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A Ligand for the Murine NK Activation Receptor Ly-49D: Activation of Tolerized NK Cells from β2-Microglobulin-Deficient Mice

Hiroshi Furukawa,* Koho Iizuka,* Jennifer Poursine-Laurent,* Nilabh Shastri, † and Wayne M. Yokoyama2‡

Mouse NK cells express inhibitory NK receptors that recognize target cell MHC class I molecules and activation receptors that are less well defined. The Ly-49D activation receptor on C57BL/6 NK cells recognizes Chinese hamster ovary cells and triggers natural killing. In this study, we demonstrate that a Chinese hamster classical MHC class I molecule is the ligand for Ly-49D in a reporter gene assay system as well as in NK cell killing assays. Ly-49D recognizes the Chinese hamster class I molecule better when it is expressed with Chinese hamster β2-microglobulin (β2m) than murine β2m. However, it is still controversial that Ly-49D recognizes H-2Dd4, as we were unable to demonstrate the specificity previously reported. Using this one ligand-one receptor recognition system, function of an NK activation receptor was, for the first time, investigated in NK cells that are tolerized in β2m-deficient mice. Surprisingly, Ly-49D-killing activity against ligand-expressing targets was observed with β2m-deficient mouse NK cells, albeit reduced, even though “tolerized” function of Ly-49D was expected. These results indicate that Ly-49D specifically recognizes the Chinese hamster MHC class I molecule associated with Chinese hamster β2m, and indicate that the Ly-49D NK cell activation receptor is not tolerized in β2m deficiency. * The Journal of Immunology, 2002, 169: 126–136.

Natural killer cells constitute a subpopulation of lymphocytes that mediate natural killing against tumor- or virus-infected cells. In recent years, it has become clear that NK cells can kill target cells that fail to express MHC class I molecules, whereas targets expressing MHC class I molecules are generally resistant to NK cell-mediated lysis. According to the “missing self” hypothesis (1–3), down-regulation of MHC class I expression may permit a pathogenic process to evade acquired specific immunity, but this would result in release of the inhibitory influence of MHC class I on NK cells, permitting NK cell lysis. The molecular basis for the missing self hypothesis is explained, in part, by MHC class I inhibitory receptors; engagement of these NK cell receptors results in the transduction of an inhibitory signal that prevents the killing of the target cells (4).

Detailed studies over the last decade have indicated that NK cell inhibitory receptors fall into two structural categories: Ig-like receptors with type I integral membrane protein orientation, as illustrated by the human killer Ig-like receptors, and mouse gp49B; and C-type lectin-like receptors with type II integral membrane orientation that include the prototypic mouse NK cell inhibitory receptor, Ly-49A. Despite the structural differences, both types of inhibitory receptors deliver negative signals via the same mechanism, involving the immunoreceptor tyrosine-based inhibitory motif (ITIM)3 in their cytoplasmic domain. Phosphorylation of the ITIM recruits and activates the tyrosine phosphatase, SHP-1, that then presumably dephosphorylates molecules in the activation cascade, leading to inhibition.

Other observations also suggested that NK cell target specificity is not solely due to the action of inhibitory receptors. Mouse NK cells have poor killing capacity against human tumor targets that are killed well by human NK cells, and vice versa, regardless of MHC class I expression on the targets. In several experimental systems, there was evidence supporting the existence of NK cell receptors that activate (5). Nevertheless, the activation receptors are not well defined. Candidate activation receptors are often highly related to the inhibitory receptors, and are encoded in the same genomic region. Putative activation receptors, however, do not contain cytoplasmic ITIMs and often contain charged residues in their transmembrane domain that facilitate association with other transmembrane molecules that can provide positive signaling action. For example, Ly-49 receptors lacking ITIMs (Ly-49D and H), but containing charged transmembrane residues, associate with the DNA-binding protein of 12 kDa (DAP12)/killer cell activating receptor-associated protein (KARAP) molecule, which transduces activation signals through its cytoplasmic immunoreceptor tyrosine-based activation motifs (6). However, the ligands are poorly understood for most NK cell activation receptors. Hence, most candidate NK cell activation receptors are orphan receptors.

3 Abbreviations used in this paper: ITIM, immunoreceptor tyrosine-based inhibition motif; β2m, β2-microglobulin; CHO, Chinese hamster ovary; DAP12, DNA-activating protein of 12 kDa; KARAP, killer cell activating receptor-associated protein; GFP, green fluorescent protein; hCD4, human CD4; LAK, lymphokine-activated killer cell; IRES, internal ribosomal entry site; lacZ, bacterial-β-galactosidase.
In previous studies, we exploited the profound differences between NK cells from C57BL/6 (B6) and BALB/c mice with respect to killing of Chinese hamster ovary (CHO) cells and ultimately defined the specificity of the Ly-49D activation receptor in B6 mice for CHO killing (7). In brief, most targets were killed equally well by B6 and BALB/c NK cells, but only B6 NK cells killed CHO cells efficiently. Subsequently, anti-Ly-49D mAb, 4E4, blocks the CHO cell killing by B6 NK cells, but does not recognize BALB/c NK cells. These data indicate that Ly-49D in B6 NK cells specifically recognizes a ligand on CHO cells and activates killing.

Although the enhanced NK cell killing of target cells lacking MHC class I expression was first demonstrated in vitro with tumor cells, this correlation has been extended to other normal cells in vivo. Con A blasts produced from β2-microglobulin (β2m)-deficient mice are more susceptible to in vitro killing by NK cells from otherwise syngeneic wild-type mice (8, 9). Moreover, bone marrow transplant grafts from β2m-deficient mice are rejected by NK cells in syngeneic wild-type hosts (10). Interestingly, however, NK cells from β2m-deficient mice do not have reactivity against autologous β2m-deficient Con A blasts, and could not reject an autologous β2m-deficient bone marrow graft, which was strongly rejected in wild-type mice. In addition, these NK cells failed to kill allogeneic Con A blasts (8, 9), and could not reject an allogeneic bone marrow graft (11). NK cells from β2m−/− mice also have depressed NK cell activity against various target cell lines (12), indicating that β2m−/− mouse NK cells are tolerant for various target cells, including MHC class I-deficient missing self target. For some targets, in vitro culture of NK cells with cytokines can abrogate this tolerance (13). These data indicate a general tolerance for autologous and allogeneic cells in β2m-deficient NK cells. The altered function of NK cells in β2m−/− mice has been explained by the higher expression levels of inhibitory NK receptors (14). However, this hypothesis cannot explain the impaired killing of β2m-deficient NK cells against MHC class I-deficient target cells, which cannot induce inhibitory signals. It is also possible that the tolerance could be due to the dysfunction or the lower expression levels of activation receptors. But, the specific functions of NK activation receptors in β2m−/− mice have not yet been analyzed.

In this study, we report the ligand of Ly-49D receptor on CHO cells. This ligand, a Chinese hamster molecule termed Hm-1-C4, resembles classical MHC class I molecules and is recognized more strongly by Ly-49D when it is expressed with Chinese hamster β2m. Identification of a ligand for an NK cell activation receptor also allowed us to examine the issue of NK cell tolerance in β2m−/− mice with respect to activation receptors.

Materials and Methods

Mice
B6, BALB/c, and β2m−/− mice backcrossed to B6 11 times were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a specific pathogen-free facility at Washington University, under supervision of veterinarians in the Division of Comparative Medicine. All experiments were approved by the Institutional Animal Studies Committee.

mAbs and cytokine
The following mAbs were used: mAb 4E4 (anti-Ly-49D mAb) (15), PK136 (anti-NK1.1 mAb), B22/49 (anti-H-2Dª mAb; gift from T. Han- sen, Washington University, St. Louis, MO) (16), and 2C11 (anti-CD3ª mAb). Anti-human CD4 (hCD4) PE mAb was purchased from BD Biosciences (Sunnyvale, CA). W6/32, anti-human HLA class I mAb, was purchased from DAKO (Glostrup, Denmark). Human IL-2 was purchased from Chiron (Emeryville, CA).

Cell lines
Plat-E was kindly provided by T. Kitamura (University of Tokyo, Institute of Medical Science, Tokyo, Japan) (17). X63Ag8-653.mIg-L-3 was kindly provided by H. Karasuyama (Tokyo Medical and Dental University, Tokyo, Japan) (18). BWZ.36 was previously described (19). BaF/3 was provided by the late M. Thomas (Washington University School of Medicine). CHO (Pro-5) was provided by P. Stanley (Albert Einstein College of Medicine, Bronx, NY). RMA was provided by K. Kärre (Karolinska Institute, Stockholm, Sweden). C1498, Daudi, Phoenix-A, and 293T cells were obtained from American Type Culture Collection (Manassas, VA). CHO cells were cultured in a MEM supplemented with penicillin G, streptomycin, t-glutamine, and 10% heat-inactivated FCS (Harlan Bioproducts for Science, Indianapolis, IN). Daudi and Phoenix-A cells were grown in DMEM supplemented with penicillin G, streptomycin, t-glutamine, and 10% FCS. Plat-E cells were cultured in DMEM supplemented with penicillin G, streptomycin, t-glutamine, and 10% FCS. BaF/3 cells were grown in the presence of 10% culture supernatant of X63Ag8-653.mIg-L-3.

RT-PCR
mRNAs were prepared from CHO cells, using Fast Track 2.0 kit (Invitro- gen, Carlsbad, CA). RACE-PCR was done using SMART RACE cDNA amplification kit (Clontech Laboratories, Palo Alto, CA) with Syrian hamster MHC class I consensus primer sets (AGTTCGTGCCTCCAGCACAG, CAGGCTTCTGTTCAAGGCCATGTAATC and GAGCGGGG GCCGTYGTTGATG, TACCCGGGAGGAGGCCGTC) (20, 21). Based on the sequences obtained from the 3′ and 5′ RACE-PCR products, sequence-specific primer sets were designed and used for the amplification of full-length Hm1-C1, C2, C5 (ACTCAAGATCGAGATGGGGCGTGGT, GTCTGATCTCCGGCTATACGGCACAG, Hm3-C1) (CGCGGG CGTGAATGGGACCATGCCGGTCTTGGTCC, GAACCTGGTCCCG GAGCGCCTGCGGAA), and Hm-C4 (ACTCAAGATCGAGATGGG TGCAAGTGGCC, GGGCAAGACATTTGTCTGACTCCTGGCTAT). Murine β2m and β2m alleles, and human, rat, and Chinese hamster β2m were amplified by PCR with specific primers (GenBank accession nos. X57112, 22, 23). Murine β2m was generated from murine β2m allele using PCR-based mutagenesis.

Retrovirus vectors and transduction
The retrovirus vectors pMX (24) and pMX-RES-GFP (pIG) (25) were kindly provided by T. Kitamura. The pMX-RES-hCD4 (pI4) was generated by replacing the IRES-GFP with IRES-hCD4 from CD4-RV vector (gift from K. Murphy, Washington University) (26). Hml1 and β2m CD- NAs from various species were subclassed into pH or pIG. The fusion constructs of Hml1 with red fluorescence protein (ClontechLaboratories) were also generated to show the ability of the molecules to be expressed on the cell surface. To introduce these cDNAs into cells, the plasmids were transfected into Plat-E or Phoenix-A packaging cell lines with Fugene6 (Roche Molecular Biochemicals, Mannheim, Germany). Target cells were incubated with the viral supernatant and 10 μg/ml polybrene (27) or in culture plates coated with Retronectin (Takara, Osaka, Japan) (28).

DAZ assay
The DAZ construct was made by fusing the mouse CD3ª chain cytoplasmic domain (aa 52–164), Ly-49A transmembrane domain (aa 40–66), and Ly-49D extracellular domain (aa 70–269). The Ly-49A transmembrane domain was introduced to avoid any confounding interaction with DAP12/ KARAP. The DA construct consists of Ly-49A transmembrane domain (aa 40–66) and Ly-49D extracellular domain (aa 70–269). These constructs were retrovirally transduced into BWZ.36 cells to generate DAZ and DA cells. One hundred thousand DAZ or DA cells were cocultivated with the same number of the target cells for 16 h in 96-well plates and lysed with 100 μl chloroform red β-galactosidase (CPRG) working solution (0.15 mM CPRG, 100 mM 2-ME, 9 mM MgCl2, 0.125% Nonidet P-40 in PBS) (19). Bacterial-β-galactosidase (lacZ) activity was determined by colorimetric assay for CPRG substrate conversion by reading absorption of each well at 495 and 630 nm for reference using 96-well microplate reader (Spectra Max Plus; Molecular Devices, Sunnyvale, CA).

Flow cytometric analysis
Cells were stained with fluorescein-conjugated mAbs, as previously described and analyzed, using FACSCalibur flow cytometer (BD Bio- sciences) (29).
Cytotoxicity assay

Lymphokine-activated killer (LAK) cells were generated, as described previously, with minor modifications (7). On day 5 or 6, adherent LAK cells were separated by mAb 4E4 and CELLection Pan Mouse IgG kit, according to the manufacturer’s instruction (Dynal Biotech, Oslo, Norway). The purity of the Ly-49D⁺ LAK cells was always >80%. The percentages of LAK A⁺ cells were similar on both sorted populations. Separated LAK cells were expanded in culture until day 9–12 and used in cytotoxicity assays that were performed in a standard 4-h ⁵¹Cr release assay. Alternatively, fresh NK cells were enriched by depletion of surface Ig⁺ B cells from total splenocytes using CELLection Pan Mouse IgG kit. Subsequently, NK cells were purified using DXS-coated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and used in a standard 4-h ⁵¹Cr release assay. Effector cells were incubated with ³¹Cr-labeled targets at various E:T ratios. After 4 h, supernatants were harvested, and their radioactivity was determined. The percent specific cytotoxicity (% cytotoxicity) was calculated using the following formula: % cytotoxicity = (experimental lysis/spontaneous lysis)/(maximum lysis/spontaneous lysis) × 100. All cytotoxicity assays were carried out in triplicate wells. Data are presented as mean ± SD.

Retrovirus-mediated gene transfer

Retrovirus-mediated gene transfer was done, as described, with minor modifications (30). Bone marrow hemopoietic precursors were isolated following i.p. injection of 5-fluorouracil (150 mg/kg). Cells flushed from femora and tibias of the BALB/c mice were cultured for 2 days in DMEM supplemented with penicillin G, streptomycin, 15% FCS, stem cell factor (100 ng/ml), IL-6 (100 ng/ml), and IL-3 (10 ng/ml). Bone marrow cells were washed and resuspended in the same medium with an equal volume of viral supernatant in the presence of polybrene and the same cytokines. This infection protocol was repeated on the following day. Recovered bone marrow cells were injected into the tail vein of 8 Gy irradiated recipient BALB/c mice. The splenocytes were harvested for LAK preparation 4 wk after the transplant.

Lung clearance assay

Lung clearance assay was performed as described (7). In brief, target cells were precultured with 2.5 µg/ml 5-fluoro-2’-deoxyuridine (Sigma-Aldrich, St. Louis, MO) and radiolabeled with ¹²⁵I-labeled 5-ido-2’-deoxyuridine (Amersham Pharmacia Biotech, Piscataway, NJ). Mice were injected i.v. with 1 × 10⁶ target cells. Mice were killed 5 h later, and the lungs were counted with a gamma counter. The percentage of the residual radioactivity was calculated as being equal to (residual radioactivity in the lungs/total injected radioactivity) × 100. Data are presented as mean ± SDs from five mice in each group.

Results

Cloning of Hm1 genes

Inasmuch as inhibitory receptors of the Ly-49 family recognize MHC class I ligands and other studies suggested that Ly-49D may recognize a murine MHC class I molecule (31), the Ly-49D ligand on CHO cells may also be an MHC class I molecule. We therefore cloned five Chinese hamster MHC class I cDNAs from CHO cells by the RACE method and named Hm1-C1-C5 (Fig. 1A, GenBank accession nos. AY064386–AY064390), reflecting Hm1 as the official designation for the hamster MHC locus. We have indicated our cDNAs as Chinese hamster origin by the “C,” and each different cDNA was numbered. Hm1-C1, C2, C4, and C5 are similar to each other and have a high degree of homology to classical mouse MHC class I genes (Fig. 1B). Hm1-C1 appears to be an alternatively spliced form of Hm1-C5, because it is identical except for the deletion of 48 bp in its cytoplasmic region. Hm1-C3 is less related to the other four molecules, but is homologous to the nonclassical mouse MHC class I molecule, Qa-1 (data not shown).

![FIGURE 1](http://www.jimmunol.org/)

Chinese hamster MHC class I (Hm1) molecules. A, Predicted amino acid sequences of Hm1 molecules compared with H-2Dd. Dashes indicate the identity with Hm1-C1, whereas asterisks indicate gaps. B, Homology of nucleotide and amino acid sequences of Hm1 molecules with each other and H-2Dd.
BaF/3 transfectants of these Chinese hamster class I molecules were produced with a bicistronic vector, allowing their expression to be monitored by expression of hCD4 from the second cistron (data not shown). To confirm the ability of these molecules to be expressed on the cell surface, fusion constructs of these class I molecules with red fluorescence protein were generated and transfected to BaF/3 cells. The expression of these molecules was confirmed by fluorescence microscopy (data not shown). However, the transfectedants were not stable enough for the use in the assay apparently, because red fluorescence protein was toxic to cells. The mAb B22/249 (anti-H-2D^d), known to bind to CHO cells (32), cross-reacts with Hm1-C4 and binds slightly to Hm1-C2, but not to the other Hm1 molecules on Hm1 transfectants (data not shown). Although BaF/3 cells were derived from BALB/c bone marrow cells (33), they express very low levels of MHC class I molecules on the cell surface (data not shown), and BaF/3 cells expressing high levels of transfected H-2D^d were not reactive with B22/249, but with anti-H-2D^d mAb 3458S (data not shown). Finally, mAb B22/249 primarily recognizes Hm1-C4 on CHO cells, as demonstrated by the expression cloning of Hm1-C4 using mAb B22/249 and magnetic beads for expression screening of retroviral constructs of a cDNA library made from CHO cells (data not shown). Thus, the Chinese hamster MHC class I cDNAs were stably expressed in BaF/3 cells.

**Specificity of Ly-49D for Hm1-C4**

To determine whether Hm1 molecules could be recognized by Ly-49D, we used a heterologous indicator cell system using an expression construct for a chimeric molecule consisting of the extracellular domain of Ly-49D with the Ly-49A transmembrane domain and cytoplasmic tail of CD3ζ. The resulting DAZ construct was stably expressed in BWZ.36 cells, containing a NF-AT repeat construct driving the expression of lacZ. Immobilized anti-Ly-49D mAb, but not other mAbs, stimulates DAZ cells to produce β-galactosidase, which can be detected by conversion of the colorimetric substrate CPRG (data not shown), indicating that the DAZ cell line is responsive to cross-linking of Ly-49D. Upon exposure of DAZ cells to the BaF/3 cells expressing the different Hm1 cDNAs, only the Hm1-C4 transfectant induced lacZ activity, suggesting that it was recognized by Ly-49D (Fig. 2A). Whereas

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**FIGURE 2.** Recognition of Hm1-C4 by Ly-49D in DAZ assay. **A**, DAZ (filled columns) or DA indicator cells (open columns) were incubated with untransfected Ba/F3 (−), various MHC class I Ba/F3 transfectants (C1 through C5), H-2D^d, Ba/F3 transfected with empty vector pI4 (−I4), CHO cells, or in the absence of targets (Target(−)), as indicated. After 16-h incubation, lacz activity was assessed with CPRG, as described in Materials and Methods. **B**, To block the recognition of Hm1-C4 transfectant or CHO cell by Ly-49D in the DAZ assay, mAb 4E4 (anti-Ly-49D, hatched columns) or isotype control (PK136, anti-NK1.1, gray columns) was added to the cell culture at the final concentration of 50 μg/ml. Control experiments without mAb were shown in A. **C**, DAZ (filled columns) or DA (open columns) cells were incubated with Ba/F3 double transfectants of various MHC class I cDNAs and piG empty control vector. **D**, DAZ (filled columns) or DA (open columns) cells were incubated with Ba/F3 double transfectants of various MHC class I cDNAs and CHOβ2m. **E**, Assessment of Ly-49D specificity against Hm1-C4 + CHOβ2m double transfectant, performed as in B. All CPRG assays were done in duplicate well, and the mean of the absorption was shown. The results are representative of three independent experiments.
lacZ activity induced by Hm1-C4 was somewhat lower than that stimulated by CHO cells, the H-2D\(^d\) transfectant did not induce any lacZ activity and no lacZ activity was induced by any other transfectant. Although the addition of mAb 4E4 (anti-Ly-49D) alone can induce weak lacZ activity in DAZ cells, the H-2D\(^d\) transfectant did not induce any lacZ activity and no lacZ activity was induced by any other transfectant. Although the addition of mAb 4E4 (anti-Ly-49D) alone can induce weak lacZ activity in DAZ cells, the lacZ activity induced by the Hm1-C4 transfectant was inhibited by mAb 4E4 (Fig. 2B). By contrast, F(ab')\(_2\) of B22/249 did not block CHO cell killing by B6 LAK cells (data not shown). Comparable results were obtained with Hm1-C4 and H-2D\(^d\) transfectants of another target cell (C1498) (data not shown). Finally, the BWZ.36 transfectants expressing the DA construct (DAZ without CD3\(^\gamma\)H9256) were not stimulated by Hm1-C4. Therefore, the DAZ assay indicated that Hm1-C4 is a potential ligand of Ly-49D.

It is possible that the other Chinese hamster MHC class I molecules could be recognized by Ly-49D, if they were expressed with Chinese hamster H9252\(^m\), as it was recently demonstrated that Ly-49A recognized H-2D\(^d\) + murine \(\beta_m\), but not H-2D\(^d\) + human \(\beta_m\) (34, 35). To investigate this possibility, double BaF/3 transfectants of Hm1 genes and CHO/H9252\(^m\) were generated by transfection of the various Hm1 cDNAs with pI4 vector and CHO/H9252\(^m\) with pIG vector, respectively, and analyzed in the DAZ assay (Fig. 2, C and D). Of the Hm1 + CHO\(\beta_m\) double transfectants, lacZ activity was induced only by the Hm1-C4 cell line. This activity was higher than that induced by the Hm1-C4 + pIG double transfectant, was similar to the level induced by CHO cells, and was inhibited by mAb 4E4 (Fig. 2E). These data indicate that none of the other Hm1 molecules are ligands for Ly-49D and that the complex of Hm1-C4 and CHO\(\beta_m\) appears to be recognized by Ly-49D to a greater extent than Hm1-C4 and murine \(\beta_m\) (see below).

**Recognition of Hm1-C4 in cytotoxicity assays**

We next investigated whether ligand specificity in the functional DAZ assay correlated with Ly-49D-mediated cytotoxicity by primary NK cells. For these studies, we exploited our previous findings that Ly-49D\(^+\) B6 LAK cells killed CHO cells (Fig. 3A), whereas Ly-49D\(^-\) B6 LAK cells did not (Fig. 3B). As predicted from the DAZ assay, Ly-49D\(^+\) B6 LAK cells killed Hm1-C4 transfectants, but not Ba/F3 (Fig. 3C). They were more efficient in killing of the Hm1-C4 + CHO\(\beta_m\) double transfectant (Fig. 3F).

![FIGURE 3. Ligand recognition in cytotoxicity assays. A, Cytotoxicity against CHO (■) and untransfected BaF/3 cells (□) by B6 Ly-49D\(^+\) LAK cells. Vertical bars show the SD in triplicate wells; SD bars smaller than the height of the symbols are not shown. B, Cytotoxicity against CHO cell (■) and BaF/3 cell (□) by B6 Ly-49D\(^-\) LAK cells. C, Cytotoxicity by B6 Ly-49D\(^+\) LAK cells against BaF/3 cells transfected with Hm1-C1 (□), C2 (○), C3 (○), C4 (▲), C5 (▲), H-2D\(^d\) (●), or p4 empty control vector (●). D, Cytotoxicity by B6 Ly-49D\(^-\) LAK cells against the same transfectants in C. E, To block Ly-49D-mediated recognition of the Hm1-C4 transfectant (square) or CHO cell (circle) by Ly-49D\(^+\) LAK cells, mAb 4E4 (anti-Ly-49D, open symbols) or isotype control (mAb PK136, anti-NK1.1, filled symbols) was added to the cell culture at the final concentration of 50 \(\mu\)g/ml. F, B6 Ly-49D\(^+\) LAK cell cytotoxicity against BaF/3 double transfectants expressing Hm1-C4 and CHO\(\beta_m\) (▲), Hml-C4 and pIG (●), p4 and CHO\(\beta_m\) (○), or p4 and pIG empty control vector (□). G, B6 Ly-49D\(^+\) LAK cell cytotoxicity against the same double transfectants in F. H, mAb 4E4 (open symbols) and isotype control mAb PK136 (filled symbols) blocking experiment, as in E, with B6 Ly-49D\(^+\) LAK cells against BaF/3 cells transfected with Hml-C4 and pIG (square) and Hml-C4 and CHO\(\beta_m\) (circle or triangle). All results in this figure are representative of three independent experiments.](http://www.jimmunol.org/Downloaded-from/article-pdf/168/1/130/1866806/1866806.pdf)
However, none of the other Hm1 transfectants were killed. Interestingly, the H-2D\(^d\) transfectants also were not killed. Killing of the Hm1-C4 and the Hm1-C4 + CHOβ\(_m\) double transfectants was due to recognition by Ly-49D because Ly-49D\(^-\) B6 LAK cells did not kill any Hm1 transfectant (Fig. 3, D and G). Furthermore, the killing of CHO cells, Hm1-C4 transfectant, and Hm1-C4 + CHOβ\(_m\) double transfectants was blocked by mAb 4E4 (Fig. 3, E and H). Therefore, we conclude that the NK cell killing assays recapitulate Ly-49D recognition of Hm1-C4, as indicated by the DAZ assay.

**Species influence of β\(_m\) on Ly-49D recognition**

Having noticed that the recognition of Hm1-C4 may be influenced by CHOβ\(_m\), we further analyzed the effects of β\(_m\) from other species on recognition by Ly-49D. To analyze this issue more carefully, we used the human Daudi target cell, which is deficient in β\(_m\) expression, and double transfectants were produced expressing Hm1-C4 with β\(_m\) from murine a, b, or c alleles, and human, rat, or Chinese hamster. Comparable transfection of the β\(_m\) cDNAs was monitored by the expression of GFP from the second cistron and detectable HLA class I molecules on the cell surface of Daudi cells, as assessed with mAb W6/32 (data not shown). Comparable transfection of the Hm1-C4 cDNA on Daudi double transfectants was monitored by B22/249 mAb (data not shown). Double transfectants of Hm1-C4 with rat or CHOβ\(_m\) induced more lacZ activity in DAZ cells than the double transfectants of Hm1-C4 with human or any of the mouse β\(_m\) alleles (Fig. 4B). Control transfectants with β\(_m\) alone did not stimulate DAZ

**FIGURE 4.** Addition of rat β\(_m\) or CHOβ\(_m\) enhances Ly-49D recognition of Hm1-C4. A, DAZ (filled columns) or DA indicator cells (open columns) were incubated with untransfected Daudi ((—)), various β\(_m\) cDNA transfectants (mouse β\(_m\) a, b, or c alleles (mβ\(_ma\), mβ\(_mb\), or mβ\(_mc\), respectively), human (hβ\(_m\)), rat (Rβ\(_m\)), Chinese hamster (CHOβ\(_m\)), empty vector pG (—JG), CHO cells, or in the absence of targets (Target(—)), as indicated. B, As in A, except that Daudi double transfectants with Hm1-C4 were used. C, To block the recognition of Daudi double transfectants or CHO cells by Ly-49D in the DAZ assay, mAb 4E4 (anti-Ly-49D, hatched columns) or isotype control (PK136, anti-NK1.1, gray columns) was added to the cell culture at the final concentration of 50 µg/ml. Control experiments without mAb were shown in A and B. D, Ly-49D\(^+\) B6 LAK cell cytotoxicity against CHO cell (■) and Daudi cell (□). E, Ly-49D\(^+\) B6 cytotoxicity against CHO cell (■) and Daudi cell (□). F, Ly-49D\(^+\) B6 LAK cell cytotoxicity against Daudi cells transfected with mβ\(_ma\) (□), mβ\(_mb\) (○), mβ\(_mc\) (△), hβ\(_m\) (■), Rβ\(_m\) (△), CHOβ\(_m\) (○), and pG empty control vector (○). G, Ly-49D\(^+\) B6 LAK cell cytotoxicity against the same transfectants as in F. H, Ly-49D\(^+\) B6 LAK cell cytotoxicity against Daudi double transfectants of Hm1-C4 with mβ\(_ma\) (□), mβ\(_mb\) (○), mβ\(_mc\) (△), hβ\(_m\) (■), Rβ\(_m\) (△), or CHOβ\(_m\) (○). I, Ly-49D\(^+\) B6 LAK cell cytotoxicity against the same double transfectants in H. All results in this figure are representative of three independent experiments.
cells, indicating lack of Ly-49D recognition of HLA class I molecules associated with any of the various β₂m (Fig. 4A). That the induced lacZ activity was specifically due to Ly-49D recognition was supported by the absence of lacZ activity in DA cells exposed to any transfectants (Fig. 4, A and B). In addition, the lacZ activity induced by double transfectants of Hm1-C4 and rat or CHOβ₂m was inhibited by mAb 4E4 (Fig. 4C). Because mAb 4E4 alone induces some weak lacZ activity in DAZ cells, presumably due to cross-linking by the soluble anti-Ly-49D preparation, there was no blocking observed of the slight lacZ activity induced by Hm1-C4 and human or mouse β₂m (Fig. 4C).

NK cell killing assays of these transfectants confirmed these results. Ly-49D⁺ B6 LAK cells killed Hm1-C4 + human, rat, or CHOβ₂m double transfectants to a greater extent than they killed Hm1-C4 + murine β₂m double transfectant (Fig. 4H). Ly-49D⁺ and Ly-49D⁻ B6 LAK cells did not kill Daudi cells (Fig. 4, D and E) or transfectants with various β₂m constructs alone (Fig. 4, F and G), and the Ly-49D⁻ B6 LAK cells did not kill any transfectants (Fig. 4I). These data indicate that Ly-49D recognizes the dimer of Hm1-C4 with rat or CHOβ₂m better than Hm1-C4 with murine β₂m.

**Specificity of Ly-49D-transduced BALB/c LAK cells**

To provide direct demonstration of the specificity of Ly-49D for Hm1-C4 in killing assays, we used a system in which the Ly-49D receptor derived from B6 was retrovirally transduced into bone marrow stem cells from BALB/c, which do not express Ly-49D. The transduced bone marrow stem cells were adoptively transferred to irradiated BALB/c hosts, and Ly-49D⁺ NK cells were then isolated for killing experiments. The expression levels of Ly-49D or DA molecules were similar between Ly-49D⁺ and DA⁺ sorted LAK cells from BALB/c mice (Fig. 5A). The Ly-49D⁺

![FIGURE 5](https://example.com/figure5.png)

**FIGURE 5.** Gene transfer of Ly-49D confers Hm1-C4 specificity. A, Sorted LAK cells from BALB/c mice were stained with anti-Ly-49D mAb and analyzed by flow cytometry. Ly-49D⁺ or DA⁺ BALB/c LAK cells were produced by isolation of Ly-49D⁺ or DA⁺ cells from spleens of animals reconstituted by transduced stem cells. Retroviral gene transduction of Ly-49D or DA construct was performed on bone marrow stem cells, which were then transplanted into syngeneic hosts. B, Cytotoxicity of Ly-49D⁺ BALB/c LAK cells was measured against CHO cell ( ), untransfected Ba/F/3 cell ( ), or Ba/F/3 transfected with Hm1-C4 ( ). C, Cytotoxicity against the same targets as in B by Ly-49D⁺ BALB/c LAK cells from the same mice used in B for generation of Ly-49D⁺ LAK cells. D, To block Ly-49D-mediated recognition of the Hm1-C4 transfectant (square) or CHO cell (circle) by Ly-49D⁺ BALB/c LAK cells, mAb 4E4 (anti-Ly-49D, open symbols) or isotype control (mAb PK136, anti-NK1.1, filled symbols) was added to the cell culture at the final concentration of 50 μg/ml. E, Cytotoxicity against the same targets as in B by DA⁺ BALB/c LAK cells. F, Cytotoxicity against the same targets as in B by DA⁺ BALB/c LAK cells from the same mice used in E. G, As in D, except BALB/c DA⁺ LAK cells were used. The results are representative of three independent experiments.
BALB/c LAK cells killed CHO cells and the Hm1-C4 transfectants, but not BaF/3 cells (Fig. 5B). This killing was completely blocked by mAb 4E4 (Fig. 5D). By contrast, Ly-49D\(^{-}\) BALB/c LAK cells did not kill the targets (Fig. 5C). As a control, BALB/c LAK cells were also prepared expressing the DA construct. Surprisingly, these NK cells also killed CHO cells (Fig. 5), but not ELAK cells were also prepared expressing the DA construct. LAK cells did not kill the targets (Fig. 5C). As a control, BALB/c LAK cells did not kill targets (Fig. 5F). These data suggest that the activating signal mediated by DAP12/KARAP may not be solely responsible for the CHO cell killing and that specific binding mediated by the DA molecule may also contribute. Nevertheless, these gene transfer data indicate that Ly-49D directly recognizes Hm1-C4.

Absence of tolerized Ly-49D function in NK cells from \(\beta_m^{-/}\) mice

Because \(\beta_m^{-}\)-deficient NK cells have a general tolerance for autologous and allogeneic cells, it was analyzed whether the function of Ly-49D is also inactive in \(\beta_m^{-/}\) mouse NK cells. To analyze fresh NK cell functions, RMA transfectants were used, because BaF/3 cells were killed by fresh splenocytes (data not shown). Ly-49D was equally expressed on B6 and \(\beta_m^{-/}\) spleen NK cells, as previously reported (36, 37) (Fig. 6A). Surprisingly, fresh NK cells from \(\beta_m^{-/}\) mice specifically killed RMA transfectants with Hm1-C4; albeit this cytotoxicity was somewhat lower than that mediated by B6 NK cells (Fig. 6, B and C), suggesting that Ly-49D function is not tolerized in \(\beta_m^{-/}\) NK cells.

FIGURE 6. In vitro and in vivo analysis demonstrating untolerized Ly-49D function in NK cells from \(\beta_m^{-/}\) mice. A, Splenocytes from B6 and \(\beta_m^{-/}\) mice were stained with anti-Ly-49D, anti-NK1.1, and anti-CD3 and analyzed by flow cytometry. Histograms for Ly-49D expression on cells gated on NK1.1\(^{-}\)CD3\(^{+}\) are shown. B and C, Cytotoxicity against the RMA mock transfectants or RMA transfectants with Hm1-C4 by freshly isolated NK cells from B6 (□) and \(\beta_m^{-/}\) (○) mice. D, To block the lysis of RMA transfectants with Hm1-C4 by freshly isolated NK cells from B6 (□) or \(\beta_m^{-/}\) (○) mice, mAb 4E4 was added to the cell culture at the final concentration of 50 \(\mu\)g/ml. As an isotype control, PK136 was used (■, for freshly isolated NK cells from B6 mice; ●, for freshly isolated NK cells from \(\beta_m^{-/}\) mice). E, Sorted LAK cells from B6 and \(\beta_m^{-/}\) mice were stained with anti-Ly-49D mAb and analyzed by flow cytometry. F and G, Cytotoxicity against the BaF/3 or Hm1-C4 transfectants in BaF/3 cell by Ly-49D\(^{-}\) B6 (□), Ly-49D\(^{-}\) \(\beta_m^{-/}\) (○) and Ly-49D\(^{-}\) \(\beta_m^{-/}\) (●) LAK cells. H, To block the lysis of Hm1-C4 transfectants by Ly-49D\(^{-}\) B6 (□) or Ly-49D\(^{-}\) \(\beta_m^{-/}\) (●) LAK cells, mAb 4E4 was added to the cell culture at the final concentration of 50 \(\mu\)g/ml. As an isotype control, PK136 was used (■, for Ly-49D\(^{-}\) B6; ●, for Ly-49D\(^{-}\) \(\beta_m^{-/}\) LAK cells). I and J, Cytotoxicity against the RMA mock transfectants or RMA transfectants with Hm1-C4 by Ly-49D\(^{-}\) B6 (□), Ly-49D\(^{-}\) \(\beta_m^{-/}\) (○), and Ly-49D\(^{-}\) \(\beta_m^{-/}\) (●) LAK cells. K, To block the lysis of RMA transfectants with Hm1-C4 by Ly-49D\(^{-}\) B6 (□) or Ly-49D\(^{-}\) \(\beta_m^{-/}\) (●) LAK cells, mAb 4E4 was added to the cell culture at the final concentration of 50 \(\mu\)g/ml. As an isotype control, PK136 was used (■, for Ly-49D\(^{-}\) B6; ●, for Ly-49D\(^{-}\) \(\beta_m^{-/}\) LAK cells). L, Lung clearance of \(^{125}\)I-labeled 5-iodo-2'-deoxyuridine-labeled RMA mock transfectants or RMA transfectants with Hm1-C4 was assessed in B6 and \(\beta_m^{-/}\) mice, untreated or treated with 200 \(\mu\)g anti-NK1.1 mAb i.v. 3 days before the assay, as indicated. Each bar represents the mean percent retention of lungs of five mice in each group. The percent retention between mAb-untreated B6 and \(\beta_m^{-/}\) mice injected with RMA transfectants with Hm1-C4 was statistically analyzed by unpaired t test, and the \(p\) value is shown.
The in vitro culture in high doses of IL-2 can abrogate the tolerance of \( \beta_m^{+/+} \) NK cells for MHC class I-deficient cells (13). The expression levels of Ly-49D were similar between Ly-49D-sorted LAK cells from B6 and \( \beta_m^{+/+} \) mice (Fig. 6E). Although Ly-49D-positive \( \beta_m^{+/-} \) LAK cells killed BaF/3 transfectants with Hm1-C4, but not BaF3 cells, the cytotoxicity was slightly lower than that mediated by Ly-49D-positive B6 LAK cells (Fig. 6, F and G). This killing of Hm1-C4 transfectants was specific because Ly-49D-negative B6 and \( \beta_m^{+/-} \) LAK cells did not kill either the transfectants or BaF3 cells (Fig. 6, F and G), and the killing was blocked by mAb 4E4 (Fig. 6H). Similarly, Ly-49D-positive \( \beta_m^{+/-} \) LAK cells also specifically killed RMA transfectants with Hm1-C4, but to a lesser degree than Ly-49D-positive B6 LAK cells (Fig. 6J). Therefore, Ly-49D function is still active in \( \beta_m^{+/-} \) LAK cells, but the lower specific killing activity of \( \beta_m^{+/-} \) NK cells using Ly-49D-Hm1-C4 interaction is not completely normalized after in vitro culture, in the absence of host environmental milieu.

In lung clearance assays of NK cell function in vivo, \( \beta_m^{+/+} \) mice were able to reject i.v. inoculated RMA cells transfected with Hm1-C4, but they retained significantly higher levels of radioactivity than B6 mice (Fig. 6L, \( p = 0.005 \)). The capacity to reject RMA transfected with Hm1-C4 is specific because \( \beta_m^{+/-} \) and B6 mice did not eliminate inoculated RMA cells. Treatment of either \( \beta_m^{+/-} \) or B6 mice with anti-NK1.1 mAb 3 days before the assay resulted in an increased retention of RMA transfected with Hm1-C4, to the level of parental RMA, consistent with a role of NK cells in specific tumor clearance from the lung. These data demonstrate that the in vivo function of Ly-49D is also active, but altered in \( \beta_m^{+/-} \) mice.

**Discussion**

We have demonstrated that Ly-49D recognizes Hm1-C4, a classical MHC class I molecule of Chinese hamster. The recognition of Hm1-C4 was enhanced when it was coexpressed with CHO\( \beta_m \). We showed this receptor-ligand specificity using four different effector cells (DAZ cells, Ly-49D+ B6 LAK cells, Ly-49D-transduced BALB/c LAK cells, and fresh NK cells) and four separate targets (BaF3, C1498, Daudi, and RMA cells) to avoid potential artifacts. Although we were unable to demonstrate Ly-49D specificity for H-2D\(^d\), as previously reported (31), these studies with a xenogeneic MHC class I ligand strongly suggest that Ly-49D recognizes a \( \beta_m \)-dependent epitope on an MHC class I molecule.

With respect to ligand specificity of Ly-49D, it is well known that Ly-49-inhibitory NK receptors recognize classical MHC class I molecules. Similar to Ly-49A recognition of H-2D\(^d\) with mouse \( \beta_m \), but not with human \( \beta_m \) (34, 35), Ly-49D also recognizes the dimer of Hm1-C4 and CHO\( \beta_m \). However, Ly-49D preferentially recognizes a xenogeneic MHC class I molecule in the context of xenogeneic \( \beta_m \), either hamster or rat, which displays only 80% amino acid homology. Although Ly-49D can recognize Hm1-C4 with murine \( \beta_m \), we could not find any difference in the recognition of Hm1-C4 with different mouse \( \beta_m \) alleles having only one amino acid difference between alleles. Double transfectants of human \( \beta_m \) and Hm1-C4 in Daudi cells were not recognized well by DAZ cell, but were killed well by Ly-49D+ B6 LAK cells (Fig. 4, B and H). This discrepancy suggests the existence of the other NK receptors on B6 LAK cells, which may recognize Hm1-C4 and human \( \beta_m \). Although these data could be interpreted to suggest that mouse or human \( \beta_m \) do not allow appropriate folding of Hm1-C4 H chain, we prefer the explanation that Ly-49D recognizes both Hm1-C4 and \( \beta_m \), analogous to Ly-49A recognition of both H-2D\(^d\) and \( \beta_m \), even though Ly-49D is thought to recognize the \( \alpha_1 \) and \( \alpha_2 \) domains of H-2D\(^d\) (38). Ly-49D may bind a dimer of Hm1-C4 and hamster or rat \( \beta_m \) with higher affinity than Hm1-C4 and mouse \( \beta_m \), consistent with structural analysis of Ly-49A complexed with its MHC class I ligand.

Interestingly, gene-transferred BALB/c LAK cells with the DA construct could kill CHO cells, but not Hm1-C4 transfected, and the killing was blocked by anti-Ly-49D mAb. This was a surprising result because DA molecules should not be able to interact with the DAP12/KARAP signaling molecule and transduce activating signals. Yet, our previous work demonstrated that the Ly-49D receptor accounted for the vast majority of the killing activity against a CHO cell line, Pro 5 (7). These data suggest that binding of Ly-49D and its ligand may facilitate the interactions of other activating receptors and ligands with lower affinities (39). However, it is also possible that the DA construct can form heterodimers with other activating Ly-49 receptors in LAK cells or that DA constructs can associate with some unknown immunoreceptor tyrosine-based activation motif-bearing adaptor molecules capable of transducing activation signals in BALB/c LAK cells.

DAP12+-NK cells kill CHO cells (40), but the killing is not blocked by mAb 4E4 (41), and the DAP12+-mice were on a mixed genetic background that may result in epigenetic effects or there may be different Ly-49 molecules involved. Indeed, the killing of another CHO cell line, CHO-K1, was not completely blocked by anti-Ly-49D mAb (data not shown). Taken together, there may be other ligands for Ly-49D in CHO cells; the current findings indicate that CHO cell killing by B6 LAK cells is mediated by a specific Ly-49D-Hm1-C4 interaction that leads to direct signaling; and specific binding may also contribute to killing independent of direct signaling.

It was reported that CHO cells were recognized by the Ly-49A or G inhibitory receptors as well as Ly-49D (42). To evaluate these possibilities, we made transfectants with an AAZ construct (Ly-49A extracellular and transmembrane domains and CD3\(^\gamma\) cytoplasmic domain chimeric construct) or a GAZ construct (Ly-49G extracellular, Ly-49A transmembrane, and CD3\(^\gamma\)cytoplasmic domain chimeric construct) in BWZ.36 cell. The AAZ cells autoreacted with each other (data not shown), suggesting that the AAZ cell recognizes H-2D\(^b\) on BWZ.36 cell, consistent with known specificity of Ly-49A for H-2D\(^b\). Evaluation of CHO reactivity by Ly-49A, therefore, could not be done by the system reported in this study. In contrast, the GAZ cell did not react to CHO cells (data not shown). Therefore, it remains controversial whether Ly-49G recognizes CHO cells, but it is unlikely to influence the other results reported in this study.

A number of functional studies suggest that Ly-49D recognizes the mouse MHC class I molecule, H-2D\(^d\). In allogeneic bone marrow transplantation assays, the Bennett group determined that rejection of transferred bone marrow from H-2D\(^d\) mice was abrogated by administration of mAb 12A8, specific for Ly-49A and Ly-49D (43). In addition, using mAb 4E5 (monospecific for Ly-49D)-sorted NK cells in killing assays against Con A blasts from different mouse strains, George et al. (44, 45) demonstrated that sorted Ly-49D+ G2- NK cells from B6 mice could kill Con A blasts from the H-2D\(^b\) or B10.BR (H-2\(^b\)) strain. Mason et al. (46) showed that the stimulation of transfected H-2D\(^b\) expressed on rat YB2/0 target cell led to DAP12/KARAP phosphorylation and production of IFN-\( \gamma \) in sorted Ly-49D+ A2- G2- NK cells from B6 mice. Nakamura et al. (32) transfected the rat NK cell tumor line, RNK16, with the Ly-49D cDNA and obtained stable transfectants (RNK.mly-49D) that gained the capacity to kill CHO cell (32). RNK.mLy-49D was used to demonstrate that Ly-49D recognizes transfected H-2D\(^b\) expressed on YB2/0, rat cell line (31). Furthermore, RNK.mLy-49D gained the capacity to kill Con A blasts from B10.D2 (H-2\(^d\)) and BALB/c, but...
not B10.BR (H-2\(^k\)), B6 (H-2\( ^b\)), or BALB.B (H-2\( ^b\)). Thus, in functional assays, Ly-49D appears to recognize H-2\( D\), but not H-2\( ^a\) or H-2\( ^b\)-encoded molecules.

In contrast, the specificity of Ly-49D for H-2\( ^d\) was not confirmed by our functional studies presented in this study, even though H-2\( ^d\) was coexpressed with rat \(\beta_2\)m (data not shown), as it was expressed on rat cell lines in Nakamura’s experiments. In addition, the Ly-49D+ BALB/c LAK cells did not kill H-2\( ^d\) transfectants in gene transfer experiments (data not shown). Hm1-C4 is not any more or less homologous to H-2\( ^d\) than the other nonreactive Hm1 molecules. In addition, H-2\( ^d\) tetramers have previously been shown not to bind Ly-49D transfectants (29, 47). H-2\( ^d\) was transfected in TAP1−/− fibroblast MEFIGAP1-27 (gift from C. Harding, Case Western Reserve University, Cleveland, OH) (48). The susceptibility of the transfectant to Ly-49A+ D− or Ly-49A+ D−-sorted B6 LAK cells was analyzed in the presence of H-2\( ^d\)-specific peptide to express H-2\( ^d\) on the cell surface. The transfected was not killed by Ly-49A+ D− LAK cells in the presence of H-2\( ^d\)-specific peptide, but was killed in the absence of the peptide, indicating that Ly-49A recognizes H-2\( ^d\) and inhibits the killing (data not shown). However, the transfectant was equally killed by Ly-49A− D− LAK cells in the presence or absence of H-2\( ^d\)-specific peptide (data not shown).

All of these data suggest that H-2\( ^d\) is not recognized by Ly-49D.

Perhaps these discrepancies may be explained by several possibilities. First, anti-Ly-49D mAbs are used to sort NK cells for in vitro study, or depletion of an NK cell subpopulation, and the expression pattern of Ly-49D may overlap with unknown NK receptors with related specificities. Although gene transfer experiments may avoid this potential problem, the use of a rodent NK cell line may permit expression of other NK cell receptors. For example, Ly-49A can be expressed on interspecies hybridoma cells between Ly-49A-negative mouse and rat cells (49). In addition, transfection of Ly-49P and W into RNK16 resulted in gaining the capacity to kill the transfectant of H-2\( ^D\) in YB2/0 cell (50, 51).

These findings suggest the possibility that the transfection of Ly-49D, P, or W in the rat cell line RNK16 caused the coexpression of other rat NK receptors recognizing H-2\( ^D\). Furthermore, the RNK16.mLy-49D transfectant could not kill H-2\( ^D\)-transfected C1498 or RMA targets and killed only the H-2\( ^D\)-transfected in YB2/0 (52). To avoid such difficulties, the receptor-ligand interaction in our system was shown using four effector cells and four target cells. Finally, a potential explanation unifying all of these data is the possibility that Ly-49D recognition of H-2\( ^D\) is dependent on the rat cell-derived peptide bound to H-2\( ^D\), a possibility that is inconsistent with peptide-independent recognition of H-2\( ^D\) by the Ly-49A inhibitory receptor. Thus, it is still controversial that Ly-49D recognizes H-2\( ^D\).

Nevertheless, the capacity to detect functional recognition of a specific ligand for an NK cell activation receptor also provided a means to evaluate NK cell activities that heretofore has been less than complete. In particular, we explored the activity of this activation receptor in the context of \(\beta_2\)m−/− mice that display NK cell tolerance, which heretofore has not been evaluated with respect to potential dysfunction or the lower expression levels of activation receptors. In the current study, we determined that NK cells from \(\beta_2\)m−/− mice kill Hm1-C4 transfectants in cytotoxicity assays in vitro and in vivo, indicating that the function of Ly-49D on \(\beta_2\)m−/− NK cells for ligand recognition and activation is still active.

On closer inspection, however, the killing by \(\beta_2\)m−/− NK cells was reproducibly somewhat lower than that mediated by wild-type NK cells, and this may be important to consider in more generalized tolerance of \(\beta_2\)m−/− NK cells. The alteration persisted after in vitro culture with IL-2, indicating that the function of Ly-49D on \(\beta_2\)m−/− NK cells appears not to be normalized by in vitro culture. Consistent with previous reports, we showed that the Ly-49D expression levels and percentages of Ly-49D− NK cells are unaltered in \(\beta_2\)m−/− mice (36, 37). Because the expression pattern of Ly-49D cannot explain the slight impairment function of Ly-49D in NK cells from \(\beta_2\)m−/− mice, our data suggest that the threshold for killing mediated by activating NK receptors may be set somewhat higher in NK cells from \(\beta_2\)m−/− mice as opposed to wild-type B6 mice.

Even though the effects on \(\beta_2\)m−/− NK cells are small, it is important to recognize that NK cells may use several different activation receptors simultaneously to kill MHC class I-deficient cells because individual NK cell does express multiple activation receptors. If several are used for killing of MHC class I-deficient cells, then killing will be due to the sum of the activities of the involved receptors. Therefore, if the activity of several of these activation receptors is somewhat impaired, perhaps some more than others, due to the “threshold” phenomenon, then the combined effect of impaired signals from activating receptors might explain the general tolerance of \(\beta_2\)m−/− NK cells.

This threshold effect could be due to several potential mechanisms. Perhaps there is a failure of tolerant NK cells to express costimulatory receptors. It is also possible that the expressed Ly-49D on NK cells from \(\beta_2\)m−/− mice is functionally impaired for conformational reasons. This could be akin to T cell anergy by antagonist peptides, or absence of costimulatory signals for T cell activation. Also, it remains possible that there is a compensatory up-regulation in the function of the inhibitory receptors in the \(\beta_2\)m−/− host environment that results in a nonspecific inhibitory influence. Thus, the mechanisms to induce the tolerance of NK cells in \(\beta_2\)m−/− mice remain somewhat ambiguous, but, based on our studies of Ly-49D, there appear not to be global defects on activation receptor expression or profound effects on activation receptor function.

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