Nonstochastic Coexpression of Activation Receptors on Murine Natural Killer Cells

By Hamish R.C. Smith,* Hubert H. Chuang,* Lawrence L. Wang,* Margarita Salcedo,‡ Jonathan W. Heusel,* and Wayne M. Yokoyama*

*From the *Immunology Program and the Rheumatology Division, the Department of Medicine and the Department of Pathology, the Center for Arthritis and Related Diseases, and the Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, Missouri 63110; and the ‡Unite de Biologie Moleculaire du Gene, Institut National de la Santé e de la Recherche Médicale, U277, Institut Pasteur, 75015 Paris, France*

Abstract

Murine natural killer cells (NK) express lectin-like activation and inhibitory receptors, including the CD94/NKG2 family of receptors that bind Qa-1, and the Ly-49 family that recognizes major histocompatibility complex class I molecules. Here, we demonstrate that cross-linking of NK cells with a new specific anti–Ly-49H mAb induced NK cell cytotoxicity and cytokine production. Ly-49H is expressed on a subset of NK cells and can be coexpressed with Ly-49 inhibitory receptors. However, unlike Ly-49 inhibitory receptors, Ly-49H is not detectable on naive splenic $CD3^+$ T cells, indicating that Ly-49H may be an NK cell–specific activation receptor. In further contrast to the stochastically expressed Ly-49 inhibitory receptors, Ly-49H is preferentially expressed with the Ly-49D activation receptor, and expression of both Ly-49H and Ly-49D is augmented on NK cells that lack receptors for Qa-1 tetramers. On developing splenic $NK1.1^+$ cells, Ly-49D and Ly-49H are expressed later than the inhibitory receptors. These results directly demonstrate that Ly-49H activates primary NK cells, and suggest that expression of Ly-49 activation receptors by NK cells may be specifically regulated on NK cell subsets. The simultaneous expression of multiple activation receptors by individual NK cells contrasts with that of T cell antigen receptors and is relevant to the role of NK cells in innate immunity.

Key words: 3D10 • cytotoxicity • Ly-49H • subset • Ly-49D

Introduction

NK cells lyse tumor or virally infected cells without prior sensitization in a process known as natural killing (1, 2). This process results in the exocytosis of granules containing perforin and granzymes that mediate target apoptosis. Upon exposure to their cellular targets, NK cells also produce cytokines such as IFN-γ, GM-CSF, and TNF-α (3). Recently, significant strides have been made to explain how NK cells recognize their targets to initiate these effector functions. One current model suggests that NK cells express two types of receptors, an activation type receptor and a second inhibitory receptor that binds MHC class I molecules and inhibits NK cell activation (4, 5). This model is consistent with the missing-self hypothesis postulating that NK cells survey tissues for normal expression of MHC class I and can be released to kill in the absence of MHC class I (6).

Over the last few years, major advances have elucidated the structure and function of the MHC class I inhibitory receptors (7–9). These receptors fall into two structural categories. NK cells express type I integral membrane, Ig-like receptors, such as the killer cell Ig-like receptors (KIRs) on human NK cells, and gp49B on mouse NK cells (10–12). These molecules are usually expressed as monomeric receptors. On the other hand, NK cells also express type II integral membrane, C-type lectin-like disulfide-linked dimeric proteins such as the homodimeric Ly-49A receptor on mouse NK cells, and the CD94/

M. Salcedo's present address is IDM Research Laboratory, Centre de Recherches Biomedicales des Cordelies, 15 rue de l'Ecole de Medicine, 75006 Paris, France.

Address correspondence to Wayne M. Yokoyama, Washington University School of Medicine, 660 South Euclid Ave., Box 8045, St. Louis, MO 63110. Phone: 314-362-9075; Fax: 314-362-9257; E-mail: yokoyama@imgate.wustl.edu

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NKG2A heterodimer on human and mouse NK cells (13–17). Despite these structural differences, all inhibitory receptors appear to mediate their functional effects through the same mechanism involving ligand-induced tyrosine phosphorylation of cytoplasmic sequences, termed immunoreceptor tyrosine-based inhibitory motifs (ITIMs)[1] (8). Phosphorylated ITIMs then recruit tyrosine phosphatases, such as SH2 domain–bearing protein tyrosine phosphatase (SHP-1), that presumably dephosphorylate molecules in the activation cascade.

Much less is known about the nature of putative NK cell activation receptors. Whereas NK cells express Fcγ-RIII (CD16) that mediates antibody-dependent cellular cytotoxicity (18), CD16 appears not to be directly responsible for most instances of NK cell activation by targets (5). Instead, leading candidates for target-specific NK cell activation receptors are molecules that are homologous to both structural types of inhibitory receptors but do not contain cytoplasmic ITIMs (5). These NK cell molecules also contain charged residues in their transmembrane domains that facilitate interaction with associated immunoreceptor tyrosine-based activation motif (ITAM)-containing signaling chains such as DAP-12 and FcϵRIγ (19, 20). As such, human killer activation receptors have been described that are homologous to the inhibitory killer cell Ig-like receptors (KIRs) and can costimulate NK cell activation when cross-linked by mAbs or exposure to target ligands (21, 22).

The C-type lectin-like receptors NKR-P1, CD94/NKG2C, Ly-49D, and Ly-49H have also been implicated in activating NK cells (20, 23–28). Although NKR-P1 appears to be expressed on all murine NK cells, it is not clear whether the additional lectin-like receptors can be expressed together on the same cell or whether expression of these molecules is regulated in some way that excludes or modifies coexpression. Whereas the ligands for NKR-P1 molecules are not clearly understood, cross-linking of rat NKR-P1 or mouse NKR-P1C (equivalent to the NK1.1 antigen) stimulates NK cell activation (29, 30). The human inhibitory CD94/NKG2A and activation CD94/NKG2C heterodimeric receptors recognize HLA-E ligands, and orthologues of these receptors recognize Qa-1 in mice (31, 32). As yet, the function of the CD94/NKG2C activation receptor has not been well studied on primary NK cells, although transfection and cross-linking of chimeric molecules containing the transmembrane and cytoplasmic domain of CD94/NKG2C can activate NK tumor cells (27). mAb cross-linking of Ly-49D on sorted NK cells as well as on transfected cell lines can induce lysis of target cells (28). Furthermore, Ly-49D is responsible for directly activating mouse NK cells to kill Chinese hamster ovary (CHO) cells, presumably by recognition of a target ligand, and Ly-49D specifically recognizes the MHC class I molecule H-2D^d on mouse targets (33, 34). However, much less is known about the properties of the Ly-49H molecule.

The Ly-49H cDNA was first identified by Takei and coworkers as a member of the Ly-49 multigene family, which now encompasses cDNAs or genes encoding the Ly-49A through Ly-49J molecules (35, 36). Like Ly-49A, most members of the Ly-49 family have ITIMs and several are known to have functions as MHC class I–specific inhibitory receptors (37). Use of serological reagents specific for certain Ly-49 family members has shown that Ly-49 family members are expressed in C57BL/6 (B6) mice by subsets of NK1.1$^+$CD3$^-$ NK cells and NK1.1$^+$CD3$^+$ cells, i.e., NKT cells (13, 38). Many of these cells express more than one Ly-49 molecule. By contrast, and with marked similarity to Ly-49D, Ly-49H lacks a cytoplasmic ITIM and contains a charged residue (arginine) in the transmembrane domain, suggesting an activating function. In support of this role, myc-tagged Ly-49H was shown to associate with DAP-12 in transfected Ba/F3 cells and to initiate early events in cellular activation after cross-linking with anti-myc antibody (39). However, the role of Ly-49H on primary NK cells remains to be established. It is not clear which NK cell effector functions Ly-49H could potentially activate or regulate, or whether stimulation through Ly-49H alone is sufficient to initiate NK cell activation. Moreover, although Ly-49H is related to the Ly-49D activation receptor and both can couple to DAP-12, it has not been determined if these two Ly-49 molecules mediate similar functions on NK cells. Finally, it is not known if NK cells can simultaneously express both Ly-49D and Ly-49H or if these molecules can be coexpressed with other lectin-like receptors such as the CD94/NKG2 family of molecules.

Detailed analyses of the serologically detectable inhibitory Ly-49 molecules (Ly-49A, Ly-49C/I, and Ly-49G) have demonstrated that each of these molecules is expressed in a developmentally regulated manner (40). These receptors are detectable on a small fraction of murine NK cells in the spleen 1–2 d after birth. Over the next 4–6 wk, NK cells expressing these receptors gradually increase to adult percentages, at which point mature NK cells can express more than one Ly-49 molecule. This coexpression of Ly-49 inhibitory molecules on a single cell appears to be due to a stochastic rather than an ordered process when analyzed by the multiplication rule for independent probabilities (37). That is, the percentage of NK cells expressing two distinct inhibitory Ly-49 molecules can be readily predicted from the product of their frequencies in the total NK cell population. These observations strongly suggest that expression of Ly-49 inhibitory receptors on NK cells is a stochastic process. However, it is not yet known if Ly-49 activation receptors are expressed by a similar mechanism.

In this report, we used a new Ly-49H–specific mAb to evaluate Ly-49H function on primary NK cells and explore its expression. These studies demonstrate that Ly-49H can directly activate primary NK cells, and that NK cells are heterogeneous with respect to expression of activation receptors. Unlike the inhibitory Ly-49 receptors,

[1]*Abbreviations used in this paper:* B6, C57BL/6; CHO, chinese hamster ovary; FCS, fetal calf serum; HRP, horseradish peroxidase; ITIM, immunoreceptor tyrosine-based inhibitory motif; NWNA, nylon wool nonadherent.

Ly-49 activation receptors as well as receptors for tetramers of the MHC class Ib Qa-1 molecule are expressed in a nonstochastic and presumably developmentally regulated manner on NK cells.

Materials and Methods

Mice. B6 and BALB/c mice were purchased from National Cancer Institute (Frederick, MD). All mice were housed in a pathogen-free barrier facility at Washington University.

Cells and Cell Lines. B cell hybridomas were grown in DMEM containing 10% FCS obtained from Harlan, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μM 2-ME at 37°C in 5% CO_2. The murine H-2^b C1498 and EL-4 cell lines were acquired from American Type Culture Collection and grown in RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μM 2-ME. The human 293T cell line, maintained in DMEM plus 10% FCS, was a gift from Dr. John Vaage (University of Oslo, Oslo, Norway). CHO-dhfr$^-$ cells and the CHO-dhfr$^-$ cell line stably expressing Ly-49A were described previously (41). Unseparated splenocytes and nylon wool nonadherent (NWNA) splenocytes were prepared as described previously (30). Neonatal splenocytes were prepared similarly, but were not passaged over nylon wool columns. For IL-2–activated NK cell culture, NWNA splenocytes were cultured in 1,000 U/ml recombinant human IL-2 (Chiron) at 5×10^5 cells/ml for 4 d. Plastic nonadherent cells were then discarded and adherent cells were depleted of T cells by antibody H57-597 (anti–TCR-β), mAb 53-6.72 (anti-CD4), and complement as described (42). The remaining cells were cultured in 1,000 U/ml IL-2 for an additional 4 d and harvested with EDTA (Versene; GIBCO BRL). The resulting IL-2–activated NK cell population was routinely 95–99% NK1.1$^+$ and <10% CD3$^+$.

cDNAs, Expression Constructs, and Transfectants. Ly-49B cDNA was PCR amplified from reverse-transcribed B6 day 7 IL-2–activated NK cell mRNA with specific sense (5′-AGGCCACATTTTAATACAAATCG-3′) and antisense (5′-CTGCTCTGTTAAGTCTGTTG-3′) primers as described previously (43) and sequenced in both directions. The B6 Ly-49C cDNA in the pAX142 expression vector and B6 Ly-49H cDNA in pBluescript KS− vector were gifts from Dr. Fumio Takei (University of British Columbia, Vancouver, Canada). The B6 Ly-49D, Ly-49E, Ly-49F, and Ly-49G2 cDNAs were reported previously (43). The B6 Ly-49I cDNA was a gift from Dr. Vinay Kumar (University of Texas Southwestern, Dallas, Texas). The Ly-49B and Ly-49I cDNAs were expressed using vector pA-puro, provided by Dr. Andrew Chan (Washington University, St. Louis). Ly-49E cDNA was mutated in the 5′ untranslated region by PCR to change a naturally occurring ATG at position 96 (43) to ATA. The Ly-49 cDNAs were ligated into the pHβAP-r-1-neo vector (44) that we modified to include an independent expression cassette of dhfr cDNA under control of an SV40 promoter following the method described in reference 41. The expressed sequence tag encoding murine DAP-12 (EST 242315) was obtained from Genome Systems, sequenced on both strands, and ligated into the eukaryotic expression vector pME-T7, a gift of Dr. John Vaage.

The chimeric Ly-49A/H cDNA was created by excising the NotI-PstI fragment of Ly-49H cDNA in pBluescript (35). This fragment encodes the Ly-49H ectodomain beginning at amino acid residue 88 (cysteine; numbering according to the sequence of the Ly-49H isoform containing the alternatively spliced cytoplasmic amino acid residues VCS [45]) and deletes the membrane-proximal 20 amino acids. This fragment was ligated to the nucleotide 374 PstI site of the Ly-49A cDNA isolated from plasmid pA1.3 (46; see Fig. 1). The resulting chimeric Ly-49A/H cDNA was sequenced on both strands and ligated into vector pA-puro for eukaryotic expression.

Mammalian cells stably expressing Ly-49 molecules were generated using standard electroporation, $Ca_3(PO_4)_2$ transfection, or lipid-mediated transfection methods. CHO cells were transfected with the pA-puro-Ly-49I expression vector together with a pSV2-dhfr expression vector as a selectable marker (44). C1498 cells were transfected with Ly-49B, Ly-49E, or chimeric Ly-49A/H expression plasmids (described above). CHO-dhfr$^-$ cells expressing Ly-49G2 were selected with methotrexate and maintained as described (41). Transient transfections of 293T cells were carried out using lipofectamine (GIBCO BRL) according to the manufacturer's protocol. 48 h after transfection, cells were harvested with Versene (GIBCO BRL) and analyzed by flow cytometry or immunoprecipitation.

Production of Anti–Ly-49H mAb. BALB/c mice were inoculated intraperitoneally with 10^7 B6-derived C1498–Ly-49A/H transfected cells emulsified in TDM-MPL adjuvant (Ribi Immunochemical) and boosted three times at 3-wk intervals. Splenic B cell hybridomas were produced by the Washington University Hybridoma Center from a single BALB/c mouse. Culture supernatants from ~450 hybridomas were screened by flow cytometry for antibody binding to a cell population containing a 1:1 ratio of normal C1498 cells and C1498–Ly-49A/H cells mixed in the same tube. Culture supernatants that specifically shifted only half the cells in the mixture were presumed to contain antibody specific for the Ly-49A/H molecule, whereas culture supernatants positively staining all cells in this mixture were assumed to contain antibodies specific for other B6 strain alloantigens. Hybridoma culture supernatants were also tested at the same time for binding to day 7 IL-2–activated B6 NK cells. This screen resulted in five independent hybridomas that were subcloned twice by limiting dilution. Each of these clones stained an identical percentage of B6 NK cells (described below), and all were IgG1 isotype as determined by ELISA (Southern Biotechnology Associates). One clone, mAb 3D10, was selected for further analysis.

Other Antibodies. Anti-CD3–Cychrome, FITC-4E5 (anti–Ly-49D), FITC-5E6 (anti–Ly-49C/I), and PE–PK-136 (anti-NK1.1) were purchased from PharMingen. Biotin-4D11 (anti–Ly-49G) and biotin-JR9 (anti–Ly-49A) were produced from hybridomas provided by Dr. John Ortaldo (National Cancer Institute, Frederick, MD) and Dr. Jacques Roland (Pasteur Institute, Paris, France), respectively. The 9E10 hybridoma (IgG1 anti–c-myc) was provided by Dr. Andrew Chan. Antibodies were purified from spent cell-free culture supernatants by affinity purification on a protein G–Sepharose or protein A–Sepharose column (Amersham Pharmacia Biotech) using standard methods.

Purified mAbs were biotinylated using *N*-hydroxysuccinimide-biotin (NHS-biotin; Pierce Chemical Co.) according to the manufacturer's instructions. FITC-3D10 was produced with FITC-celite (FITC isomer 1; Calbiochem) as described (47).

To produce a rabbit anti–Ly-49A NH_2 terminus antiserum, a synthetic peptide encoding the first 15 amino acids of Ly-49A with an added COOH-terminal cysteine (MSEQEVTYSMVRFHKC) was coupled to KLH by the maleimidobenzoyl-*N*-hydroxysuccinimide method (47). Rabbit antisera to this immunogen were raised by Antibodies Incorporated according to standard protocols. Antiserum 1298 binds the Ly-49A NH_2 terminus and cross-reacts with the NH_2 termini of Ly-49B, C, D, E, G, H, and I by immunoprecipitation analysis (data not shown).

Cell Surface Biotinylation and Immunoprecipitations. Cell surface biotinylation, cell lysis, and immunoprecipitation were carried out essentially as described (47). Lysates were stored at −70°C if not used immediately. For immunoprecipitations, 1–2 μl of antiserum 1298 or 5 μg of purified mAb was added to a volume of lysate equivalent to 2×10^6 cells with 20 μl of a 50% slurry of protein A–Sepharose (Amersham Pharmacia Biotech). mAb 9E10 (IgG1, anti–c-myc) was used as a control for mAb 3D10 (also IgG1). 5 μg of purified rabbit anti–mouse antibody (Caltag) was included in immunoprecipitations with these IgG1 mAbs to increase binding to protein A–Sepharose. Immunoprecipitates were washed three times in lysis buffer, separated by SDS-PAGE, and transferred to solid membrane (Immobilon; Millipore). Blots were blocked in TBS-T (20 mM Tris, 0.9% wt/vol NaCl, 0.05% Tween 20, pH 7.4) containing 1% BSA and washed in TBS-T alone. Biotinylated proteins were visualized with avidin–horseradish peroxidase (HRP) conjugate and enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech) and photographic film (XK-1; Eastman Kodak Co.).

Redirected Lysis Assay. The redirected lysis assay using ^{51}Cr-labeled human Daudi B cell lymphoma targets was carried out as described (30). Purified antibodies were used at a final concentration of ~2 μg/ml and were added to wells containing target cells immediately before adding effector cells. Percent specific lysis was calculated as described previously (30).

Cytokine Production Assay. Stimulation of IL-2–activated NK cells was performed essentially as described (3). 96-well flat-bottomed tissue culture plates (Falcon; Becton Dickinson) were coated with goat anti–mouse $F(ab')_2$ antibody at 10 μg/ml for ~15 h at room temperature, washed three times, and blocked with 2% BSA in PBS. Triplicate wells were then incubated with anti–Ly-49H mAb, PK136 (anti-NK1.1) positive control mAb, or isotype control mAb 9E10 at 10 μg/ml for 4–8 h, and washed three times. 10^5 IL-2–activated NK cells were plated in each well and incubated at 37°C for 8 h. Culture supernatants were harvested and assayed for GM-CSF and IFN-γ by ELISA (Endogen). In each experiment, ELISA standard curves were derived using recombinant cytokines, permitting quantitation of cytokine concentrations.

Generation of Qa-1^b Tetramers. The bacterial strain expressing the mouse β2-microglobulin gene was supplied by Dr. David Margulies (National Institutes of Health, Bethesda, MD). Bacteria expressing Qa-1^b was provided by M. Salcedo. Soluble MHC complexes were produced as described previously (48, 49). In brief, after isopropyl-β-d-thiogalactopyranoside (IPTG) induction of the bacteria, inclusion bodies were isolated and dissolved in urea. Refolding in vitro was around a Qdm peptide (AMAPRTLLL) synthesized by Macromolecular Resources (Fort Collins, CO). After FPLC purification on a Superdex 75 column (Amersham Pharmacia Biotech), complexes were enzymatically biotinylated using BirA enzyme (Avidity). Free biotin was removed using a monoQ column (Amersham Pharmacia Biotech), and tetramerized with streptavidin-PE (PharMingen) at a 4:1 molar ratio.

Flow Cytometric Analysis. Adherent cells from IL-2–activated NK cell cultures were harvested with Versene (GIBCO BRL), and washed three times in FACS® buffer (HBSS without phenol red, 3% FCS, 0.1% wt/vol NaN_3). To block nonspecific Fc receptor binding, IL-2–activated NK cells and B6 splenocytes were first incubated with mAb 2.4G2 (50) at 5 μg/ml in FACS® buffer for 15 min before addition of antibodies. For antibody staining, 5×10^5 cells were incubated with antibodies at 1–5 μg/ml in 96-well V-bottomed plates (Nalge Nunc International) for 30 min on ice, then washed three times. Secondary reagents avidin-PE, avidin-Cychrome, and avidin-allophycocyanin (PharMingen) were used at optimum final concentrations as determined by titration experiments. During flow cytometry, cells were gated according to forward and side scatter and cell surface marker expression (as indicated in the text). Where indicated, dead cells were excluded by gating with propidium iodide staining (47). Fluorescence data from 10^4 to 10^5 gated events were collected.

Statistical Analysis of Ly-49 Receptors on B6 NK Cells. B6 NWNA splenocytes were analyzed by four-color flow cytometry. At least 10^4 gated NK1.1$^+$CD3$^-$ cells were analyzed for expression of Ly-49H and a second Ly-49 molecule (as indicated in each figure). For example, Ly-49H$^+$A$^+$, Ly-49H$^+$A$^-$, Ly-49H$^-$A$^+$, and Ly-49H$^-$A$^-$ cell populations were determined and association of Ly-49H with Ly-49A was analyzed by the χ^2 test of independence.

Results

Generation of a Cell Line Stably Expressing Chimeric Ly-49A/H Protein. The Ly-49H cDNA predicts a charged residue in the deduced transmembrane domain, suggesting that efficient cell surface expression of Ly-49H requires association with additional molecules such as DAP-12, as described by Lanier and colleagues during the progress of this work (19). To express the extracellular domain of B6 Ly-49H in the absence of any associated molecules, we exploited the fact that the related Ly-49A molecule can be readily expressed at the cell surface. We therefore created a chimeric Ly-49A/H cDNA encoding the cytoplasmic, transmembrane, and first 19 extracellular amino acids of Ly-49A fused to the ectodomain of the B6 allotype of Ly-49H beginning in the predicted Ly-49H stalk region at amino acid residue 88 (Fig. 1 A, and Materials and Methods). This chimeric Ly-49A/H cDNA was placed under control of the chicken β-actin promoter and stably expressed in the murine C1498 cell line. To evaluate expression of the Ly-49A/H molecule, lysates from cell surface biotinylated C1498–Ly-49A/H cells were immunoprecipitated with rabbit polyclonal anti–Ly-49A NH_2 terminus peptide antiserum 1298, and immunoprecipitates were visualized on Western blots with avidin-HRP conjugate (Fig. 1 B). A biotinylated protein band migrating at 85 kD under nonreducing SDS-PAGE was immunoprecipitated from Ly-49A/H transfected cell lysates but not in the absence of antiserum or from untransfected C1498 cells. Under reducing conditions, this band migrated at 45 kD, consistent with the expression of the Ly-49A/H molecule as a disulfide-linked homodimer. Similar detection and migration of Ly-49A protein immunoprecipitated from control EL-4 cell lysates by antiserum 1298 confirmed the antiserum specificity and reducing conditions. A 27-kD protein also detected in this analysis under reducing conditions was nonspecific because it was present in lysates of both C1498 and C1498–Ly-49A/H cells, as well as in mock immunoprecipitations from C1498–Ly-49A/H lysates (Fig. 1 B, lane 2). These results indicated that the Ly-49A/H molecule is expressed as a stable, disulfide-linked cell surface ho-

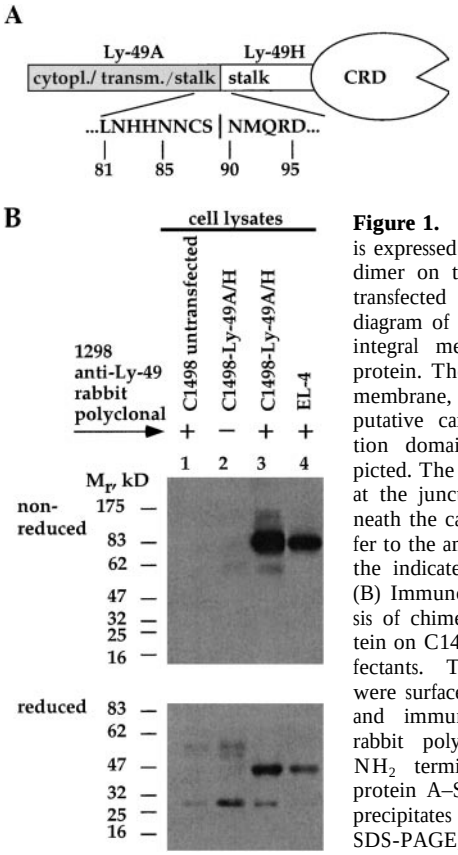

Figure 1. Chimeric Ly-49A/H is expressed as a disulfide-linked dimer on the surface of stably transfected cells. (A) Schematic diagram of the chimeric type II integral membrane Ly-49A/H protein. The cytoplasmic, transmembrane, stalk regions, and the putative carbohydrate recognition domain (CRD) are depicted. The amino acid sequence at the junction is depicted beneath the cartoon. Numbers refer to the amino acid position in the indicated Ly-49 sequences. (B) Immunoprecipitation analysis of chimeric Ly-49A/H protein on C1498 Ly-49A/H transfectants. The indicated cells were surface biotinylated, lysed, and immunoprecipitated with rabbit polyclonal anti-Ly-49A NH_2 terminus antibody, and protein A–Sepharose. Immunoprecipitates were separated by SDS-PAGE, transferred to nylon membrane, and visualized after Western transfer with avidin-HRP conjugate and enhanced chemiluminescence (ECL). Upper gel, non-reducing conditions; lower gel, reducing conditions.

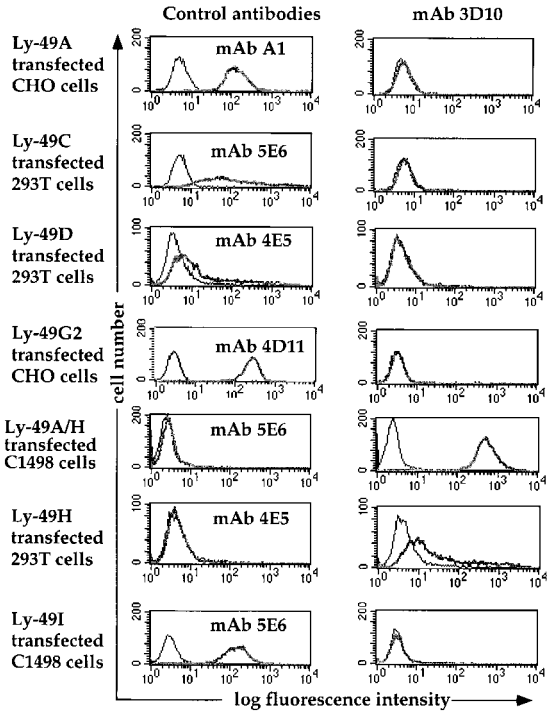

Figure 2. mAb 3D10 specifically binds Ly-49H and does not cross-react with Ly-49A, C, D, G2, or I. Flow cytometric analysis was conducted with cell lines transfected with cDNAs encoding the indicated Ly-49 molecules for binding to mAb 3D10. Expression of each transfected cDNA was verified with control mAbs as indicated (left panels). Dead cells were excluded from the analysis by propidium iodide. mAb 3D10 specifically recognized cells expressing Ly-49H or the chimeric Ly-49A/H molecule, and was unreactive with cells expressing Ly-49A, C, D, G2, or I (right panels). For the Ly-49D and Ly-49H transfections, a cDNA expression construct encoding the murine DAP-12 molecule was cotransfected.

modimer, consistent with the structure of native Ly-49 molecules.

Specificity of mAb 3D10. The C1498–Ly-49A/H transfectant was used as an immunogen to produce BALB/c anti–Ly-49H mAbs, resulting in the generation of candidate mAb 3D10. The specificity of mAb 3D10 for Ly-49 molecules was evaluated by flow cytometric or immunoprecipitation analysis of a panel of cell lines transfected with cDNAs for the B6-derived Ly-49A, B, C, D, E, G2, H, or I molecules. Expression of transfected Ly-49 molecules for which specific antibody was available was confirmed by flow cytometry. Since antibodies specific for Ly-49B and Ly-49E are not available, expression of these transfected molecules was demonstrated by immunoprecipitation with the rabbit anti–Ly-49 NH_2 terminus polyclonal antiserum 1298. In these analyses, we were unable to detect expression of Ly-49F protein with antiserum 1298 that recognizes the identical NH_2-terminal 14 amino acids (the peptide region against which the antibody was raised) as the deduced Ly-49F peptide. Cloning of two independent Ly-49F cDNAs and transfection analysis in three separate cell lines also failed to result in surface expression. Although the Ly-49F cDNA contains an open reading frame, it is not currently clear whether the Ly-49F cDNA we reported previously (43) encodes a mature Ly-49 molecule, requires additional molecules for expression, or represents a pseudogene or sterile transcript. Therefore, we evaluated the reactivity of mAb 3D10 for all other detectable, known Ly-49 molecules.

In flow cytometric analysis, mAb 3D10 specifically stained the C1498–Ly-49A/H cell line and 293T cells transiently expressing Ly-49H (Fig. 2), the latter demonstrating that mAb 3D10 recognizes the full-length B6 Ly-49H molecule. mAb 3D10 was unreactive with transfectants expressing Ly-49A, C, D, and G2. Under conditions of very high Ly-49I expression in transiently transfected cells, or high concentrations of mAb 3D10 (50 μg/ml), we observed weak cross-reactivity of mAb 3D10 for Ly-49I (data not shown). However, CHO cells stably overexpressing Ly-49I at levels greater than Ly-49I expression on B6 splenocytes were unreactive with mAb 3D10 in flow cytometry under conditions used for subsequent analyses (Fig. 2). Similarly, no reactivity was detected to lysates from Ly-49B and Ly-49E transfected cells in immunoprecipitation analysis (Fig. 3). Antiserum 1298 specifically immunoprecipitated protein migrating at ~75-kD from these Ly-49B and Ly-49E transfected cell lysates as well as from Ly-49A–expressing control

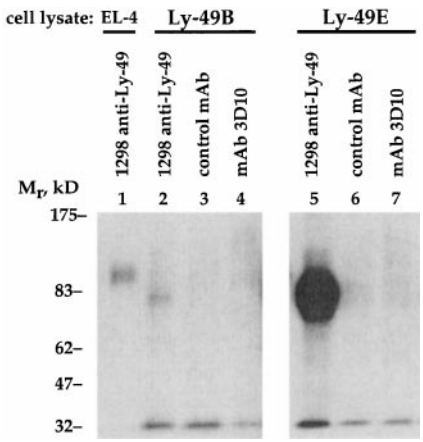

Figure 3. Immunoprecipitation analysis of mAb 3D10 with lysates from Ly-49B transfected cells and Ly-49E transfected cells. Cells were surface labeled with biotin, lysed, and immunoprecipitated with anti–Ly-49A rabbit antiserum 1298 (lanes 1, 2, and 5), isotype control mAb (lanes 3 and 6), or mAb 3D10 (lanes 4 and 7) as described in the legend to Fig. 1.

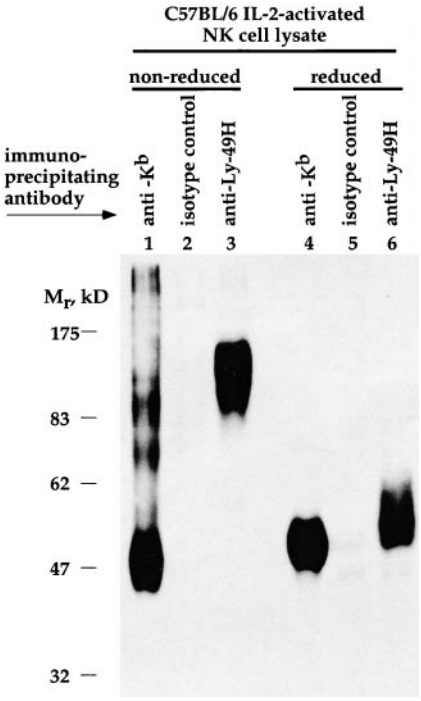

Figure 4. Ly-49H is expressed on NK cells as a disulfide-linked dimer. Lysates from cell-surface biotinylated B6 IL-2–activated NK cells were immunoprecipitated with the indicated antibodies as described in the legend to Fig. 1. mAb 3D10 specifically immunoprecipitates a disulfide-linked dimer that migrates at 110 kD under nonreducing conditions and 55 kD upon reduction.

EL-4 cell lysates (Fig. 3), confirming expression of each of these Ly-49 proteins. These results indicate that mAb 3D10 specifically recognizes Ly-49H and, under the conditions used here, does not cross-react with any other known, expressible Ly-49 proteins.

Although mouse DAP-12 has been reported to be required for expression of Ly-49H at the cell surface (39), in our analysis using 293T cell transient transfection assays, cotransfection of mouse DAP-12 was not necessary for detectable cell surface expression of Ly-49H by mAb 3D10 staining of Ly-49H transfected cells (data not shown). However, cotransfection of mouse DAP-12 cDNA augmented expression of Ly-49D and Ly-49H molecules approximately twofold in 293T cells and was therefore included in the Ly-49H (and Ly-49D) transfection assays presented here (Fig. 2). Taken together, these results indicate that mAb 3D10 recognizes a mouse DAP-12–independent epitope on Ly-49H.

To examine the expression of Ly-49H on primary NK cells, mAb 3D10 was used in further immunoprecipitation analysis of IL-2–activated NK cells (Fig. 4). mAb 3D10 specifically immunoprecipitated a protein species that migrated as a single band at ~110 kD under nonreducing conditions. Upon reduction, this protein band shifted to a single species migrating at ~55 kD, suggesting that Ly-49H is expressed as a disulfide-linked homodimer on the surface of B6 IL-2–activated NK cells. This expression pattern and quaternary structure are consistent with that observed for all Ly-49 molecules examined to date (5). Inasmuch as only one molecular species was observed, these data further suggest that mAb 3D10 is immunospecific for Ly-49H.

Anti–Ly-49H Induces Redirected Lysis. We next examined the ability of the anti–Ly-49H mAb 3D10 to activate primary NK cells to kill the human $Fc\gamma R^+$ Daudi B lymphoma cell line in the redirected lysis assay (Fig. 5). mAb 3D10 specifically induced killing of Daudi cells in a dose-dependent manner. By contrast, addition of control mAbs A1 (anti–Ly-49A) or 9E10 (IgG1 isotype control) did not increase specific lysis of Daudi target cells when compared with medium alone. Interestingly, the addition of positive

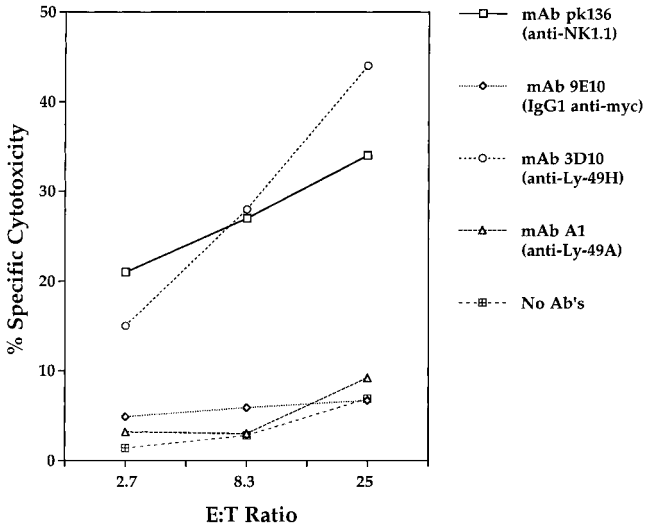

Figure 5. Anti–Ly-49H mAb redirects lysis of human Daudi cells. B6 IL-2–activated NK cells were incubated for 4 h at 37°C with 10^4 ^{51}Cr-labeled target cells at E/T ratios of 2.7, 8.3, and 25 in the presence of the antibodies indicated.

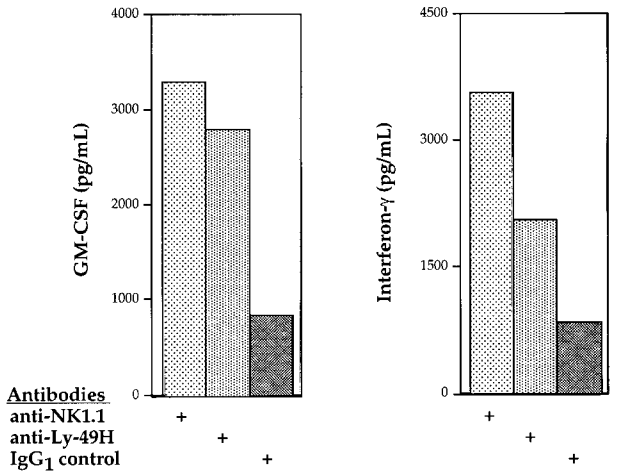

Figure 6. Anti–Ly-49H induces cytokine production by B6 IL-2–activated NK cells. Plate-bound anti-NK1.1 (mAb PK136), anti–Ly-49H (mAb 3D10), or isotype control mAb were used to stimulate IL-2–activated NK cells for 8 h in 96-well flat-bottomed plates. Culture supernatants were assayed by ELISA for levels of GM-CSF and IFN-γ. Stimulation of IL-2–activated NK cells by anti–Ly-49H mAb induces production of GM-CSF (left) and IFN-γ (right) by IL-2–activated NK cells. Positive control anti-NK1.1 mAb also induces production of GM-CSF and IFN-γ, whereas minimal induction of cytokine expression was observed in wells containing control mAb.

control mAb PK136 (anti-NK1.1) augmented lysis of Daudi cells in a manner similar to the addition of mAb 3D10, even though $3D10^+$ cells constitute a subpopulation of NK cells whereas NK1.1 is expressed on all NK cells (see below and Discussion). These data show that cross-linking of Ly-49H is sufficient to activate NK cell cytotoxicity, demonstrating that Ly-49H is an NK cell activation receptor on primary NK cells.

Direct Ly-49H Cross-Linking Activates Cytokine Production. As a further test of the capacity of Ly-49H to activate functions of primary NK cells, we examined the effect of cross-linking Ly-49H on the production of cytokines. Plate-bound anti–Ly-49H augmented the production of GM-CSF and IFN-γ by IL-2–activated NK cells, similar to the effect of anti-NK1.1 mAb (Fig. 6). The lower levels of cytokines produced by Ly-49H cross-linking compared with NK1.1 cross-linking are consistent with the expression of Ly-49H on a subpopulation of NK cells (see below), whereas the NK1.1 molecule is expressed on >98% of NK cells in this particular experiment (data not shown, and see Discussion). The effect of plate-bound anti–Ly-49H mAb was specific, as the amount of cytokines produced in cultures containing anti–Ly-49H mAb was also markedly higher than in culture wells containing irrelevant IgG1 control antibody. These results demonstrate that cross-linking of Ly-49H can activate primary NK cells to produce the cytokines GM-CSF and IFN-γ.

Expression of Ly-49H on B6 NK Cells. Flow cytometric analysis using mAb 3D10 with freshly isolated unfractionated splenocytes indicated that approximately half of $NK1.1^+CD3^-$ cells expressed Ly-49H (data not shown). Similarly, NWNA splenocytes revealed that ~56% of $NK1.1^+CD3^-$cells were reactive (Fig. 7 A, top left; and Table I). Expression of Ly-49A, Ly-49C/I, Ly-49D, and Ly-49G on this population was consistent with previous reports (Table I; reference 37). After 7 d of culture in IL-2, the percentage of NK cells expressing Ly-49H was maintained at 52% (Fig. 7 A, top right; and Table I), demonstrating that the total fraction of NK cells expressing Ly-49H is stable under these culture conditions.

Ly-49H was not detectable on $CD3^+$ cells in freshly isolated splenocytes (data not shown), in preparations of fresh NWNA splenocytes, or in cultured IL-2–activated NWNA cells that had not been depleted of $CD3^+$ cells (Fig. 7 A, bottom left and right, respectively). Examination of Ly-49D expression using the 4E5 mAb gave similar re-

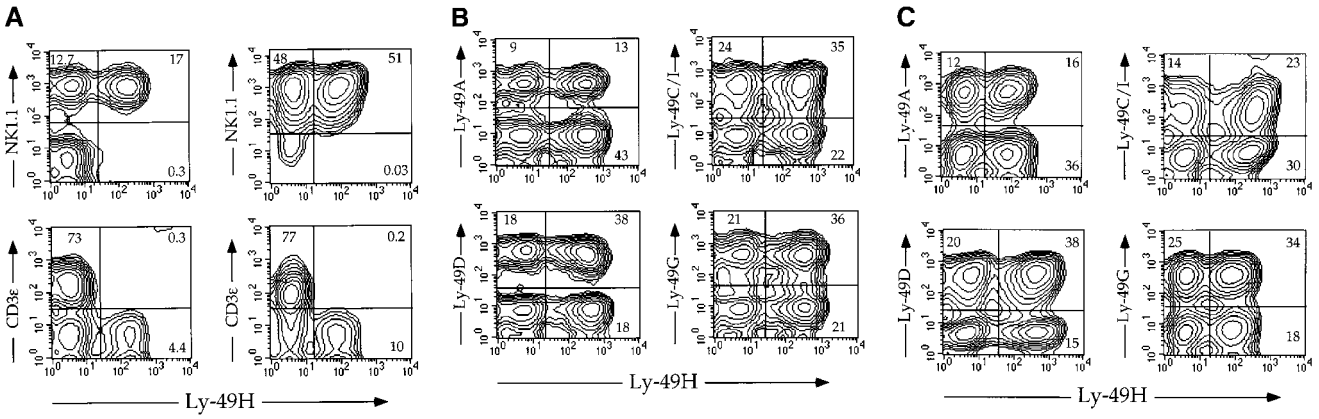

Figure 7. Flow cytometric analysis of Ly-49H expression. (A) Ly-49H vs. NK1.1 or CD3ε expression on freshly isolated NWNA splenocytes (left side) or $CD3^-$ IL-2–activated NK cells (right side) (the bottom right panel contains IL-2–activated NK cells that were not depleted of $CD3^+$ cells). (B) Coexpression of Ly-49H with Ly-49A (mAb A1), Ly-49D (mAb 4E5), Ly-49C/I (mAb 5E6), and Ly-49G (mAb 4D11) on gated $NK1.1^+CD3^-$ freshly isolated NWNA splenocytes. (C) Coexpression of Ly-49H with Ly-49A, D, C/I, and G on $CD3^-$ IL-2–activated NK cells. For each contour plot, the percentage of cells in each quadrant is indicted in the appropriate quadrant.

Table I. *Distribution of Ly-49 Molecules on NK Cells*

	Ly-49A	Ly-49C/I	Ly-49D	Ly-49G	Ly-49H	Total Ly-49D and Ly-49H
	%	%	%	%	%	%
Splenic NK cells	22 ± 0.1	57 ± 3.8	57 ± 1.1	57 ± 2.4	56 ± 2.1	75 ± 1.7
IL-2–activated NK cells	27 ± 0.5	35 ± 3.3	59 ± 1.6	57 ± 2.7	52 ± 0.2	NA
Ly-49H$^+$ splenocytes	23 ± 0.1	62 ± 1.6	68 ± 1.3	62 ± 2.8	NA	NA
Ly-49H$^-$ splenocytes	21 ± 0.9	54 ± 2.5	40 ± 0.3	50 ± 2.5	NA	NA
Qa-1 receptor$^+$ IL-2–activated NK cells	23 ± 3.1	43 ± 5.2	38 ± 5.9	48 ± 0.9	45 ± 18	54 ± 6.7
Qa-1 receptor$^-$ IL-2–activated NK cells	25 ± 3.9	55 ± 4.8	72 ± 6.2	61 ± 2.1	67 ± 16*	87 ± 3.5

Mean percentages ± SD from three independent cell preparations and analyses of NK1.1$^+$CD3$^-$ NWNA splenocytes or day 7 NK1.1$^+$CD3$^-$ IL-2–activated NK cells, as indicated. Differences between Ly-49H$^+$ and Ly-49H$^-$ NK cells are significant by χ^2 test of independence, $P < 0.025$. NA, not applicable.

*In a single experiment, Ly-49H expression was lower than generally observed, but the average difference between Ly-49H expression on Qa-1$^+$ and Qa-1$^-$ cells was 22 ± 3.8%.

sults (data not shown), demonstrating that Ly-49D is also absent on CD3$^+$ cells in these cultures. These observations indicate that both Ly-49H and Ly-49D are NK cell–specific receptors, unlike the inhibitory Ly-49A, Ly-49C/I, and Ly-49G receptors, which are also expressed on CD3$^+$ cells (usually NK1.1$^+$CD3$^+$) (51).

We also analyzed the coexpression of other Ly-49 family members with Ly-49H on NK cells. Ly-49H is coexpressed on the Ly-49A, Ly-49C/I, and Ly-49G inhibitory receptor subsets on fresh NWNA CD3$^-$ spleen cells (Fig. 7 B, and Table I). After 7 d of culture in IL-2, the coexpression of Ly-49H with each of the serologically detectable Ly-49 receptors changed minimally (Fig. 7 C), further supporting the conclusion that Ly-49H expression on NK cells is stable after IL-2 activation. Furthermore, with respect to Ly-49 activation receptors, flow cytometric analysis of total Ly-49D and Ly-49H expression on B6 NK1.1$^+$CD3$^-$ NWNA cells using mAbs 3D10 (anti–Ly-49H) and 4E5 (anti–Ly-49D) together indicated that 75% of cells express either Ly-49D, Ly-49H, or both Ly-49 activation receptors (Table I).

Careful studies of the serologically detectable Ly-49 inhibitory receptors have previously shown that the percentage of NK cells expressing two Ly-49 inhibitory receptors can be approximated by the product of the individual frequencies of each receptor in the total NK cell population, termed the product rule for independent events (37). A parallel approach to this analysis is to separate total NK cells into Ly-49H$^+$ and Ly-49H$^-$ subsets and measure the expression of a second Ly-49 molecule on each subset. This approach also permits statistical analysis by the χ^2 test of independence. We analyzed gated NK1.1$^+$CD3$^-$ NWNA splenocytes according to Ly-49H and Ly-49A expression, generating a dot plot of four NK cell subsets that were Ly-49H$^+$A$^+$, Ly-49H$^+$A$^-$, Ly-49H$^-$A$^+$, and Ly-49H$^-$A$^-$. This analysis determined that Ly-49A is expressed on 23% of Ly-49H$^+$ cells and on 21% of Ly-49H$^-$ cells, suggesting that expression of Ly-49A is independent of Ly-49H on NK cells (Table I), compatible with a stochastic model for expression of Ly-49 inhibitory receptors.

On the other hand, when this method was applied to coexpression of Ly-49C/I, Ly-49D, or Ly-49G with Ly-49H, these molecules were expressed on a greater percentage of Ly-49H$^+$ cells than on Ly-49H$^-$ cells (Table I). The Ly-49C/I proteins were expressed on 62% of Ly-49H$^+$ cells and 54% of Ly-49H$^-$ cells, and Ly-49G was also expressed on 62% of Ly-49H$^+$ cells and 50% of Ly-49H$^-$ cells. Most strikingly, Ly-49D was preferentially expressed on 68% of Ly-49H$^+$ cells, whereas only 40% of Ly-49H$^-$ cells express Ly-49D (Table I). Taken together, these data suggest that the stochastic expression of Ly-49 inhibitory receptors also appears to apply to coexpression of Ly-49A and Ly-49H on NK cells. By contrast, there were small but consistent increases in the NK cell expression of the Ly-49C/I and Ly-49G proteins with Ly-49H, and there was a marked increase in Ly-49D expression on Ly-49H$^+$ NK cells compared with Ly-49H$^-$ NK cells, suggesting that control of Ly-49H (and Ly-49D) expression may not be explained by a stochastic model.

Expression of Ly-49 Activation and Inhibitory Molecules with Qa-1 Receptors. Receptors for the MHC class Ib molecule Qa-1 on murine NK cells have previously been shown to be members of the lectin-like NKG2/CD94 family (52). Recombinant Qa-1 tetramers containing bound Qdm peptide specifically bind the heterodimeric CD94/NKG2A, CD94/NKG2C, and the CD94/NKG2E complexes (52). Since we observed preferential coexpression of Ly-49 activation receptors on a subset of NK cells, we used tetramers of Qa-1/Qdm peptide complexes to compare expression of CD94/NKG2 molecules with Ly-49 activation and inhibitory molecules. Flow cytometric analysis of each serologically detectable Ly-49 molecule with Qa-1 receptors on

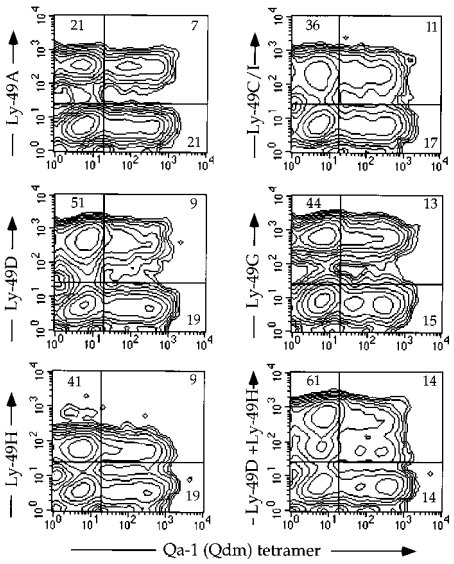

Figure 8. Flow cytometric analysis of Ly-49 activation and inhibitory molecule expression with Qa-1 receptors on IL-2–activated NK cells. Coexpression of tetrameric recombinant Qa-1 folded with Qdm peptide with Ly-49A (mAb A1), Ly-49C/I (mAb 5E6), Ly-49G (mAb 4D11), Ly-49D (mAb 4E5), Ly-49H (mAb 3D10), and both Ly-49D and Ly-49H. For each contour plot, the percentage of cells in each quadrant is indicated in the appropriate quadrant.

$NK1.1^+CD3^-$ IL-2–activated NK cells revealed Ly-49 expression on both Qa-1 $receptor^+$ and Qa-1 $receptor^-$ NK cell subsets (Fig. 8, and Table I). Ly-49A appears to be similarly distributed on both Qa-1 $receptor^+$ and Qa-1 $receptor^-$ NK cell subsets, whereas Ly-49C/I and Ly-49G expression is moderately diminished on Qa-1 $receptor^+$ cells compared with cells lacking expression of Qa-1 receptors. Thus, Ly-49 A expression and Qa-1 receptor expression appear to be independent of each other, but expression of Ly-49C/I and Ly-49G may be moderately influenced by Qa-1 receptor expression or vice versa.

By contrast, the Ly-49D and Ly-49H activation molecules are expressed on a markedly smaller fraction of the Qa-1 $receptor^+$ NK cell subset than the Qa-1 $receptor^-$ subset (Fig. 8, and Table I). This preferential distribution of Ly-49 activation receptors on Qa-1 $receptor^-$ cells is similar to the preferential coexpression of Ly-49D and Ly-49H on an NK cell subset, indicating that NK cells may undergo developmental steps that partially favor or segregate certain NK cell receptor phenotypes.

Ly-49D and Ly-49H Expression on Developing $NK1.1^+$ Splenocytes. Given the observed coexpression of the Ly-49D and Ly-49H activation molecules with inhibitory Ly-49 molecules, we sought to determine how these molecules are expressed in ontogeny on $NK1.1^+$ splenocytes with respect to the inhibitory Ly-49A, C/I, and G molecules. In flow cytometric analysis, Ly-49D and Ly-49H were not detectable on day 17 fetal thymocytes or fetal liver (data not shown). However, Ly-49D and Ly-49H were detected on 2.6 and 1.4% of $NK1.1^+$ splenocytes, respectively, on postnatal day 3, compared with 5.3% of $NK1.1^+$ splenocytes observed to express the Ly-49G inhibitory receptor (Fig. 9), consistent with previous studies of Ly-49G (40). The $Ly\text{-}49D^+$ subset of $NK1.1^+$ cells was consistently larger than the $Ly\text{-}49H^+$ subset, particularly from days 3–21 postpartum (Fig. 9). These observations suggest that Ly-49D is expressed shortly before Ly-49H in this interval of $NK1.1^+$ cell development in the spleen. In addition, the Ly-49D and Ly-49H activation receptors took longer to reach near-adult percentages on $NK1.1^+$ cells than the inhibitory receptors, especially in the first 21 d postpartum (Fig. 9). After this point, the percentage of cells expressing Ly-49D and Ly-49H continues to increase, whereas the expression of the inhibitory receptors reaches a plateau, resulting in a greater fraction of $NK1.1^+$ splenocytes expressing Ly-49D or Ly-49H at day 50 postpartum than any one of the inhibitory receptors studied. Taken together, these results suggest that Ly-49D and Ly-49H receptor expression on $NK1.1^+$ cells lags behind Ly-49G,

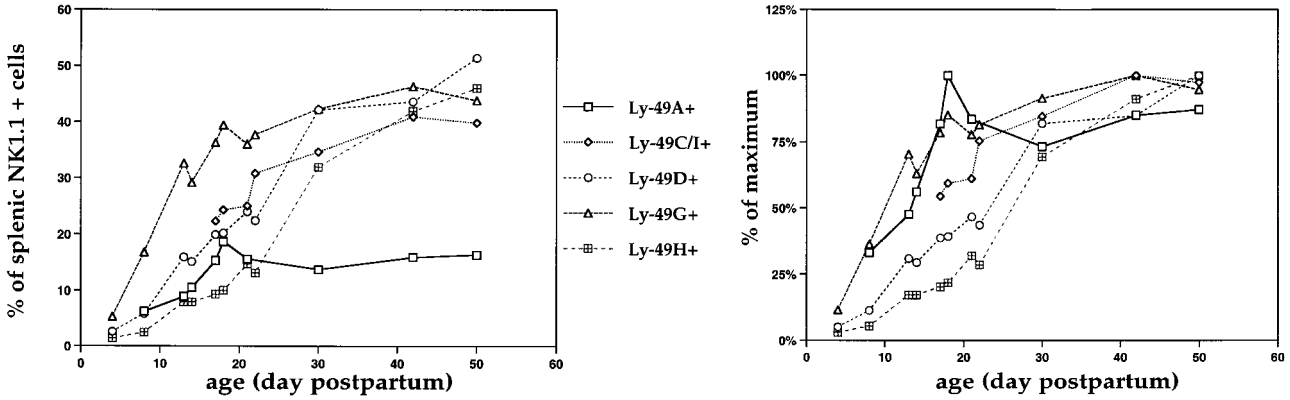

Figure 9. Development of Ly-49 activation and inhibitory receptor expression on $NK1.1^+$ splenocytes. Single cell suspensions of splenocytes were stained with anti-NK1.1 (mAb PK136) and the indicated antibodies and analyzed by flow cytometry for expression of the Ly-49A, Ly-49C/I, Ly-49D, Ly-49G, and Ly-49H molecules. Left, percentage of splenic $NK1.1^+$ cells expressing the indicated Ly-49 molecule as a function of time after birth. Right, the same data set but plotted as the percentage of maximum expression observed for each Ly-49 molecule as a function of time after birth.

Ly-49C/I, and Ly-49A inhibitory receptor expression in the postpartum period.

Discussion

An activating role for the Ly-49H molecule had been previously suggested by studies of Ba/F3 pro-B cell transfectants in vitro, showing that an epitope-tagged Ly-49H molecule could induce initial biochemical events associated with cellular activation (39). By producing an mAb that specifically binds the Ly-49H molecule and does not cross-react with other Ly-49 family members, we have shown directly that cross-linking of Ly-49H on B6 IL-2–activated NK cells is sufficient to induce NK cell cytotoxicity and production of the immunoregulatory cytokines IFN-γ and GM-CSF.

Other NK cell receptors, such as the CD16, NK1.1, murine Ly-49D, and human KAR molecules, can induce NK cell cytotoxicity or cytokine production, indicating that NK cells may use multiple different receptors to achieve an activated state (20, 25–28). The simultaneous expression of multiple activation receptors by individual NK cells is strikingly different from the expression of a single rearranged TCR on an individual T cell that is exquisitely specific for cognate antigen in the context of MHC molecules. NK cell expression of numerous different activation NK cell receptors, presumably specific for distinct ligands, is probably relevant to the role of NK cells in early, innate immunity (Fig. 10). Despite a smaller total population than T lymphocytes, large numbers of NK cells need to be triggered immediately without clonal expansion for significant effector responses. This type of "amplification" would be possible if many NK cells express the same receptor and if an individual NK cell expresses multiple receptors that could respond to different stimuli. By contrast, activation and expansion of appropriate antigen-specific T cell clones require several days. Hence, detailed understanding of NK cell receptors, such as Ly-49H, and their ligands should provide physiological insight into the role of NK cells in innate immunity.

Some of the structurally distinct NK cell activation receptors apparently transduce signals through the same signaling chain. For example, both Ly-49D and Ly-49H can transduce extracellular signals via DAP-12 (39). Since we demonstrate that an individual NK cell can express both Ly-49D and Ly-49H, the Ly-49D$^+$H$^+$ NK cell may be triggered by the ligands for either Ly-49D or Ly-49H with potentially similar outcomes. Importantly, under conditions in which cross-linking of either Ly-49D or Ly-49H alone is insufficient to activate an NK cell, it is conceivable that simultaneous cross-linking of both receptors could produce a synergistic effect resulting in NK cell activation. The importance of this synergistic signaling hypothesis is that a target cell could trigger an individual NK cell by expression of either high levels of a single ligand for any expressed NK cell receptor or low levels of ligands for multiple NK cell receptors. Further analysis of these hypotheses will permit detailed evaluation of NK cell function.

The observation that cross-linking of Ly-49H can activate NK cell cytotoxicity and cytokine production in vitro suggests that NK cells may use the Ly-49H receptor to detect infected or damaged cells in vivo, presumably by signaling through the associated DAP-12 molecule. We observed similar levels of cytotoxicity induced by cross-linking Ly-49H compared with cross-linking NK1.1, whereas NK cells stimulated through Ly-49H produced less IFN-γ and GM-CSF than NK1.1-stimulated cells. One explanation for these differences is that in the redirected lysis assay, mAb 3D10 (IgG1 isotype) binds the human Daudi cell line FcRs with higher affinity than PK136 (IgG2a isotype) (53), resulting in a more efficient induction of cytotoxicity. By contrast, stimulation of NK cells through NK1.1 and the associated FcR common γ chain could be more effective at inducing cytokine release than stimulation through DAP-

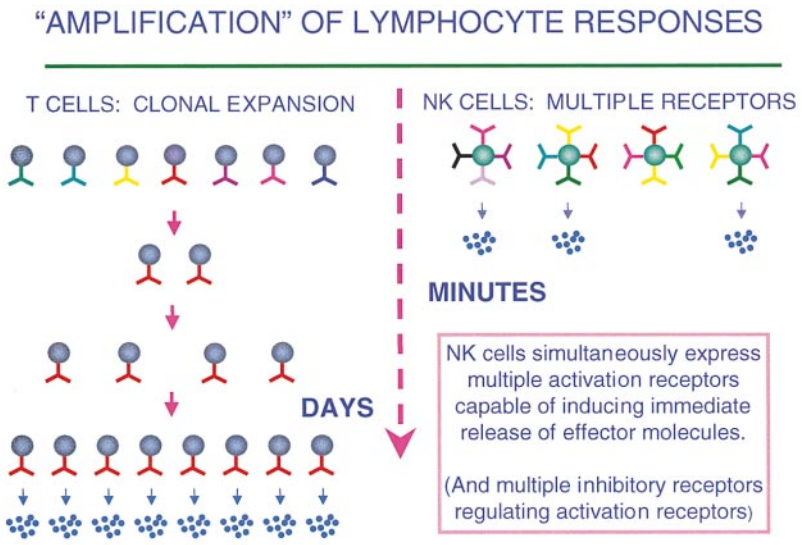

Figure 10. A model contrasting amplification of effector cell responses between T and NK cells. Expression of NK cell activation receptors on overlapping subsets could increase the repertoire of activation receptors expressed by a single NK cell. In contrast to T lymphocytes that require days to clonally expand antigen-specific cells into an effective population, NK cells respond to target cell ligands immediately. In the model shown here, expression of NK cell activation receptors on overlapping subsets could potentially increase the range of ligands a single NK cell could recognize, thereby activating a larger fraction of the NK cell population in response to ligand. If NK cell activation receptors were expressed exclusively of each other, the number of NK cells that could respond to target cell expression of ligands would be reduced.

12–linked Ly-49H. It is also possible that in the cytokine assay, the different amounts of cytokines produced are due to NK1.1 expression on essentially all cells compared with Ly-49H expression on approximately half the NK cells. Finally, our studies using antibody cross-linking of Ly-49H cannot totally exclude inadvertent cross-linking of other molecules such as CD16 in the cytokine production assay we carried out. However, the specific induction of cytokine production by addition of Ly-49H mAb but not by control mAb indicates that even if Ly-49H synergizes with a second NK cell receptor in this assay, Ly-49H nevertheless functions to activate NK cells.

Our analysis of Ly-49 expression on NK cell subsets describes a wide range of NK cell phenotypes with respect to Ly-49. The current understanding of NK cells does not immediately suggest a unified mechanism responsible for this diverse distribution. Previous studies of Ly-49 inhibitory receptor expression on NK cell subsets have demonstrated that expression of these molecules on the same NK cell appears to be stochastic rather than a regulated process (40). The data we present here demonstrate that the fraction of Ly-49H$^+$ NK cells expressing Ly-49A is essentially the same as the fraction of Ly-49H$^-$ NK cells expressing Ly-49A, implying that Ly-49A expression is not influenced by Ly-49H expression and vice versa. This relationship also appears to occur between Ly-49A and expression of Qa-1 receptors. Therefore, these data suggest that the coexpression of Ly-49H with the Ly-49A inhibitory receptor and the coexpression of Qa-1 receptors with Ly-49A are also stochastic, consistent with previous studies of the Ly-49 inhibitory receptors (37).

By contrast, our observations of Ly-49H expression with other Ly-49 receptors are not readily explained by a stochastic mechanism. We observed a modest but consistent augmentation of expression of the Ly-49C/I and Ly-49G receptors on Ly-49H$^+$ cells compared with Ly-49H$^-$ cells. This same increase in Ly-49C/I and Ly-49G expression was evident on Qa-1 tetramer$^-$ cells compared with Qa-1 tetramer$^+$ cells. These differences in Ly-49C/I and Ly-49G expression are statistically significant when analyzed by the χ^2 test of independence. However, we favor the interpretation that these differences may not be biologically meaningful in the functional activity of Ly-49H$^+$ versus Ly-49H$^-$ NK cell subsets or Qa-1 tetramer$^+$ and Qa-1 tetramer$^-$ subsets. It is possible that these slight disparities in Ly-49C/I and Ly-49G expression arise from the order in which Ly-49 genes are transcribed during NK cell ontogeny, and do not necessarily reflect a later selection for specific Ly-49 inhibitory receptors to be coexpressed with Ly-49H or in the absence of receptors for Qa-1.

A more pronounced difference was apparent in the distribution of the Ly-49D activation receptor with Ly-49H. The expression of Ly-49D on 57% of NK1.1$^+$CD3$^-$ cells and the observation that 68% of Ly-49H$^+$ NK cells express Ly-49D suggest a positive association between these two activation receptors. Expression of Ly-49D on only 40% of Ly-49H$^-$ NK cells further implies that the lack of Ly-49H expression on a cell correlates with the lack of Ly-49D expression. Similarly, both Ly-49D and Ly-49H expression was augmented on NK cells that lacked expression of receptors for Qa-1. Furthermore, 87% of NK cells that did not bind Qa-1 tetramers expressed either Ly-49D or Ly-49H compared with 54% of NK cells that expressed receptors for Qa-1. These results indicate that a mechanism exists in B6 mice that selects coexpression of Ly-49D and Ly-49H on an NK cell and appears to preferentially express Ly-49D and Ly-49H on NK cells that lack receptors for Qa-1. Alternatively, regulation could be by inhibition of Ly-49D expression on a Ly-49H$^-$ cell, or inhibition of Ly-49 activation molecules on cells expressing receptors for Qa-1. One explanation for these findings is that the expression of the DAP-12 molecule may be regulated in NK cells. Superimposed on this regulation, the expression of either Ly-49D, Ly-49H, or both is obviously favored on a DAP-12$^+$ NK cell, whereas lack of DAP-12 expression in NK cells would presumably not permit Ly-49D and Ly-49H expression. If this mechanism was operative in even a fraction of NK cells, this might result in the skewing observed here. Alternatively, the total number of DAP-12–linked receptors on a given NK cell could be regulated such that it is maintained within a narrow range. NK cells might therefore have the potential to express a diverse repertoire of DAP-12–associated receptors, including Ly-49D, Ly-49H, or CD94/NKG2C. Once a certain threshold of DAP-12–mediated signaling was achieved during NK cell ontogeny, further expression of activation receptors would be turned off. This model would therefore predict that Ly-49D$^+$ or Ly-49H$^+$ cells are more likely to express additional DAP-12–associated receptors such as CD94/NKG2C compared with Ly-49D$^+$H$^+$ NK cells. Many other mechanisms are conceivable that could act at any of several stages during NK cell ontogeny. Importantly, the notable result is that a B6 NK cell that expresses one Ly-49 activation receptor is more likely to express a second Ly-49 activation receptor and less likely to express receptors for the MHC class Ib Qa-1 molecule.

A recent report using single cell PCR to measure Ly-49 expression on individual NK cells reported that 16% of a total of 62 NK cells analyzed expressed Ly-49H, and that 45% express Ly-49D (54). By contrast to the results reported here, in which we find 56% Ly-49H$^+$ NK cells and 57% Ly-49D$^+$ NK cells in the spleen by flow cytometry, the PCR analysis also found no positive association with Ly-49D and Ly-49H on a single NK cell. One explanation for this discrepancy is that RNA expression may not correlate directly with protein expression. Furthermore, determining the accuracy of single cell PCR detection of a given RNA transcript is difficult due to sensitivity and specificity issues. We conclude that while the single cell PCR analysis of individual NK cells is a valuable contribution to our understanding of the total Ly-49 repertoire, the flow cytometric analysis we present here is a more reliable determination of Ly-49D and Ly-49H on splenic NK cells.

Ly-49 inhibitory receptors are currently thought to maintain tolerance to self by binding to MHC molecules and preventing NK cell lysis (37). Expression of these inhibitory

Ly-49 molecules in the mouse has previously been shown to be detectable by flow cytometry 1–2 d after birth and to increase to adult levels over the next 6–8 wk (40). Our observations that the Ly-49D and Ly-49H activation receptors are expressed at approximately the same time during development of $NK1.1^+$ cells but at a lower frequency suggest a model in which a developing NK cell is more likely to express an inhibitory Ly-49 receptor before commencing expression of a Ly-49 activation receptor. However, we have not yet determined whether each $NK1.1^+$ cell expressing Ly-49D and/or Ly-49H also expresses a Ly-49 inhibitory receptor. In addition, $CD3^+$ cells were not excluded from this analysis, so it is possible that the $NK1.1^+$ populations analyzed may also include rare splenic $NK1.1^+$ T lymphocytes expressing inhibitory Ly-49 molecules. Nevertheless, our analysis of $NK1.1^+$ splenocytes suggests that expression of Ly-49D and Ly-49H activation receptors initially lags behind inhibitory receptor expression. Between days 30 and 50 postpartum, the expression of Ly-49D and Ly-49H by splenic $NK1.1^+$ cells apparently increases at a somewhat faster rate than the inhibitory receptors in this population, resulting in larger subsets of $NK1.1^+$ cells expressing Ly-49D or Ly-49H by day 50 than any one of the inhibitory receptors. By this time, however, most NK cells express one or another inhibitory receptor. Although further analysis of single NK cells is necessary for detailed understanding, these results are consistent with a model in which a developing NK cell expresses a Ly-49 inhibitory receptor for self-MHC to maintain tolerance before expressing a Ly-49 activation receptor.

These considerations are also relevant to the missing-self hypothesis. One prediction of this hypothesis is that NK cells should not express activation receptors that could be triggered by self-ligands in the absence of inhibitory self-MHC–specific receptors. As we have observed here, this prediction is applicable to developing NK cells, such that developing NK cells gain the capacity to be inhibited by self before they acquire the ability to be activated. Another corollary is that if an activation receptor is expressed before the inhibitory receptors, the activation receptor is unlikely to be specific for self-molecules. As such, the NKR-P1C (NK1.1) activation receptor may not be specific for self-ligands. Therefore, as our detailed knowledge of NK cell receptors grows, such considerations are necessary for a complete understanding of the NK cell receptor repertoire.

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