rejection of BALB/c BM cells was confirmed by examining the cells repopulating the spleens on day 8 post-transplantation. We detected RAE-1-positive cells, predominately co-expressing CD43, Ly-6C/G, and CD11b in the spleens of CB6F1 mice treated with NKG2D mAb (**Fig. 2b**); however, many fewer cells were recovered from the cIg-treated mice and very few of these cells expressed RAE-1 (**Fig. 2b**). These data indicated that rejection of RAE-1-positive BALB/c BM cells in CB6F1 mice is efficiently prevented by either the depletion of NK cells or by blocking the NKG2D receptor.

In the BALB/c into CB6F1 hybrid resistance model, the F1 recipient mice express both H-2^d and H-2^b, whereas the donor BM cells express only H-2^d. We sought to determine whether reactivity against H-2^d on the parental donor cells was necessary for NKG2D-dependent graft rejection. To address this question, BALB.B (H-2^b) BM was transplanted into C57BL/6 (H-2^b) recipients. The C57BL/6 recipients rejected BALB.B BM; however, graft rejection was prevented either by blocking NKG2D or by depletion of the NK cells in the recipients (**Supplementary Fig. 2**). Therefore, NKG2D-dependent rejection of BM does not require incompatibility between the MHC of the donor and recipient.

Genetic control of NKG2D-ligand expression

Because we observed that C57BL/6 BM cells, unlike BALB/c cells, expressed little or no NKG2D ligands (**Fig. 1a**), we investigated the role of NKG2D in hybrid resistance in CB6F1mice against C57BL/6 parental BM grafts. CB6F1 mice rejected C57BL/6 BM cells, and this was prevented by depletion of NK cells in the recipient by treatment with the depleting NK1.1 mAb (**Fig. 3**). However, NKG2D mAb blockade had no effect on rejection of C57BL/6 parental BM in the CB6F1 recipient, in contrast to the ability of

NKG2D mAb to allow engraftment of BALB/c parental BM in these recipients (**Fig. 2a**). These results clearly indicate that both NKG2D-dependent and NKG2D-independent mechanisms are operative in NK cell-mediated BM rejection, and that these are genetically regulated.

Differential expression of the NKG2D ligands by BM cells in different mouse stains may be an important consideration in transplantation and influence whether NKG2D plays a potential role in NK cell-mediated rejection. As shown in **Fig. 1**, NKG2D ligands could be detected on freshly isolated BM cells from BALB/c, but not C57BL/6 mice. Consistent with the finding that RAE-1 is expressed on proliferating BALB/c BM progenitor cells *in vivo* (**Fig. 1g**), we have also observed that RAE-1 expression is up-regulated by culturing BALB/c BM *in vitro* with interleukin-3 (IL-3), stem cell factor (SCF), and interleukin-6 (IL-6). Therefore, we have examined the expression of RAE-1, H60, and MULT1 on *in vitro* cultured BM cells from several different mouse strains (including C57BL/6, BALB/c, BALB.B, C3H, CBA, CBA/J, DBA/2, FVB, and NK1.1 congenic NOD). **Table 1** summarizes the percentages of *in vitro* cultured BM cells expressing the NKG2D ligands and the amounts of ligand present on the cell surface (represented as the mean fluorescence intensity). BALB/c and BALB.B expressed the highest frequency of cells expressing NKG2D ligands and the highest expression of RAE-1; C57BL/6 demonstrated the lowest frequency of NKG2D ligand-bearing BM cells – other strains were similar to C57BL/6 or were intermediate. These results suggest that the NKG2D ligands are genetically regulated and may influence transplantation differently in different donor-recipient combinations.

NK cells reject RAE-1-transgenic BM cells

To evaluate whether or not expression of RAE-1 on C57BL/6 or CB6F1 BM cells would cause rejection of syngeneic BM grafts, we generated transgenic mice expressing RAE-1ε

driven by a human β-actin promoter that would result in RAE-1ε expression in all tissues. **Fig. 4a** shows the expression of RAE-1ε on freshly isolated BM cells from C57BL/6 RAE-1ε transgenic mice, which is similar to the amounts of RAE-1 present on the repopulating BALB/c BM cells (**Fig. 1d,e**).

We tested freshly isolated BM cells from the RAE-1ε transgenic C57BL/6 mice as targets for IL-2-activated syngeneic, non-transgenic NK cells in a standard *in vitro* cytotoxicity assay. As shown in **Fig. 4b**, activated NK cells killed freshly isolated RAE-1ε transgenic C57BL/6 BM cells, but not RAE-1-negative non-transgenic C57BL/6 BM cells. Cytotoxicity was blocked by NKG2D mAb, demonstrating that the killing was NKG2D-dependent. In accordance with the *in vitro* results, irradiated non-transgenic C57BL/6 mice rejected BM cells from RAE-1ε transgenic C57BL/6 donors; rejection was prevented in mice treated with the neutralizing NKG2D mAb, but not control Ig (**Fig. 4c**). Similar results were obtained when the RAE-1ε transgenic C57BL/6 were crossed with BALB/c mice and RAE-1ε transgenic CB6F1 BM was grafted into non-transgenic CB6F1recipients (data not shown). The RAE-1ε transgenic CB6F1 BM cells, unlike non-transgenic CB6F1 BM cells (**Fig. 1c,e**), expressed high amounts of RAE-1ε and were rejected by the syngeneic non-transgenic CB6F1 recipients (data not shown). Rejection was prevented by administration of the neutralizing, non-depleting NKG2D mAb or by depletion of NK cells with NK1.1 mAb (data not shown). Collectively, these findings demonstrate that C57BL/6 and CB6F1 NK cells can reject H-2 identical BM cells, provided that the cells express RAE-1.

DAP10 and DAP12 in NKG2D-mediated BM rejection

In mice, alternative RNA splicing of NKG2D transcripts generates two protein isoforms called NKG2D-S and NKG2D-L. NKG2D-L is expressed predominantly in resting NK

cells and associates with the DAP10 adapter protein, whereas NKG2D-S is induced by activation of NK cells and associates with either DAP10 or DAP12 (ref. [14]). To determine whether DAP10 or DAP12 or both adapters are involved in NKG2D-mediated rejection, BM cells from RAE-1ε transgenic C57BL/6 mice were transplanted into irradiated wild-type, DAP10-deficient, and DAP12-deficient C57BL/6 recipients. Mice were injected with ^{125}I-UdR on day 5 and spleens were harvested and counted on day 6. Compared with wild-type C57BL/6 mice, DAP10-deficient C57BL/6 mice demonstrated impairment in their rejection of the RAE-1ε transgenic C57BL/6 BM graft (**Fig. 5a**). By contrast, DAP12-deficient C57BL/6 recipients rejected the RAE-1ε transgenic C57BL/6 BM more efficiently that the DAP10-deficient C57BL/6 mice, although slightly less well than wild-type C57BL/6 mice (**Fig. 5b**). Wild-type, DAP10-deficient, and DAP12-deficient C57BL/6 mice all failed to reject the RAE-1ε transgenic C57BL/6 BM graft when treated with the depleting NK1.1 mAb or with the non-depleting, neutralizing NKG2D mAb. These results indicate a predominant role of DAP10, and a lesser role of DAP12, in NKG2D-dependent BM rejection.

Defective graft rejection in RAE-1ε transgenic mice

We have reported previously that *in vitro* activation of NK cells from NOD mice induces expression of RAE-1, which results in ligand-dependent modulation of NKG2D on the NK cells[24]. Analysis of the expression of NKG2D on the surface of NK cells from the RAE-1ε transgenic C57BL/6 mice revealed lower expression of NKG2D than present on NK cells from wild-type mice (**Fig. 6a**). Although the amount of NKG2D on NK cells in the RAE-1ε transgenic C57BL/6 was substantially diminished, the number of NK cells in the spleens and the expression of NK1.1, Ly-49D, Ly-49A, Ly-49C/I, Ly-49F/I/C/H, and Ly-49G2 on the NK cells were similar to wild-type NK cells (data not shown). To examine whether

NKG2D function is impaired in RAE-1ε transgenic NK cells, we performed an antibody-redirected cytotoxicity assay using cIg, NKG2D mAb and NK1.1 mAb. Although NK1.1-dependent cytotoxic activity of RAE-1ε transgenic NK cells was identical to that of wild-type C57BL/6 NK cells, NKG2D-dependent cytotoxicity was impaired in RAE-1ε transgenic NK cells (**Fig. 6b**).

The RAE-1ε transgene is driven by a β-actin promoter in these transgenic mice; therefore, in these animals the NK cells co-express both ligand and receptor. To determine whether wild-type (non-transgenic) NK cells are inactivated *in vivo* by constant exposure to NKG2D ligands, we generated BM chimeras by transplanting wild-type Ly5.2 congenic C57BL/6 BM into lethally irradiated RAE-1ε C57BL/6 (Ly5.1) transgenic recipients. Three months after transplantation, the number of NK cells in the spleens and the expression of NK1.1 (**Fig. 6c**), Ly-49D, Ly-49A, Ly-49 C/I, Ly-49F/I/C/H and Ly-49G2 (not shown) in Ly5.2 BM into RAE-1ε transgenic mice were similar to that in Ly5.2 BM -> wild-type C57BL/6 mice. In contrast, NKG2D expression on NK cells was diminished in Ly-5.2 BM -> RAE-1ε transgenic mice (**Fig. 6c**). Consistent with the diminished expression of NKG2D on the NK cells, NKG2D-dependent cytotoxic activity was impaired in Ly-5.2 BM into RAE-1ε transgenic mice, as determined by an *in vitro* antibody-redirected cytotoxicity assay (**Fig. 6d**). Furthermore, we investigated whether the chimeric Ly-5.2 BM into RAE-1ε transgenic mice reject the RAE-1ε transgenic C57BL/6 BM cells. As expected, the NK cells that developed in the chimeric wild-type Ly-5.2 C57BL/6 BM into wild-type Ly5-.1 C57BL/6 mice efficiently rejected the transplanted RAE-1ε transgenic BM cells (**Fig. 6e**). In contrast, NK cells that developed in the chimeric Ly-5.2 C57BL/6 BM into RAE-1ε transgenic mice failed to reject the transplanted RAE-1ε transgenic BM cells (**Fig. 6e**). These findings indicated that NKG2D modulation of NK cells is caused by the interaction with irradiation-resistant recipient RAE-1 expressing cells

in vivo, and that this results in impairment of NKG2D function *in vivo*.

To investigate whether F1 hybrid resistance is affected by the diminished expression of NKG2D on NK cells in the RAE-1ε transgenic C57BL/6 mice, we crossed the transgenic mice with BALB/c mice and tested the RAE-1ε transgenic CB6F1 mice for their ability to reject parental BALB/c BM cells. Unlike wild-type CB6F1 mice, the RAE-1ε transgenic CB6F1 mice failed to reject BALB/c BM cells (**Fig. 6f**). Moreover, treatment with NK1.1 mAb or NKG2D mAb did not influence the ^{125}I-UdR incorporation of BALB/c BM cells in the RAE-1ε transgenic CB6F1 recipients; however, depleting NK cells or blocking NKG2D allowed engraftment of BALB/c BM cells in wild-type CB6F1 recipients. These findings, together with the NKG2D mAb blocking experiments, implicate NKG2D as an important component in F1 hybrid resistance against BALB/c BM grafts.

DISCUSSION

In this study, we provide evidence that NKG2D is involved in hybrid resistance – the rejection of parental BM grafts by F1 recipients. RAE-1, an NKG2D ligand, is expressed on BM cells in BALB/c mice and its expression is up-regulated on progenitor cells repopulating irradiated CB6F1recipients. Rejection of parental BALB/c BM cells by CB6F1recipients was blocked by a neutralizing, non-depleting NKG2D mAb. By contrast, repopulating C57BL/6 BM cells express little NKG2D ligand, and rejection of parental C57BL/6 BM by CB6F1 was NKG2D-independent, demonstrating the existence of both NKG2D-dependent and NKG2D-independent mechanisms of hybrid resistance. Furthermore, we have shown that NK cells can reject syngeneic C57BL/6 BM grafts, if these otherwise normal BM cells express sufficient amounts of NKG2D ligand. Therefore, in some situations, NKG2D ligand expression alone is sufficient to serve as a barrier for resistance to BM transplantation.

Several factors appear to regulate hybrid resistance. Activating and inhibiting Ly49 receptors for MHC class I have been proposed to influence NK cell-mediated BM rejection; however, many of the experiments implicating Ly49 in hybrid resistance have used mAbs that deplete NK cells expressing these receptors, rather than blocking specific receptor – ligand interactions[12,25-27]. NK cells in wild-type mice can reject BM grafts from syngeneic β2-microglobulin-null donors; therefore, in this case, NK cells apparently use an as yet undefined MHC class I-independent activating receptor for the detection and elimination of BM cells[28]. Nonetheless, there is evidence that the activating Ly49D receptor expressed in C57BL/6 mice recognizes H-2^d (refs. [25,29,30]), and in some cases this may contribute to hybrid resistance, although Ly49D apparently does not contribute to rejection of parental BM by CB6F1 recipients[27].

Genetic regulation of the NKG2D ligands is also complex. C57BL/6 and BALB/c mice have different genes encoding the NKG2D ligands, and they are regulated differently in the two strains. Here, we show that BALB/c BM cells constitutively express low amounts of RAE-1 and these NKG2D ligands are up-regulated during expansion of proliferating progenitor cells in irradiated recipients. By contrast, the *Raet1* genes in C57BL/6 mice are more tightly regulated and demonstrate much lower expression on BM cells. It is interesting to note that expression of RAE-1 on CB6F1 BM cells appears to behave more like C57BL/6, although further studies are required to define the regulation of these genes in homozygous and heterozygous hosts. Additionally, our findings have revealed the existence of both NKG2D-dependent and NKG2D-independent mechanisms of hybrid resistance, with CB6F1 rejection of parental BALB/c BM representing the former and parental C57BL/6 BM representing the latter mechanism, respectively. Earlier work[31] reported that CB6F1 mice reject C57BL/6 parental BM cells more strongly than BALB/c parental BM grafts, suggesting the possibility that NK cell-mediated rejection of BALB/c

BM may require costimulation by signals through the NKG2D receptor, whereas the as yet unidentified mechanism of CB6F1rejection of C57BL/6 parental BM cells is sufficient without NKG2D activation. The complexity of hybrid resistance may be explained by the fact that different mouse strains express different activating and inhibitory Ly49 receptors, different H-2 ligands, and different NKG2D ligands, which may be regulated in a distinct fashion depending on the genetic background.

The *Raet1* genes were identified first by their expression in mouse embryonic tissues[19], but they are silent in most tissues in adult mice. Our present study indicates that expression of RAE-1 is up-regulated on the BALB/c donor hematopoietic cells repopulating an irradiated host. Examination of the phenotype of the cells repopulating the spleens of the recipients indicated that the RAE-1-bearing cells are predominantly myeloid-lineage progenitors. We did not detect RAE-1 on BM cells isolated from 5-FU-treated BALB/c mice, and in the reconstituted recipients only a minor proportion of the RAE-1-positive cells expressed Sca-1 and c-kit, which are previously defined markers of HSCs. Furthermore, we did not detect RAE-1 expression on the population of freshly isolated BM cells that contains HSCs (gating on lineage marker-negative, Thy1-low, Sca1-high BM cells, unpublished observation). Thus, it appears likely that RAE-1 is predominantly present on the expanding progenitor cells, rather than the long-term HSCs. Prior studies have also suggested that HSCs are not direct targets of NK cell-mediated rejection of BM grafts[32]. In the early embryo, RAE-1 is expressed on cells in several tissues, including the brain, heart, limb buds and brachial arches[19]. Whether NKG2D ligands will be expressed on the progeny of stem cells in tissues or organs other than hematopoietic cells is of interest and may have implications in the transplantation of stem cells into immunocompetent adults.

METHODS

Mice

6-8 week old C57BL/6, BALB/c, CBA/JCr, FVB/NCr, C3H/HeJCr, DBA/2NCr, and CB6F1 mice were purchased from the National Cancer Institute Animal Program, and BALB.B mice were purchased from Jackson Laboratories. NK1.1-congenic NOD mice were generously provided by A. Bendelac (University of Chicago). RAE-1ε transgenic mice were generated as described previously and were backcrossed onto the C57BL/6 background[33]. DAP12-deficient mice on the C57BL/6 background (backcrossed 9 generation) were described previously[34], and DAP10-deficient mice were generated from C57BL/6 embryonic stem cells (JHP, unpublished). All experiments were performed according to the guidelines of the UCSF Committee on Animal Research and the Schering Plough BioPharma Committee on Animal Research.

Reagents, cytokines and antibodies

Mouse NKG2D mAb, clone CX5 (rat IgG1 isotype), was generated as described previously[24]. Mouse NKG2D, clone 191004 (rat IgG2a isotype), was generated in a collaborative project with J.P. Houchins (R&D Systems, Minneapolis, MN). All NKG2D mAbs recognize the NKG2D extracellular domain and efficiently block the binding of NKG2D to its ligands. For *in vivo* injection, we used NKG2D mAb CX5 and NK1.1 mAb PK136 that did not contain detectable endotoxin (<0.3 pg/injection). The NKG2D mAb CX5 is a blocking antibody that does not deplete NKG2D-bearing NK cells or T cells when injected *in vivo*[23,35,36] (**Supplementary Fig 1**). Control rat IgG was purchased from Sigma. Pan-RAE-1 mAb (clone 186107, rat IgG2b isotype), H60 mAb (clone 205310) and MULT1 mAb (clone 237104) were generated in a collaborative project with J.P. Houchins (R&D Systems)[35,36]. Other antibodies were purchased from BD PharMingen or

eBiosocience. Mouse IL-3, stem cell factor, and IL-6 were purchased from R&D Systems.

BM transplantation

The method has been described previously[25]. Briefly, mAb treatments (200 μg/mouse) were performed 2 days before BM transfer, and all recipients were treated with poly I:C (Sigma, 200 μg/mouse) to boost NK cell-mediated graft rejection[37] one day before injection of BM cells. On day 0, mice were irradiated by exposure to lethal doses (11Gy) of ^{137}Cs gamma irradiation, and then 4 x 10^5 or 4 x 10^6 BM cells were injected intravenously (i.v.). 5 days after transfer, the mice were given 26 μg of 5-fluoro-2' - deoxyuridine (Sigma) i.v. to suppress endogenous thymidine synthesis[25]. Thirty min later, the mice were given 3 μCi of 5-[^{125}I]iodo-2'-deoxyuridine (Amersham Life Science) i.v. On day 6, the spleens were removed from recipient mice and counted by using a gamma counter.

Generation of BM chimeric mice

The method has been described previously[38]. Briefly, 1x10^7 Ly5.2 C57BL/6 BM cells were transferred intravenously into NK cell-depleted and irradiated recipient mice (absorbed dose of radiation =11 Gy). During reconstitution, mice were maintained on antibiotics.

Preparation of NK cells

NK cells were enriched as described previously[39]. Briefly, spleen cells were incubated with CD4 mAb (clone GK1.5) and CD8 mAb (clone 53-6.7), and thereafter these cells were mixed with magnetic beads coated with goat anti-mouse Ig and goat anti-rat Ig (Advanced Magnetic, Inc.). CD4, CD8, and surface Ig (sIg)-positive cells were removed by magnetic cell sorting.

Flow Cytometric Analysis

To detect NKG2D ligands, we used the extracellular domain of mouse NKG2D fused to human IgG1 Fc (mNKG2D-Ig)[16]. A phycoerythrin (PE)-conjugated goat anti-human IgG Fcγ fragment (Jackson ImmunoResearch) was used as a second step reagent. One million cells were stained with 0.5 μg of mNKG2D-Ig and with 0.25 μg of other mAbs. To determine which NKG2D ligands were expressed, cells were stained with a biotinylated pan RAE-1 mAb that recognizes all five known RAE-1 proteins (i.e. RAE-1α, β, γ, δ and ε), biotinylated H60 mAb or MULT1 mAb. PE-conjugated streptavidin or allophycocyanine (APC)-conjugated streptavidin was used to detect biotinylated mAbs. For detection of NKG2D, one million cells were stained with 0.25 μg biotinylated or PE-labeled NKG2D mAb (clone 191004). Cells were co-stained with CD43, Ly6C/G, CD11c, B220, CD3, TER119, NK1.1 and CD49d (DX5) mAbs. The cells were incubated with mAbs for 20 min and washed with PBS containing 0.01% NaN_3. Cells were analyzed by using a FACSCalibur (Becton Dickinson). Viable lymphocyte populations were gated based on forward and side scatter profiles and by lack of propidium iodide staining.

Cytotoxic Assay

mAb-mediated redirected cytotoxicity assays were performed as described previously[40]. For effector cells, C57BL/6 NK cells cultured for 7 days in RPMI-1640 medium containing 10% FCS and 2000 U/ml recombinant human IL-2 (National Cancer Institute Biological Resources Branch Pre-clinical Repository). Target cells were labeled with 50 μCi of $Na_2(^{51}Cr)O_4$ for 2 h at 37°C in RPMI-1640 medium containing 10 % FCS, washed three times with medium, and used in cytotoxicity assays. ^{51}Cr-labeled target cells (5 x 10^3) and effector cells were mixed in U-bottomed wells of a 96-well microtiter plate at the indicated E/T ratios in triplicate. After 4 h incubation, the cell-free supernatants were collected and

the radioactivity was measured in a Micro-beta counter (Wallac). The spontaneous release was less than 15% of the maximum release. The percentage of specific ^{51}Cr release was calculated according to the following formula: % Specific lysis =(experimental – spontaneous) release x 100 / (maximal – spontaneous) release.

Acknowledgements

We thank N. Killeen and J. Dietrich of the UCSF Comprehensive Cancer Center Transgenic and Targeted Mutagenesis Shared Resource for generation of RAE-1ε transgenic mice, and all Lanier lab members for technical assistance and critical discussion, and T. Sasazuki for helpful discussion. NIH grant CA95137 supported this work. A Human Frontier Science Program Long-term Fellowship, the Uehara Memorial Foundation, the Irvington Institute Foundation and UCSF Liver Center Pilot & Feasibility Grant P30-DK26743 supported K.O. L.L.L. is an American Cancer Society Research Professor.

COMPETING INTERESTS STATEMENT

These studies were supported in part by Schering Plough BioPharma.

Figure legends

Figure 1. RAE-1 is expressed on BALB/c BM cells, but not on C57BL/6 BM cells. **(a)** Freshly isolated BALB/c BM cells were stained with a mouse NKG2D-human Ig Fc fusion protein (NKG2D Ig) or control human Ig (cIg). To detect the binding of NKG2D-Ig, a PE-conjugated anti-human IgG was used as a second step antibody. The dotted line represents cIg staining and the thick line shows NKG2D ligand expression on BM cells. (**b)** C57BL/6 BM cells were stained with biotinylated pan RAE-1 mAb, biotinylated H60 mAb, biotinylated MULT1 mAb or a biotinylated isotype-matched cIg, and then were stained with PE-conjugated streptavidin. The dotted line shows the cIg staining and the thick line shows RAE-1, H60 and MULT1 expression on BM cells. (**c, d)** CB6F1 recipients were treated with NK1.1 mAb on day -2. On day 0, recipients were irradiated (11 Gy) and then reconstituted with BALB/c or CB6F1 BM cells ($4x10^6$). On day 7, cells from the recipient spleens were isolated and analyzed as in **a, b**. The dotted line represents cIg staining and the thick line shows NKG2D ligand (**c**), RAE-1, H60 and MULT1 (**d**) expression on BM cells. Numbers represent the mean fluorescence (arbitrary linear units) of the stained cells (**a-d**). (**e, f)** BM cells were transferred into irradiated recipients pretreated with NK1.1 mAb. Cells were isolated and stained as in **c**. (**g)** BALB/c BM cells were transferred into irradiated CB6F1mice that were pretreated with NK1.1 mAb. 6 days after transfer, BrdU was injected into mice. 2 h or 12 h later, cells from recipient spleens were collected and stained with pan-RAE-1 mAb and anti-BrdU. **(h**) BM cells from 5-FU-treated BALB/c mice were transferred into irradiated CB6F1mice that were pretreated with NK1.1 mAb. 8 days post-transfer, cells were isolated and analyzed as in **c,e**. In panel **a-d**, numbers in upper right corner of the histograms represent the mean fluorescence intensity (arbitrary linear units) of the cells stained with NKG2D-Ig or the specific mAbs. In panel **e-h**, >98% of cells stained with cIg were in the lower left quadrant (not shown), and the

percentage of cells in each quadrant is displayed. Results shown in **a-h** were reproducible (at least 2 independent experiments), and representative data are presented.

Figure 2. NKG2D blocks BALB/c BM into CB6F1 hybrid resistance.
(a) $4x10^6$ BM cells were transferred into irradiated CB6F1 recipients, mice were injected with ^{125}I-UdR on day 5 and spleens were harvested and counted on day 6. Black bars show ^{125}I-UdR uptake of spleens in BALB/c BM into CB6F1mice and white bars show uptake of radiolabel in CB6F1 BM into CB6F1recipients. Mice were treated with the non-depleting, neutralizing NKG2D mAb or the NK cell-depleting NK1.1 mAb (200 μg/mouse on day –2), as indicated. Results are the mean $\pm$ s.d. cpm (5 mice per group). The experiment was performed twice with comparable results. **(b)** Phenotype of BALB/c donor cells repopulating irradiated CB6F1recipients treated with NKG2D mAb or control Ig. Mice were treated as described in **a**, and splenocytes were harvested on day 8 post-transplantation. Cells were stained and data are presented as described in **Fig. 1**. Results shown were reproducible (at least 2 independent experiments), and representative data are presented.

Figure 3. NKG2D-independent rejection of C57BL/6 parental BM grafts in CB6F1recipients.
$4x10^6$ BM cells were transferred into irradiated CB6F1recipients, mice were injected with ^{125}I-UdR on day 5 and spleens were harvested and counted on day 6. Black bars show ^{125}IUdR uptake of spleens in C57BL/6 BM into CB6F1mice and white bars show uptake of radiolabel in CB6F1 BM into CB6F1 recipients. Mice were treated with the non-depleting, neutralizing NKG2D mAb or the NK cell-depleting NK1.1 mAb (200 μg/mouse on day

–2), as indicated. Results are the mean ± s.d. cpm (5 mice per group). Results shown were reproducible (at least 2 independent experiments), and representative data are presented.

Figure 4. Rejection of syngeneic BM cells expressing RAE-1.
(a) Freshly isolated BM from wild-type C57BL/6 and RAE-1ε transgenic C57BL/6 mice were stained with cIg or pan-RAE-1 mAb. Data are presented as described in **Fig. 1**. (**b)** Freshly isolated BM from wild-type C57BL/6 and RAE-1ε transgenic C57BL/6 mice were used as targets in a standard *in vitro* cytotoxicity assay using IL-2-activated wild-type NK cells as effectors in the presence of cIg or NKG2D mAb (clone 191004). MAbs were used at 10 μg/ml. (**c**) $4x10^6$ RAE-1ε transgenic C57BL/6 BM cells were transferred into irradiated C57BL/6 recipients, mice were injected with ^{125}I-UdR on day 5 and spleens were harvested and counted on day 6. Black bars show ^{125}I-UdR uptake of spleens in RAE-1ε transgenic BM into C57BL/6 mice and white bars show uptake of radiolabel in wild-type C57BL/6 BM into C57BL/6 recipients. Mice were treated with the non-depleting, neutralizing NKG2D mAb or the NK cell-depleting NK1.1 mAb (200 μg/mouse on day –2), as indicated. Results are the mean ± s.d. cpm (5 mice per group). All experiments were performed at least twice with comparable results.

Figure 5. Rejection of RAE-1ε transgenic C57BL/6 BM in DAP10-deficient and DAP12-deficient C57BL/6 recipients.
$4x10^6$ RAE-1ε transgenic C57BL/6 BM cells were transferred into irradiated wild-type C57BL/6 recipients (black bars), DAP10-deficient C57BL/6 recipients (white bars in **a**), or DAP12-deficient C57BL/6 recipients (white bars in **b**). Mice were injected with ^{125}I-UdR on day 5 and spleens were harvested and counted on day 6. Mice were treated with the non-depleting, neutralizing NKG2D mAb or the NK cell-depleting NK1.1 mAb (200

μg/mouse on day –2), as indicated. Results are the mean ± s.d. cpm (5 mice per group). Results shown were reproducible (at least 2 independent experiments), and representative data are presented.

Figure 6. Impairment of bone marrow rejection in RAE-1ε transgenic mice. **(a**) Splenocytes from wild-type and RAE-1ε transgenic C57BL/6 mice were stained with pan-RAE-1 mAb (left panels) or NKG2D and NK1.1 mAb (right panels). RAE-1 expression was analyzed on spleen cells, and NKG2D expression was analyzed by gated on $NK1.1^+$ cells. Thin lines show cells stained with cIg; thick lines show RAE-1 or NKG2D specific staining. Numbers represent the mean fluorescence (arbitrary linear units) of the stained cells. **(b)** Enriched NK cells were prepared from wild-type or RAE-1ε Tg C57BL/6 mice that were injected i.p. with poly I:C (100 μg/mouse) one day before harvesting the splenocytes. Monoclonal antibody-dependent re-direct killing assays against CD32-transfected 721.221 target cells were performed as described [40] by using control Ig (cIg), NKG2D, or NK1.1 mAb. **(c)** Ly 5.2 C57BL/6 BM cells ($1x10^7$/mouse) were transferred into irradiated wild-type (WT) or RAE-1ε Tg C57BL/6 mice. Three months after transplantation, the expression level of NKG2D (left panels) and NK1.1 (right panels) was analyzed on splenic NK cells (gated on $CD3^-$, $NK1.1^+$ lymphocytes). Thin lines show cells stained with cIg; thick lines show RAE-1 or NKG2D specific staining. Numbers represent the mean fluorescence intensity (arbitrary linear units) of the stained cells. **d.** Enriched NK cells were prepared from the spleen of Ly5.2 C57BL/6 BM into RAE-1 Tg and Ly5.2 C57BL/6 BM into C57BL/6 mice injected i.p. one day before with poly I:C (100 μg/mouse). mAb-dependent re-directed cytotoxicity assays were performed as in **Fig. 4b**. **(e)** Black bars show ^{125}I-UdR uptake in spleens of $RAE\text{-}1^+$ Tg BM cells into chimeric mice

(Ly5.2 C57BL/6 BM into wild-type C57BL/6) and white bars show uptake of radiolabel in spleens of RAE-1^+ Tg BM cells into chimeric mice (Ly5.2 C57BL/6 BM into RAE-1 Tg chimeric mice). **(f)** Impaired hybrid resistance in RAE-1ε transgenic CB6F1 mice. $4x10^6$ BALB/c BM cells were transferred into irradiated recipients, mice were injected with ^{125}I-UdR on day 5 and spleens were harvested and counted on day 6. Black bars show ^{125}I-UdR uptake of spleens in BALB/c BM into wild-type CB6F1mice, white bars show uptake of radiolabel in BALB/c BM into RAE-1ε transgenic CB6F1recipients, and gray bars show CB6F1BM into CB6F1 mice. Mice were treated with the non-depleting, neutralizing NKG2D mAb or the NK cell-depleting NK1.1 mAb (200 μg/mouse on day –2), as indicated. Results are the mean $\pm$ S.D. cpm (5 mice per group). All experiments were performed at least twice with comparable results.

Supplementary Figure 1. NKG2D mAb CX5 does not deplete NK cells *in vivo.* C57BL/6 mice were injected with 200 μg NKG2D mAb CX5 or PBS (control) on day 0. Splenocytes were harvested on day 2 and day 5 and stained with FITC-conjugated NK1.1 mAb (x-axis) and PE-conjugated NKG2D mAb (y-axis). The percentage of NK1.1^+ NK cells (4% of cells in all panels) was unaffected by *in vivo* treatment with NKG2D mAb CX5, whereas there was evidence for modulation of NKG2D (that is expression of NKG2D on the NK cells) in the CX5 mAb-treated mice. Data shown are representative of several experiments in C57BL/6, BALB/c and NOD mice treated with CX5 mAb.

Supplementary Figure 2. H-2 incompatibility is not required for NKG2D-dependent BM rejection.

$4x10^5$ C57BL/6 or BALB.B BM cells were transferred into irradiated C57BL/6 recipients, mice were injected with ^{125}I-UdR on day 5 and spleens were harvested and counted on day

6. Black bars show ^{125}I-UdR uptake of spleens in BALB.B BM into C57BL/6 mice and white bars show uptake of radiolabel in C57BL/6 BM into C57BL/6 recipients. Mice were treated with the non-depleting, neutralizing NKG2D mAb or the NK cell-depleting NK1.1 mAb (200 μg/mouse on day –2), as indicated. Results are the mean $\pm$ s.d. cpm (5 mice per group). Experiments were performed twice with comparable results.

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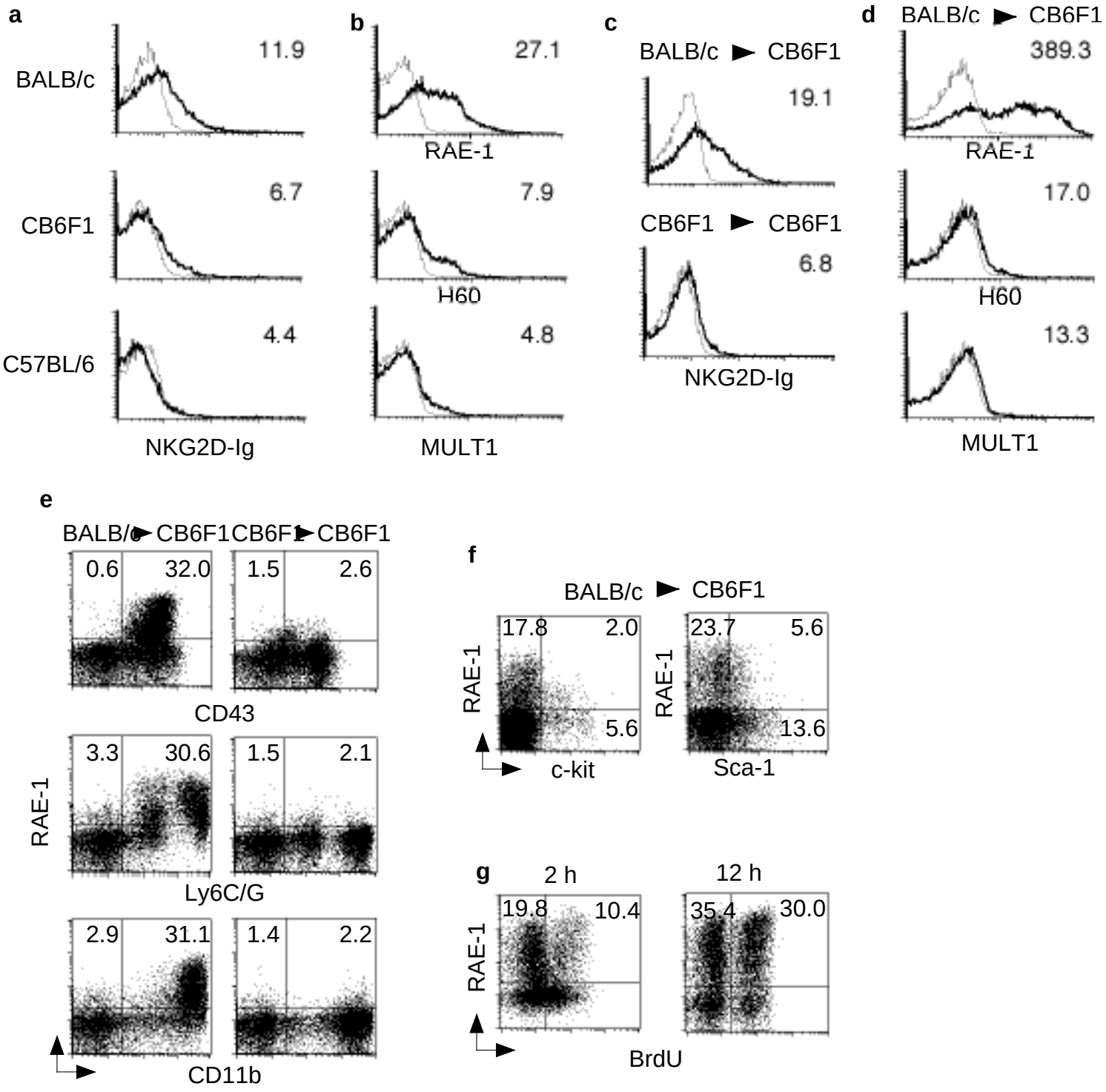
Strain	RAE-1	H60	MULT1
C57BL/6	3% (MFI = 81)	<2%	<2%
BALB/c	14% (124)	5% (36)	<2%
BALB.B	12% (93)	5% (33)	<2%
C3H	4% (118)	<2%	3% (53)
CBA/J	3% (58)	<2%	3% (52)
DBA/2	3% (59)	<2%	3% (42)
FVB	3% (97)	<2%	<2%
NK1.1 NOD	7% (87)	10% (45)	<2%

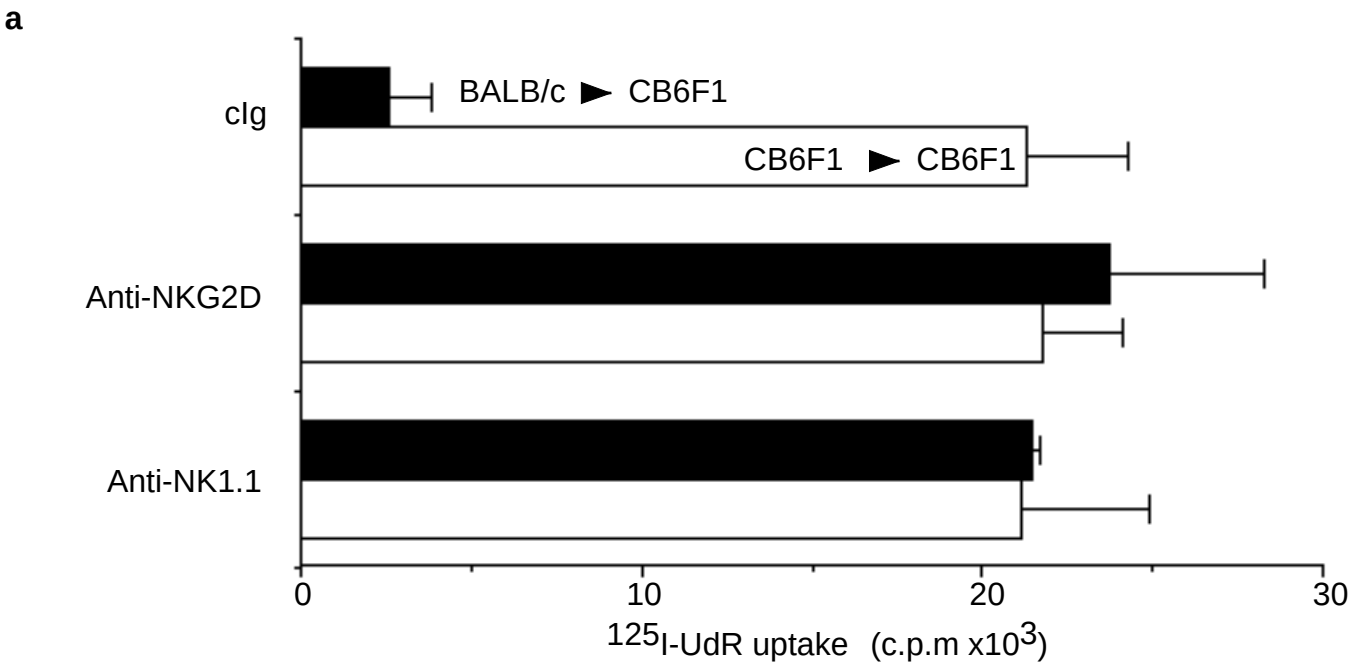
Table 1. Expression of NKG2D ligands on *in vitro* cultured bone marrow cells.

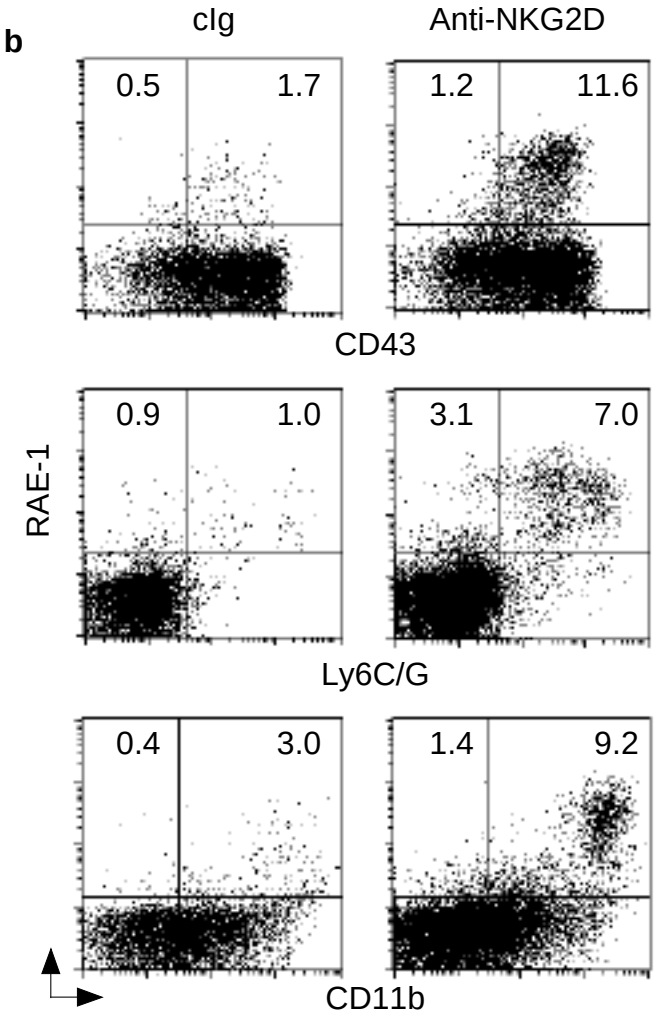
Bone marrow cells isolated from the indicated mouse strains were cultured (5 x 10^6 cells/ml in RPMI-1640 + 10% FCS) for 4 days with mouse IL-3 (10 ng/ml), stem cell factor (10 ng/ml), and IL-6 (10 ng/ml), harvested and then stained with control Ig or RAE-1, H60 or MULT1 mAb. The percentages of cells expressing each of the NKG2D ligands are shown, and the mean fluorescence intensity (MFI) (gating on positive cells) is indicated in parentheses.

Ogasawara Figure 1



Ogasawara Figure 2





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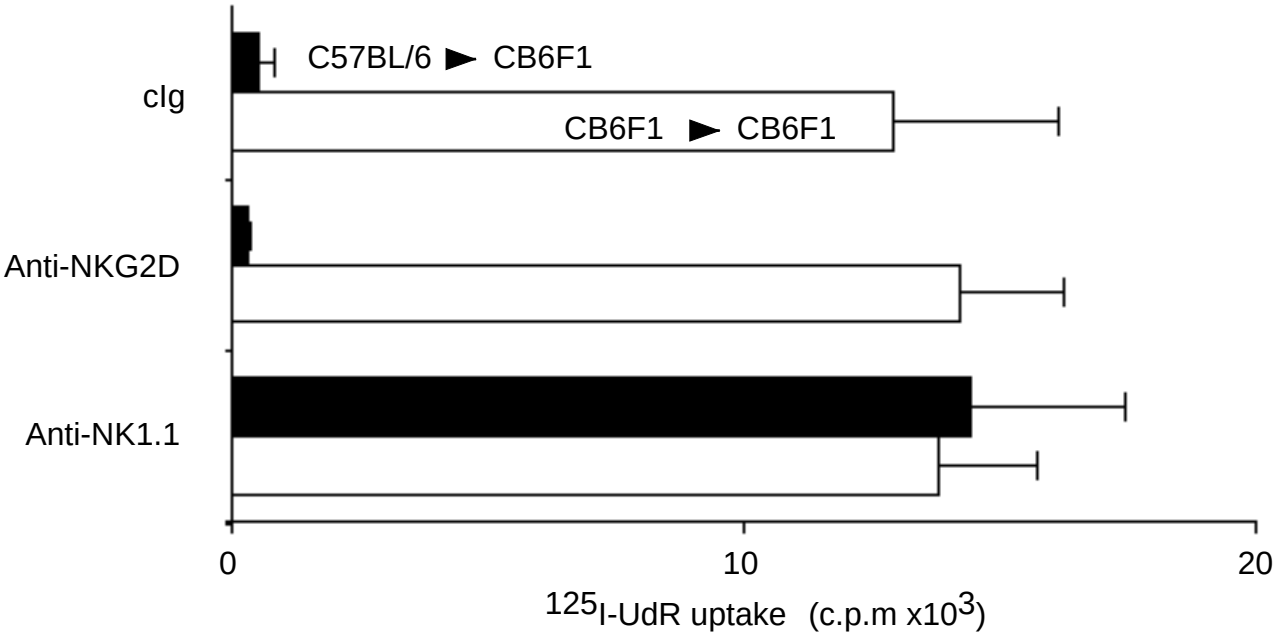

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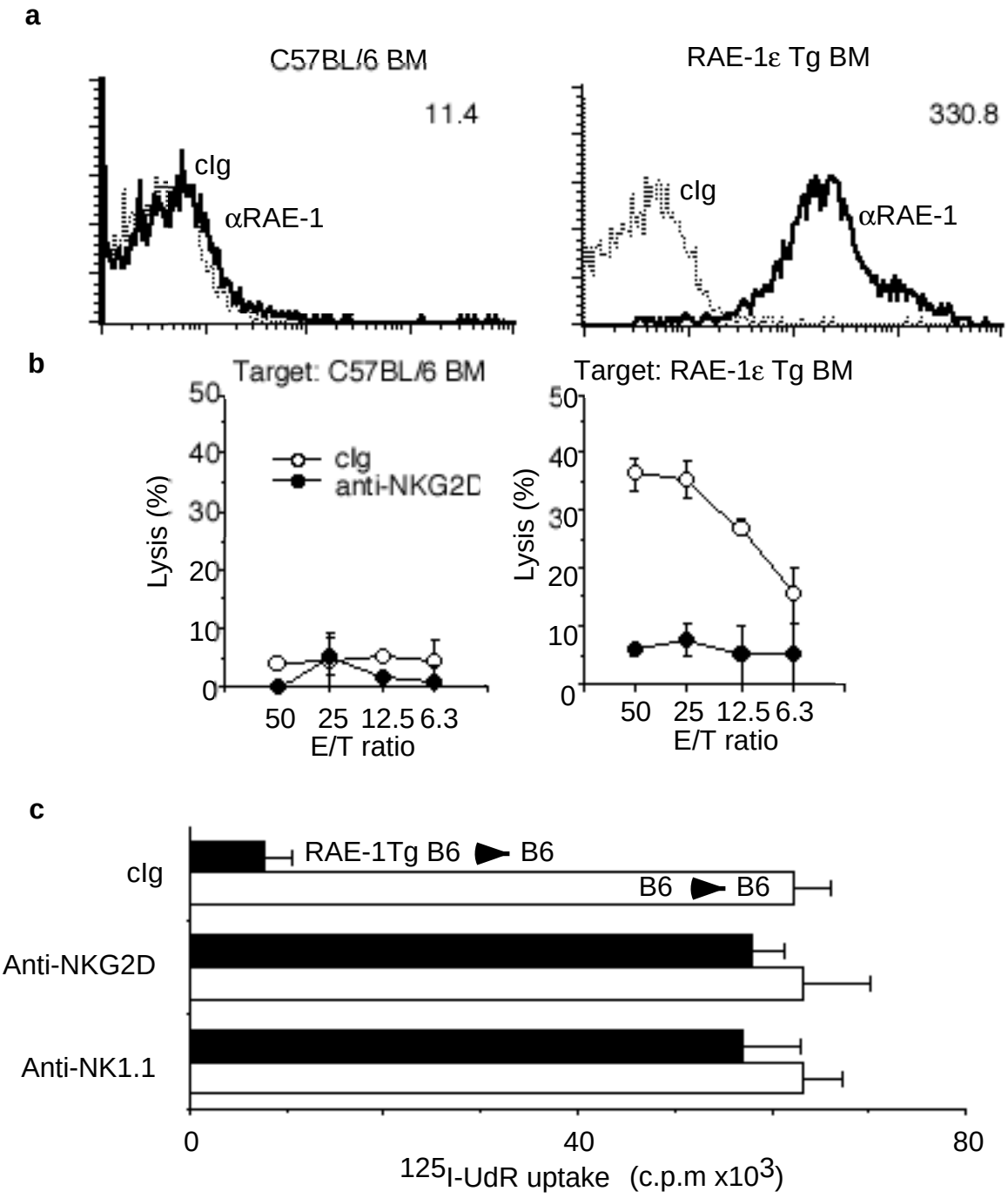

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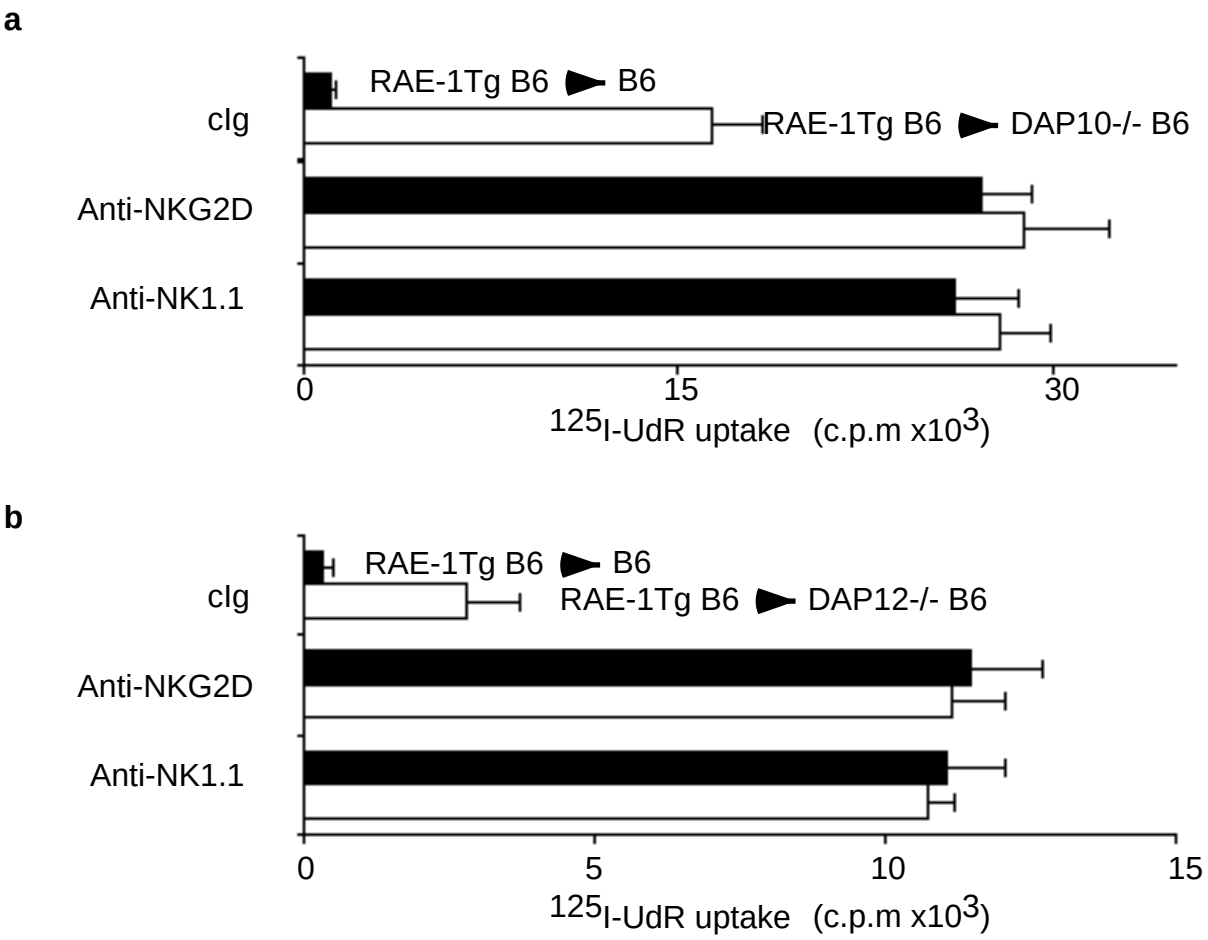

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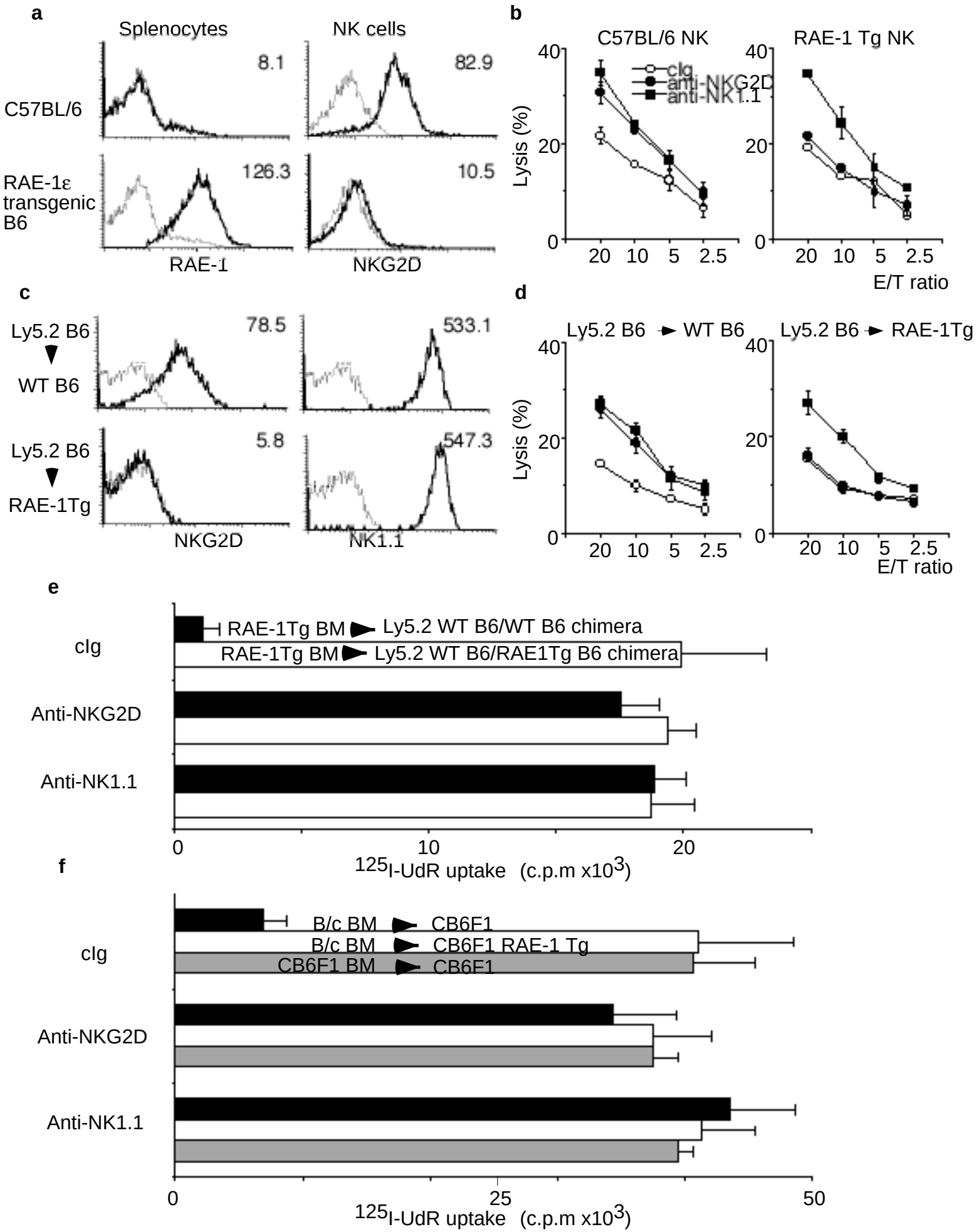
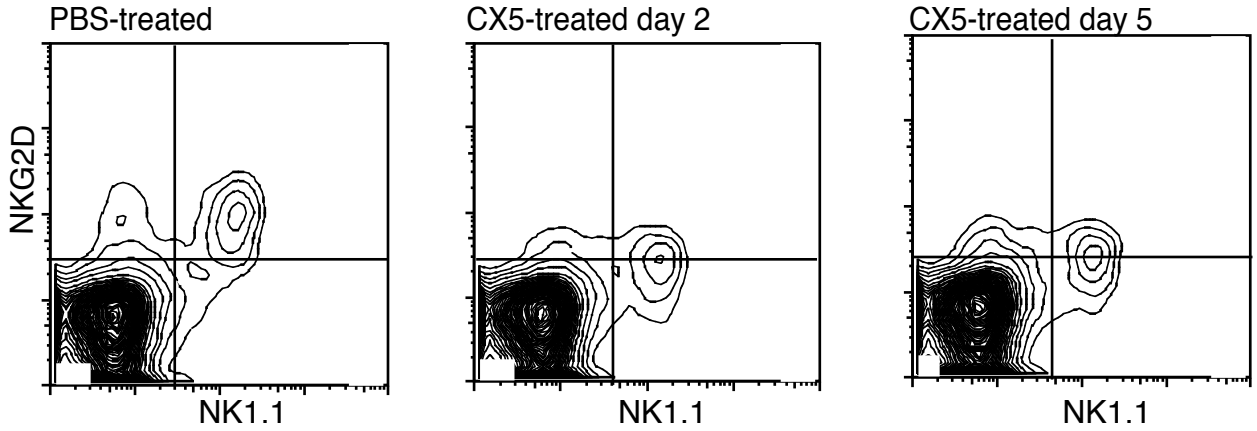


Ogasawara Supplementary Figure 1

Ogasawara Supplementary Figure 1



Ogasawara Supplementary Figure 2