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Mouse CD94 Participates in Qa-1-Mediated Self Recognition by NK Cells and Delivers Inhibitory Signals Independent of Ly-49

Noriko Toyama-Sorimachi,2,a** Yuriko Taguchi,† Hideo Yagita,‡ Fujiko Kitamura,† Akemi Kawasaki,‡ Shigeo Koyasu,§ and Hajime Karasuyama*†

Inhibitory receptors expressed on NK cells recognize MHC class I molecules and transduce negative signals to prevent the lysis of healthy autologous cells. The lectin-like CD94/NKG2 heterodimer has been studied extensively as a human inhibitory receptor. In contrast, in mice, another lectin-like receptor, Ly-49, was the only known inhibitory receptor until the recent discovery of CD94/NKG2 homologues in mice. Here we describe the expression and function of mouse CD94 analyzed by a newly established mAb. CD94 was detected on essentially all NK and NK T cells as well as small fractions of T cells in all mouse strains tested. Two distinct populations were identified among NK and NK T cells, CD94bright and CD94dull cells, independent of Ly-49 expression. The anti-CD94 mAb completely abrogated the inhibition of target killing mediated by NK recognition of Qa-1/Qdm peptide on target cells. Importantly, CD94bright but not CD94dull cells were found to be functional in the Qa-1/Qdm-mediated inhibition. In the presence of the mAb, activated NK cells showed substantial cytotoxicity against autologous target cells as well as enhanced cytotoxicity against allogeneic and “missing self” target cells. These results suggest that mouse CD94 participates in the protection of self cells from NK cytotoxicity through the Qa-1 recognition, independent of inhibitory receptors for classical MHC class I such as Ly-49. The Journal of Immunology, 2001, 166: 3771–3779.

Natural killer cells play important roles in a first line of defense against viral infections and tumor development where they exert cytotoxicity and cytokine production (1, 2). It has been an intriguing question how NK cells discriminate self from nonself, because they do not express Ag-specific receptors such as TCRs or B cell receptors on their surface (3). Although the mechanism of target recognition by NK cells is not fully understood, significant advances have been made in the recent decade. The first clue came from the observations that there was an inverse correlation between the susceptibility of target cells to NK cell-mediated lysis and the expression of MHC class I on their surface (4–6). This led to the hypothesis that NK cells express a receptor(s) for MHC class I, which delivers signals to negatively regulate cytotoxic activity of NK cells to prevent self-killing (7). Recently, indeed, a number of receptors responsible for the target recognition of NK cells have been molecularly identified in both human and rodents (8–16).

NK receptors for MHC class I have been categorized into three families based on their structures. One is the killer cell inhibitory receptors belonging to Ig superfamily, expressed in humans but not in rodents (8–10). The second is the lectin-like Ly-49 homodimeric receptors expressed in rodents but not in humans (11, 12, 15). The third is the lectin-like heterodimers consisting of CD94 and NKG2 subunits, which were first identified and have been characterized extensively in human (17, 18). Accordingly, it was initially thought that human and rodent NK cells used distinct systems to recognize MHC class I. However, this turned out not to be the case, because recent studies demonstrated that homologues of CD94/NKG2 heterodimers exist in rodents as well (19, 20). Thus, rodent NK cells appear to use multiple mechanisms to survey MHC class I expression on target cells as observed in human NK cells.

A unique feature of human CD94/NKG2 heterodimers is that they recognize HLA-E, a nonclassical MHC class I molecule (18, 21–24). HLA-E is ubiquitously expressed and stabilized on cell surface by binding signal peptides of classical MHC class I molecules in a TAP-dependent manner (25, 26). Therefore, NK cells can monitor the biosynthesis of highly polymorphic MHC class I molecules as well as TAP function by recognizing a relatively nonpolymorphic sequence of signal peptides (21–24, 27, 28). Intriguingly, the treatment of human NK clones with anti-CD94 mAbs provided complex consequences. Some NK clones were activated by the treatment, whereas other clones from the same donor were inhibited or unaffected (Ref. 29, for review see Ref. 17). Recent studies have provided a clue to solve this puzzling issue. CD94 is paired with some members of NKG2 family, which consists of at least six different members (30, 31). NKG2A, B, and F contain immunoreceptor tyrosine-based inhibitory motif (ITIM)3

3 Abbreviations used in this paper: ITIM, immunoreceptor tyrosine-based inhibitory motif; β2m, β2-microglobulin; iEL, intestinal intraepithelial lymphocyte; HA, hemagglutinin; CHO, Chinese hamster ovary.

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in their cytoplasmic portions and, therefore, transmit inhibitory signals. In contrast, NKG2C, D, and E lack cytoplasmic ITIM but associate with DAP12 or DAP10 transmitting activation signals (32–34).

In contrast to human CD94, the study of mouse CD94 as a functional NK receptor has been hampered by the lack of Abs that can specifically recognize CD94 and interfere with its possible functions. Recently, mouse CD94/NKG2 heterodimer reconstituted on COS7 cell transfectants was shown to bind to a nonclassical MHC class I molecule Qa-1β, a functional counterpart of HLA-E (35, 36). Furthermore, it was demonstrated that expression of Qa-1β on target cells led to inhibition of target cell lysis by NK cells (35). These results suggested that CD94/NKG2 heterodimer on mouse NK cells could function as an inhibitory receptor for Qa-1 recognition and functions as an inhibitory receptor independent of CD94 and NKG2A. Thus, our results provided that primary NK cells indeed express and use CD94 for the recognition and functions of Qa-1 on target cells. However, no direct evidence has been provided that primary NK cells indeed express and use CD94 for the Qa-1 recognition and the following inhibitory signaling. In the present study, we established a novel mAb specific to mouse CD94 and used it to explore the expression of mouse CD94 and evaluate its function on primary NK cells. Our studies demonstrate that mouse CD94 is expressed not only on NK cells but also on NK T cells and subsets of T cells. Importantly, the anti-CD94 mAb completely blocked the inhibition of target cell lysis mediated by the NK recognition of Qa-1. Furthermore, the mAb induced NK-mediated lysis of autologous target cells and enhanced NK-mediated lysis of allotypic and missing self target cells. Thus, our results provide direct evidence that CD94 is involved in NK target recognition and functions as an inhibitory receptor independent of Ly-49 family members. A possible implication of CD94 in self tolerance of NK cells is discussed.

Materials and Methods

Animals

C57BL/6 and BALB/c mice were purchased from Shimizu Laboratory Animal Center (Hamamatsu, Japan). C57BL/6 β2-microglobulin−/− (β2m−/−) (37), C57BL/6 rag2−/−, and C3H rag2−/− mice (38) were obtained from Taconic Farms (Germantown, NY). B10.D2 mice and Armenian hamsters were obtained from Sankyo (Tokyo, Japan). All experiments were performed according to the Guidelines for Animal Use and Experimentation as set out by our institutions.

Cell culture

Cells were cultured in RPMI 1640 medium supplemented with 10% FCS (lot FKB09; Mitsubishi Kasei, Tokyo, Japan), 10 mM HEPEs, 2 mM l-glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, 1% (v/v) nonessential amino acids (100×; Flow Laboratories, Irvine, U.K.), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Monoclonal Abs

Biotin-conjugated A1 (anti-Ly-49A), SW-5E6 (anti-Ly-49C/I), and FITC-conjugated PK136 (anti-NK1.1), DX5 (anti-panNK), H57-597 (anti-TCRβ), GL3 (anti-TCRγ), 6A8.6F10.1A6 (anti-Qa-Ⅰβ), and APC-conjugated RM4-5 (anti-CD4), 53-6 (anti-CD8α), and PerCp-conjugated 2C11 (anti-CD3ε) were purchased from Pharmingen (San Diego, CA).

Preparation of NK cells

Single-cell suspension was prepared from spleens of 6- to 8-wk-old mice. To prepare NK cell-rich fraction, splenocytes were depleted of surface IgG B cells by using MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, cells were incubated with sheep anti-mouse IgG-conjugated magnetic beads (Dynabeads DB11002; Dynal, Oslo, Norway) in MACS buffer solution (0.5% BSA, 2 mM EDTA in PBS, pH 7.2) for 15 min on ice, and then the beads bound to magnetic beads were removed by MACS. Recovered unbound cells (surface IgG splenocytes) were used as effector cells in the killer assay. For preparation of IL-2-activated NK cells, splenocytes were cultured in complete medium containing 2 nM recombinant human IL-2 (400 U/ml; Shionogi, Tokyo, Japan) for 5 days. For preparation of poly(I:C)-activated NK cells, 100 μg of poly(IC) (Sigma P-0913; Sigma, St. Louis, MO) was i.p. injected to mice, and spleen cells were isolated 18 h after the injection.

Preparation of mAb specific for NK cells

We noticed that NK cells from C57BL/6 bm/bm mice (39) did not express Ly-49C/I recognized by SW-5E6 mAb in contrast to those from C57BL/6 mice (data not shown). Ly-49C/I-deficient progeny without brachymorphic phenotype was established by further backcross onto C57BL/6 mice and designated as C57BL/6 del/C. Armenian hamsters were immunized at weekly intervals by footpad injection of IL-2-activated NK cells (1–2 × 105 cells/animal) prepared from C57BL/6 del/C. CFA was included in the first inoculum. After three immunizations, the booster injection was made in the footpad 2 days before the fusion. Popliteal lymph node cells were fused with murine P815 myeloma cells (40) using PEG1500 (Boehringer Mannheim, Mannheim, Germany) and cultured with medium containing hypoxanthine/aminopterin/thymidine and rIL-6 (41). Hybridomas were screened by their ability to stain splenic NK cells and to induce enhanced cytotoxicity against syngeneic or allogeneic lymphoblast target cells.

Construction and transfection of expression vectors for mouse CD94 and NKG2A

RNA was prepared from IL-2-activated spleen cells, and PCR-based cloning was conducted using primers designed for amplifying full-length cDNA of CD94 and NKG2A tagged with hemagglutinin (HA) and Myc, respectively (19, 42, 43), which were cloned into pcDNA3.1 vector (Invitrogen, Groningen, The Netherlands). COS7 cells were transiently transfected with the vector plasmids by electroporation method. After 48 h, cell lysates were prepared as described previously (44), and incubated with Yui13 mAb or control hamster IgG. The immunoprecipitates were subjected to SDS-PAGE, followed by immunoblot analysis with biotinylated anti-HA mAb (12CA5) and HRP-conjugated streptavidin, detected by an ECL system (Amersham Pharma Biotech, Buckinghamshire, U.K.).

Flow cytometric analysis

Cells (1 × 106) were pretreated for 20 min on ice with 20% heat-inactivated normal hamster serum and 2.4G2 mAb (45) to block FcR-mediated nonspecific binding of Abs. Subsequently, cells were incubated with 10 μg/ml FITC-conjugated or biotin-conjugated Abs for 30 min followed by incubation with PE- or APC-conjugated streptavidin for 15 min on ice. Flow cytometry was performed with FACS Calibur, and data were analyzed with the CellQuest program (Becton Dickinson, San Jose, CA). Intestinal intraepithelial lymphocytes (IEL) used for flow cytometric analysis were prepared as described previously (46).

Cell sorting

Surface Ig+ spleen cells from C57BL/6 mice were prepared as described above. To prepare the NK-enriched fraction for cell sorting, surface Ig+ cells were incubated with DX5-conjugated microbeads (Miltenyi Biotec), and then the cells bound to microbeads were collected by AutoMACS (Miltenyi Biotec). Collected cells were incubated with FITC-conjugated NK1.1 mAb and biotin-conjugated Yui13 mAb followed by incubation with PE- or APC-conjugated streptavidin. Cell sorting was performed with EPICS Elite (Beckman Coulter, Fullerton, CA) to isolate NK1.1+ CD94null and NK1.1+ CD94bright, and purity was checked with FACS Calibur.

Semi-quantitative RT-PCR analysis

Total RNA was prepared from freshly sorted NK1.1+ CD94bright and NK1.1+ CD94null cells by using Isogen (Wako, Tokyo, Japan), and template cDNA was synthesized by using oligo(dT) primer. The integrity of mRNAs and successful cDNA synthesis were verified for each sample by monitoring transcription factor IID (TFIID). Amplified PCR products were subjected to electrophoresis on agarose gels.

Killer assay

A standard 4-h 51Cr-release assay was performed to examine cytotoxic activity of cells. Con A blasts were prepared by stimulation of lymph node cells with 5 μg/ml Con A for 48 h. Chinese hamster ovary (CHO) cells stably expressing QA-1β (CHO/Qa-1β) were prepared by introducing full-length mouse QA-1β cDNA in pcDNA3.1 vector into CHO cells, and the cell surface expression of QA-1β was verified by staining with anti-QA-1β mAb. These cells were labeled with 51Cr (3700 KIU/105 cells) for 45 min at 37°C and used as target cells in the killer assay. HPLC-purified Qa-1 determinant modifier; AMAPRTLLL (47) and OVA (SINFEKL) peptides (48) were added at the concentration of 100 μM to 31Cr-labeled target cells plated in V-bottom 96-well plates at 104 cells/well. After 90
Results

Establishment of a mAb specific to mouse CD94

With the aim of establishing mAbs specific for novel NK receptors, hamsters were immunized with IL-2-activated mouse NK cells as described in Materials and Methods. Hybridomas were prepared from lymph node cells of immunized animals, and their supernatants were screened for the reactivity to NK cells by flow cytometric and functional analyses. One of the selected mAbs, designated Yuri3, was further studied to identify its target Ag.

Flow cytometric analysis revealed that in spleen of C57BL/6 mice the majority of cells stained with Yuri3 expressed NK markers such as DX5 and NK1.1 (Fig. 1A). Essentially all DX5<sup>+</sup> and NK1.1<sup>+</sup> cells were stained with Yuri3. Interestingly, two distinct populations were observed among DX5<sup>+</sup> and NK1.1<sup>+</sup> cells with regard to the expression levels of Yuri3 Ag. Approximately half of the cells expressed Yuri3 Ag at high levels while the other half expressed at lower levels. For the flow cytometric analysis, cells were pretreated with anti-FcγRIII mAb 2.4G2 and normal hamster serum to prevent nonspecific binding of Ab. Indeed, control hamster IgG did not stain DX5<sup>+</sup> and NK1.1<sup>+</sup> spleen cells (data not shown). Therefore, low levels of Yuri3 Ag detected on the half of NK1.1<sup>+</sup> cells appear to be significant and specific. Yuri3 Ag was detected on DX5<sup>+</sup> cells not only from C57BL/6 mice but also from all other mouse strains tested (Fig. 1B). Both Yuri3 Ag<sup>bright</sup> and Yuri3 Ag<sup>dull</sup> populations were observed in all strains analyzed, though levels of the Yuri3 Ag varied among different strains. This broad reactivity of Yuri3 across the mouse strains is distinct from the strain-specific reactivity of SW-5E6 mAb recognizing certain allotypes of Ly-49C/I and A1 mAb recognizing C57BL/6 allotype of Ly-49A (Fig. 1B). These results suggested that Yuri3 reacts with an invariant molecule expressed on NK cells or recognizes a common epitope shared by molecules carrying allelic polymorphism such as Ly-49. To explore the latter possibility, the correlation in the expression of Yuri3 Ag and Ly-49 was examined by three-color flow cytometry (Fig. 1C). Both Ly-49C/I<sup>+</sup> and Ly-49C/I<sup>−</sup> fractions among the NK1.1<sup>+</sup> cells were found to express Yuri3 Ag. Proportion of Yuri3 Ag<sup>bright</sup> and Yuri3 Ag<sup>dull</sup> populations was comparable in the two fractions. This was also true for Ly-49A<sup>+</sup> vs Ly-49A<sup>−</sup> fractions and Ly-49G<sup>+</sup> vs Ly-49G<sup>−</sup> fractions among the NK1.1<sup>+</sup> cells (Fig. 1C, lower panels, and data not shown). Therefore, Yuri3 Ag is apparently distinct from Ly-49C/I, Ly-49A, and Ly-49G.

Human CD94 is an invariant component of CD94/NKG2A-F heterodimers (17, 18). Though the gene coding for mouse CD94 was already identified (19, 20), the expression of mouse CD94 protein on primary NK cells has not been characterized yet. To test the possibility that Yuri3 recognizes mouse homologue of CD94, the reactivity of Yuri3 to COS7 cells transfected with mouse CD94 cDNA alone or together with mouse NKG2A cDNA was examined. As shown in Fig. 2A, Yuri3 stained CD94-transfectants as well as CD94/NKG2A double transfectants but not from mock transfectants (Fig. 2B). From these results, we concluded that Yuri3 mAb recognizes mouse CD94.

Expression of mouse CD94 not only on NK cells but also on NK T cells and subsets of T cells

The expression of mouse CD94 in various lymphoid subsets was analyzed by multicolor flow cytometry with Yuri3 Ab. In spleen, NK1.1<sup>+</sup> cells include CD3<sup>−</sup> NK population and CD3<sup>dull</sup> NK T population. Essentially all cells in both populations were found to express CD94 (Fig. 3A). Though NK T cells appeared to express lower levels of CD94 than NK cells, CD94<sup>bright</sup> and CD94<sup>dull</sup> populations were detectable at the similar frequency (~50%) each in both cell types. In thymus, the vast majority of NK1.1<sup>+</sup> cells express intermediate levels of CD3, therefore belong to NK T cell lineage (Fig. 3B, left). CD94 was also detected on these thymic NK

**FIGURE 1.** A molecule(s) recognized by Yuri3 mAb is predominantly expressed on DX5<sup>+</sup> and NK1.1<sup>+</sup> cells in spleen from various mouse strains independent of Ly-49. A. Spleen cells from C57BL/6 mice were stained with biotin-Yuri3 (revealed by PE-streptavidin) in combination with FITC-DX5 or anti-NK1.1 mAb. B. Splenocytes from indicated mouse strains were enriched for NK and T cells by eliminating surface Ig<sup>+</sup> cells using magnetic cell sorting, and stained with FITC-DX5 in combination with biotin-Yuri3, anti-Ly-49C/I, and anti-Ly-49A mAbs revealed by APC-streptavidin. C. Surface Ig<sup>+</sup> splenocytes from C57BL/6 mice were stained with FITC-anti-NK1.1 and biotin-Yuri3 (revealed by APC-streptavidin) in combination with PE-anti-Ly-49C/I or anti-Ly-49A mAbs. NK1.1<sup>Ly-49<sup>−</sup></sup> and NK1.1<sup>Ly-49<sup>+</sup></sup> populations were gated as shown in left panels, and the profile of Yuri3 staining (solid line) on each population was displayed as histograms overlaid with control staining with hamster IgG (dotted line; right panels).
Anti-HA epitope Ab. CD94 protein in the immunoprecipitates was detected by western blotting separated by 5–20% SDS-PAGE under reducing conditions. HA-tagged control hamster IgG (lanes 2, 4), and soluble lysates were incubated with Yuri3 mAb (lanes 1 and 2). Detergent-soluble lysates were incubated with Yuri3 mAb (lanes 3 and 4), or empty vector (lanes 5 and 6). After 48 h, cells were collected with PBS containing 0.05% EDTA and lysed with lysis buffer solution containing 1% Nonidet P-40.

Approximately 2% of CD3⁺NK1.1⁻ T cells in the spleen were found to express relatively low levels of CD94 (Fig. 3A, lower panels). Analysis of CD4 and CD8 expression revealed that ~70% of CD94⁺ T cells were CD4⁺CD8⁻ and 20–30% of them were CD4⁻CD8⁺. These CD94⁺ T cells were predominantly αβT cells (data not shown). To examine whether γδT cells also expressed CD94, the expression of CD94 on iIEL was analyzed (Fig. 3C). In our analysis, numbers of αβT cells and γδT cells were almost equal in iIEL. In both populations, 2–4% of cells were found to express low levels of CD94. In contrast, Ly-49A⁺ cells and Ly-49C/I⁺ cells were found predominantly in the αβT cell population.

Ontogenetical expression of CD94 on mouse NK cells

Previous studies demonstrated that Qa-1 binding cells exist in fetal NK cells (49, 50). This suggests that fetal NK cells may express CD94/NKG2A heterodimers. Therefore, we analyzed the expression of CD94 during ontogeny of NK cells in fetal and neonatal liver from C57BL/6 mice. CD94⁻⁺ cells were detected among NK1.1⁺ cells in the fetal liver as early as on day 14 of gestation. Proportion of CD94⁺ cells among NK1.1⁺ cells appeared to increase progressively during ontogeny. CD94 was detected on 10% of NK1.1⁺ cells in the fetal liver on day 14 of gestation and 53% of NK1.1⁺ cells in liver on day 5 after birth. In contrast, the expression of Ly-49 such as Ly-49A was hardly detectable on fetal liver cells as reported previously (51–53). These results indicated that CD94 is expressed on NK cells very early during ontogeny before the expression of known Ly-49 molecules.

Effect of MHC class I on CD94 expression

It has been demonstrated that the expression of mouse Ly-49 is influenced by the expression of MHC class I (54–56). To examine
whether this is also true for CD94 expression, levels of CD94 and Ly-49 expression on NK cells were compared between C57BL/6 and C57BL/6 $\beta_2m^{-/-}$ mice. In accord with previous reports (55, 56), the expression of Ly-49A was up-regulated in C57BL/6 mice (Fig. 5, left panels). The ratio of CD94$^{\text{bright}}$ vs CD94$dull$ population was significantly higher in C57BL/6 $\beta_2m^{-/-}$ mice than in C57BL/6 mice (Fig. 5, left panels). Levels of CD94 expression in each population were also slightly higher in C57BL/6 $\beta_2m^{-/-}$ mice. Thus, the expression of CD94 on NK cells appears to be affected by the expression of $\beta_2m$-associated MHC class I molecules.

Involvement of CD94 in protection of target cells from NK cell cytotoxicity through Qa-1 recognition

Previous studies using soluble Qa-1 tetramers and an anti-mouse NKG2 mAb clearly demonstrated that NK recognition of Qa-1 inhibits target cell lysis and that NKG2 on mouse NK cells is involved in the Qa-1 recognition (35, 36). However, no direct evidence has been provided that mouse CD94 is involved in the Qa-1 recognition and the following inhibitory signaling in primary NK cells. To address this issue, the effect of anti-CD94 mAb Yuri3 on the recognition of Qa-1 and cytotoxic activity of primary NK cells was examined by using CHO cells stably transfected with Qa-1$^+$ cDNA as target cells. In the absence of Qdm peptide (AMA PRTL), Qa-1$^+$ transfecteds were lysed as efficiently as untransfected CHO cells by activated NK cells from poly(I:C)-treated C57BL/6 mice (Fig. 6A, ○ and □). Preincubation of Qa-1$^+$ transfecteds with Qdm peptides resulted in substantial inhibition of their lysis by NK cells (Fig. 6A, ●). In contrast, preincubation of untransfected CHO cells with Qdm peptides had no effect on their lysis (Fig. 6A, ▼). These results are consistent with the previous report showing that murine NK cell cytotoxicity was inhibited by Qa-1$^+$ expressed on human T2 target cells only when Qa-1$^+$ was associated with Qdm peptide (35). Notably, in the presence of Yuri3 mAb, NK activity against Qa-1$^+$ transfecteds preincubated with Qdm peptides was enhanced up to the level comparable to that against Qa-1$^+$ transfecteds preincubated with control OVA peptides (SIINFEKL) (Fig. 6B). This indicates that the inhibition of cytotoxic activity mediated by NK recognition of Qa-1/Qdm was completely canceled by Yuri3. Control hamster IgG did not show such an effect. Moreover, Yuri3 showed no detectable effect on NK activity against Qa-1$^+$ transfecteds preincubated with control OVA peptides or on the lysis of P815 target cells (data not shown). Furthermore, Yuri3 did not enhance YAC 1 killing or IFN-γ production in NK cells (data not shown). Therefore, the effect of Yuri3 is likely due to the blocking of NK recognition of Qa-1$^+$/Qdm.

FIGURE 4. CD94 is expressed on NK1.1$^+$ cells in fetal and neonatal liver. Ontogeny of NK receptors on NK cells in the fetal and neonatal livers was examined by two-color flow cytometry. Mononuclear cells were isolated from livers of fetus and newborn mice at the indicated developmental points and stained with FITC-anti-NK1.1 mAb or anti-Ly-49A mAb A1 (revealed by APC-streptavidin). Percentage of CD94$^{\text{bright}}$NK1.1$^+$ cells in liver MNCs is indicated.

FIGURE 5. Expression of CD94 is affected by the presence of $\beta_2m$-associated molecules. Surface Ig$^+$ splenocytes were prepared from C57BL/6 and C57BL/6 $\beta_2m^{-/-}$ mice and stained with FITC-anti-NK1.1 in combination with biotin-anti-CD94 (left panels) or anti-Ly-49A (right panels). Biotin-mAbs were revealed by PE-streptavidin. Profiles of CD94 and Ly-49A expression on NK1.1$^+$ cells are displayed as histograms.

FIGURE 6. The anti-CD94 mAb abrogates inhibition of target cell lysis mediated by NK recognition of Qa-1/Qdm complex. A. Surface Ig$^+$ spleen cells from poly(I:C)-treated C57BL/6 mice were used as effector cells. As target cells, CHO cells stably transfected with Qa-1$^+$ (CHO/Qa-1) and parental CHO cells were labeled with $^{51}$Cr and then incubated with 100 μM Qdm peptide or equal volume of PBS at room temperature for 90 min before the effector cells were added. Cytotoxic assay was performed at indicated effector/target ratios. Data are indicated as mean ± SD of the triplicate wells. B. The effector cells were incubated with anti-CD94 mAb Yuri3 or control hamster IgG (each 25 μg/ml) at room temperature for 10 min before being mixed with $^{51}$Cr-labeled CHO/Qa-1 transfecteds that had been preincubated with Qdm peptide or control OVA peptide (each 100 μM) at room temperature for 90 min. Data are indicated as mean ± SD of the triplicate wells.
Qa-1\textsuperscript{a}/Qdm rather than active stimulation of cytotoxic activity of NK cells. Taken together, it is strongly suggested that CD94 is involved in NK recognition of Qa-1\textsuperscript{a}/Qdm to deliver inhibitory signals.

**CD94\textsuperscript{bright} but not CD94\textsuperscript{dull} NK population functions in Qa-1/ Qdm-mediated inhibition**

To clarify functional difference of CD94\textsuperscript{bright} and CD94\textsuperscript{dull} cells in Qa-1/Qdm-mediated inhibitory function, these two populations isolated from C57BL/6 spleen cells were evaluated for their cytotoxicity against CHO transfectants. Sorted cells (>95% purity) were cultured with IL-2 for 4 days. Transition either from CD94\textsuperscript{bright} cells to CD94\textsuperscript{dull} cells or vice versa was not observed during culture even though levels of CD94 expression were upregulated in both cell types, most likely due to the blastic change of cells in the presence of IL-2 (Fig. 7A). CD94\textsuperscript{bright} and CD94\textsuperscript{dull} cells showed comparable cytotoxicity to CHO cells preincubated with Qdm, and Yuri3 mAb had no significant effect on their cytotoxicity (Fig. 7B, right panel). In contrast, when Qa-1\textsuperscript{a} transfectants preincubated with Qdm were used for target cells, CD94\textsuperscript{bright} cells showed much less cytotoxicity than CD94\textsuperscript{dull} cells did. The addition of Yuri3 mAb greatly enhanced the cytotoxicity of CD94\textsuperscript{bright} cells, whereas it had no significant effect on the cytotoxicity of CD94\textsuperscript{dull} cells. These results indicate that the Qa-1/Qdm-mediated inhibition of NK cytotoxicity observed in bulk NK cells as shown in Fig. 6B is attributed to CD94\textsuperscript{bright} cells but not CD94\textsuperscript{dull} cells. To know whether such functional difference in CD94\textsuperscript{bright} and CD94\textsuperscript{dull} cells is due to difference of NKG2 expression, we examined transcription of NKG2 in these two populations by semiquantitative RT-PCR. PCR primers were set within well-conserved sequences of known NKG2A, C, and E as described previously (36). Template cDNAs were serially diluted in 2-fold. The integrity of mRNAs and successful cDNA synthesis were verified for each sample by monitoring transcription factor IID.

**The anti-CD94 mAb induces autologous target killing and enhances allogeneic and missing self target killing**

We next explored possible roles of CD94 in the protection of self cells from NK cell cytotoxicity by using autologous target cells in NK assay. Activated NK cells from poly(I:C)-treated C57BL/6 mice showed significant cytotoxic activity against Con A blasts prepared from allogeneic BALB/c mice whereas they showed very little activity against Con A blasts prepared from syngeneic C57BL/6 mice regardless of the absence or presence of control hamster IgG (Fig. 8, A and B, □). However, in the presence of Yuri3 mAb, C57BL/6 NK cells showed significant cytotoxicity against syngeneic C57BL/6 target cells (Fig. 8A, □). The induction of cytotoxic activity against syngeneic target cells in the presence of Yuri3 was also observed in the combination of BALB/c-mice-derived NK and target cells (Fig. 8C). The effect of Yuri3 was further analyzed on NK activity against allogeneic or missing self target cells in the context of classical MHC class I. C57BL/6 NK cells showed much higher cytotoxic activity against allogeneic BALB/c target cells in the presence of Yuri3 than in the presence of control IgG (Fig. 8B). Such enhancement of NK activity in the presence of Yuri3 was also observed in a missing self combination, that is, (C57BL/6 rag2\textsuperscript{−/−} × C3H rag2\textsuperscript{−/−})\textsuperscript{F1} NK cells against C57BL/6 target cells (Fig. 8D). Thus, Yuri3 mAb induced NK-mediated lysis of syngeneic target cells and enhanced NK-mediated lysis of allogeneic and missing self target cells. These results suggest that CD94 functions as an inhibitory receptor on NK cells independent of inhibitory receptors for classical MHC class I such as Ly-49 family members.

**Discussion**

Previous studies demonstrated that approximately half of mouse NK cells bound to Qa-1 tetramers (35, 36, 57). A similar proportion of NK cells was shown to be stained with an anti-NKG2 mAb that reacts with NKG2A, NKG2C, and NKG2E (36). The mAb
completely inhibited the binding of Qa-1 tetramers to mouse NK cells (36). These results clearly demonstrated that NKG2 on primary NK cells is responsible for the QA-1 binding. Notably, cytotoxic activity of QA-1-binding NK cells was inhibited by the expression of QA-1/Qdm complex on target cells (35), indicating that the QA-1 receptor on NK cells functions as an inhibitory receptor. QA-1 tetramer binding analysis using COS7 cells transfected with cDNA coding for CD94 and NKG2 revealed that only CD94/NKG2 heterodimer, but not CD94 or NKG2A alone, was able to bind to QA-1 tetramers (35, 36). Therefore, it was assumed that NKG2 expressed on NK cells is associated with CD94 to form an inhibitory receptor for QA-1. However, the expression and function of mouse CD94 in primary NK cells had never been directly demonstrated due to the lack of appropriate Abs specific to mouse CD94. Thus, it could not be formally ruled out that a molecule other than CD94 forms an inhibitory receptor for QA-1 in association with NKG2 on primary NK cells.

The present study with the newly established anti-mouse CD94 mAb Yuri3 provided direct evidence that CD94 on mouse NK cells is indeed involved in the recognition of QA-1 on target cells and functions as an inhibitory receptor. Importantly, the anti-CD94 mAb completely blocked the inhibition of target killing mediated by the NK recognition of QA-1. This indicates that the QA-1 recognition is operated primarily by CD94 complex on NK cells. In combination with the previous observation that the anti-NKG2 mAb completely inhibited the binding of QA-1 tetramers to mouse NK cells, we conclude that CD94/NKG2 complexes are indeed the predominant, if not the only, QA-1 receptors on mouse NK cells. NKG2 family consists of several members including recently identified NKG2C and NKG2E (36). Although CD94/NKG2A, CD94/NKG2C, and CD94/NKG2E were all shown to bind to QA-1, NKG2C and NKG2E do not contain ITIM-like sequences in their cytoplasmic tails, unlike NKG2A. Instead, they contain a positively charged residue in their transmembrane domains like human NKG2C and NKG2E, suggesting that they function as activating receptors. In our study, the expression of QA-1/Qdm complex on target CHO cells led to the inhibition of NK activity, and the inhibition was canceled by the anti-CD94 mAb. Therefore, the functional balance between inhibitory and activating CD94/NKG2 complexes appears to be biased toward inhibition of NK activity at least in our experimental conditions. This is consistent with the previous report that NKG2A transcripts are at least 20-fold more abundant than NKG2C and NKG2E transcripts in IL-2-stimulated NK cells (36).

The pattern of CD94 expression in different NK subsets defined by Ly-49 expression demonstrated in our study as well as the QA-1 binding activity in those subsets (57, 58) indicate the independent expression of two distinct inhibitory receptors, CD94/NKG2 and Ly-49 families, on mouse NK cells. Our results further suggest that these two receptors are functionally independent as well and that both are necessary for the protection from self-killing. In combination of C57BL/6 NK cells and BALB/c target cells, Ly-49 such as Ly-49C/I on NK cells does not transduce inhibitory signals in the absence of H-2b on target cells, resulting in NK cell-mediated target killing (59). Although C57BL/6 and BALB/c mice are allogeneic in terms of classical MHC class I, they share a nonclassical MHC class I QA-1b, and the sequence of Qdm peptide is found in the leader peptide of both H-2Dd and H-2Dd (60). Therefore, one may assume that the CD94/NKG2 complex on C57BL/6 NK cells is potent to recognize QA-1/Qdm complex on BALB/c target cells and to transduce inhibitory signals. If this is the case, NK cells are not fully activated in this combination. Indeed, NK activity was further enhanced in the presence of the anti-CD94 mAb. This was also true for NK activity in missing self combination in which target cells expressed the same QA-1b as did NK cells. The mAb itself did not induce IFN-γ production in NK cells and had no effect on the lysis of YAC 1 and P815 target cells (data not shown). Therefore, the effect of Yuri3 is likely due to the blocking of NK recognition of QA-1b rather than active stimulation of NK cytotoxicity. These results strongly suggest that CD94/NKG2 functions as an inhibitory receptor independent of Ly-49. Moreover, it appears that the inhibitory signal through CD94/NKG2 alone is not sufficient for the prevention of NK cell-mediated killing when the inhibitory signal through Ly-49 is absent. In contrast, the lack of the inhibitory signals through CD94/NKG2 alone seems sufficient to induce NK cell-mediated lysis of target cells, because the anti-CD94 mAb induced autologous target killing, where the inhibitory signal through Ly-49 is supposed to be delivered. Thus, two different systems are necessary for full protection from self-killing, one that recognizes classical MHC class I, and the other that recognizes nonclassical MHC class I. The functional importance of activating type CD94/NKG2 receptors in allorecognition remains to be further investigated. Recent study suggested that activating type Ly-49D receptor is responsible for the ability of H-2^d/Ft hybrid mice to reject H-2^d/d parental bone marrow cells (hybrid resistance) (61). In this context, it would be interesting to study possible involvement of activating type CD94/NKG2 receptors in hybrid resistance and alloreactivity.

Two distinct subpopulations were identified in both NK and NK T cells on the basis of levels of CD94 expression. Though all NK and NK T cells were found to express CD94, approximately half of them in spleen had higher expression of CD94 than the other half. We demonstrated that CD94bright cells and CD94^{null} cells were functionally different and that the former but not the latter was involved in the QA-1/Qdm-mediated inhibition of NK cytotoxicity. Previous studies showed that approximately half of the NK cells reacted with an anti-NKG2 mAb and possessed QA-1 binding activity that was completely inhibited by the mAb (36). Taken together, it is strongly suggested that CD94bright cells correspond to the QA-1-bound, anti-NKG2 mAb-reactive population while CD94^{null} cells correspond to cells that do not possess the
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Qa-1- and anti-NKG2 mAb-binding activity. However, our semi-quantitative RT-PCR analysis demonstrated that NKG2 genes were transcribed not only in CD94bright but also in CD94dull cells. To clarify structural and functional differences of CD94 receptor molecules on CD94bright and CD94dull cells, the following remains to be determined: 1) What kinds of NKG2 gene(s) are transcribed in CD94dull cells? 2) Are the NKG2 gene(s) transcribed in CD94dull cells translated into mature NKG2 proteins and expressed on cell surface? 3) If some NKG2 molecules are expressed on CD94dull cells, do they associate with CD94 on the cell surface? 4) What is physiological function of CD94dull cells?

Proportion of the CD94bright subpopulation among NK cells was significantly higher in C57BL/6 $\beta_2$-m$^{-/-}$ mice than in C57BL/6 mice, implying that the expression of CD94 on NK cells is influenced by the expression of $\beta_2$-m-associated MHC class I, most likely Qa-1. On the contrary, it was previously reported that NK cells from TAP$^{-/-}\beta_2$m$^{-/-}$ mice and C57BL/6 control mice showed similar Qa-1 binding patterns, with respect to both the percentage of cells stained with Qa-1 tetramers and the intensity of staining (57). Further studies are needed to elucidate mechanism and functional significance of regulation of CD94 expression.

CD94 expression was detected on fetal liver NK cells as early as on day 14 of gestation, that is, before the expression of known Ly-49 family members such as Ly-49A became detectable (62). In contrast to adult NK cells, there was no clear discrimination of CD94bright and CD94dull populations in fetal NK cells. Instead, CD94^NK1.1bright and CD94^NK1.1dull populations were distinguishable. The percentage of the former population increased as development proceeded. The relationship between these two populations in fetal NK cells and the two populations found in adult NK cells (CD94bright and CD94dull) remains to be clarified. Because previous studies demonstrated that Qa-1 binding cells were detected in fetal liver NK cells (49), one may assume that at least some of CD94 expressed on fetal NK cells is functional in recognition of Qa-1. It is an intriguing possibility that CD94 on fetal NK cells is involved in the immunological regulation of maternal-fetal interaction.

Besides NK and NK T cells, a small fraction of $\alpha$BT and $\gamma$T cells was also found to express CD94 as in humans (17). Among iIEL, CD94^ cells were detected in both $\alpha$BT and $\gamma$T cell subsets at the similar frequency (2–4%) while Ly-49A$^b$-m$^{-/-}$ T cells blasts by natural killer cells from normal but not from $\beta_2$m$^{-/-}$ mice: nonresponsiveness controlled by $\beta_2$m$^{-/-}$ bone marrow in chimeric mice. Proc. Natl. Acad. Sci. USA 88:10332.


