Expression of Ly49E and CD94/NKG2 on Fetal and Adult NK Cells[1]

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Murine NK cells express inhibitory receptors belonging to the Ly49 and CD94/NKG2 family. Ly49E and CD94 are the only NK cell receptor transcripts detectable in fetal NK cells. Still unproved is the surface expression of Ly49E on NK cells. Here we generated two novel mAbs, a mAb recognizing Ly49E with cross-reactivity to Ly49C, and a mAb against NKG2A/C/E. Ly49E was immunoprecipitated as a disulfide-linked homodimer with 46-kDa subunits. Removal of N-linked carbohydrates revealed a 31-kDa protein backbone. NKG2A was immunoprecipitated as a 38-kDa protein. Although the frequency of fetal NK cells expressing Ly49E was higher than 25%, it decreased drastically from 2 wk after birth. Phenotypic analysis showed that ~90% of fetal NK cells and ~50% of adult NK cells express high levels of CD94/NKG2. The remaining 50% of adult NK cells expressed low surface levels of CD94/NKG2. Expression of Ly49E and CD94/NKG2 was not restricted to NK cells, but was also observed on NK T and memory T cells. Functional analysis showed that sorted Ly49E$^+$ and CD94/NKG2$^+$ fetal NK cells could discriminate between MHC class I-positive and MHC class I-negative tumor cells. We also demonstrated that Ly49E becomes phosphorylated following pervanadate stimulation of fetal NK cells. The expression levels of Ly49E and CD94/NKG2 were similar in wild-type compared with β_2-microglobulin$^{-/-}$ mice. In conclusion, generation of mAbs against Ly49E and NKG2 extended the phenotypic and functional characterization of NK cells. *The Journal of Immunology,* **2001, 166: 4302–4311.**

Natural killer cells are capable of lysing tumor and viral-infected cells that lack MHC Ags (1, 2). NK cells also mediate "hybrid resistance" in which MHC-homozygous parental bone marrow grafts are rejected by F_1 hybrids (3, 4). The discovery and cloning of receptors that recognize polymorphic MHC class I molecules and transduce inhibitory signals to NK cells revealed a molecular basis for the function of NK cells. NK cell receptors can be divided into three families. One family contains the killer cell inhibitory receptors (KIR)[3] (3) expressed on human NK cells (5). The second family consists of lectin-like CD94/NKG2A-F heterodimeric receptors that are expressed in humans and rodents (5–9). The third family has been identified in mice and contains the Ly49 receptors. In C57BL/6 mice, the Ly49 family consists of 14 highly related genes (Ly49A-N) encoding lectin-like glycoproteins expressed as disulfide-linked homodimers (10–14). The inhibitory receptors of the distinct families contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) sequence in their cytoplasmic domain. Phosphorylation of tyrosine in the ITIM sequence recruits the intracellular protein phosphatase SHP-1 and eventually results in inhibition of NK cell activity (15–18). CD94/NKG2C and CD94/NKG2D, Ly49D and Ly49H, and KIR2DS NK cell receptors lack ITIM sequences in their cytoplasmic tail. These receptors are activating rather than inhibiting because they associate with adaptor proteins, DAP10 or DAP12, which transmit activating signals to NK cells (19–23).

The human and murine CD94/NKG2 heterodimers have been implicated in the recognition of the nonclassical MHC class I molecules, HLA-E and Qa-1^b, respectively (9, 24–27). Similar to HLA-E, the murine homolog Qa-1^b binds TAP-dependent peptides derived from MHC class I signal sequences (28). It has recently been shown, using either Qa-1^b tetramers or an anti-NKG2 mAb, that ~50% of adult murine NK cells express CD94/NKG2 receptors (9, 27, 29). These CD94/NKG2$^+$ NK cells do not lyse target cells expressing Qa-1^b (27, 30).

Several murine inhibitory Ly49 receptors have been defined by mAbs, which allowed phenotypic analysis and characterization of their ligand specificities. Similar to human KIRs, murine Ly49 receptors have been shown to recognize different classical MHC class I molecules (5, 31–33). Inhibitory Ly49 receptors are expressed on subsets of NK cells that partially overlap (12, 34–37). Because Ly49A$^+$ NK cells are present in B6 (H-2^b) mice that do not express the MHC class I ligand of Ly49A (H-2^d), the Ly49A$^+$ NK cells in B6 mice would be autoaggressive (38–40). Therefore, it is accepted that each NK cell should express at least one inhibitory receptor that recognizes a self-MHC class I molecule to maintain self-tolerance (39, 40). However, during ontogeny, expression of the different Ly49 receptors on NK cells is a late event. From fetal life until the first days after birth, few or no splenic NK cells express Ly49A/C/G2 or I. Starting 1 wk after birth the different Ly49 members are gradually expressed, reaching adult levels at 6–8 wk of life (30, 41–43). Because NK cells from fetal and newborn mice can discriminate between MHC class I-positive and -negative target cells (41, 44, 45), class I-specific inhibitory receptors have to be expressed by these NK cells. It has been shown that

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[3] Abbreviations used in this paper: KIR, killer cell inhibitory receptor; FCA, flow cytometric analysis; FD, fetal day; ITIM, immunoreceptor tyrosine-based inhibitory motif; WT, wild-type; β_2m, β_2-microglobulin; HEK-T cells, human embryonic kidney T cells.

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35–50% of fetal and 95% of neonatal NK cells express Qa-1^b receptors (30, 46, 47). The lysis of target cells by fetal and newborn Qa-1^b receptor-positive NK cells is inhibited by the nonclassical class I molecule Qa-1^b (30, 46, 47). In addition, all the available evidence suggests that CD94/NKG2 molecules are the only Qa-1^b receptors on NK cells (9), implying that these inhibitory receptors control the lytic activity of part of fetal and newborn NK cells. However, surprisingly, also Qa-1^b receptor-negative fetal NK cells have been shown to distinguish between wild-type (WT) and class I-deficient tumor cells (47), raising the question of how these cells are controlled. We and others have demonstrated that although fetal NK cells are negative for the majority of the different members of the Ly49 family, these cells do express high levels of mRNA for the putatively inhibitory Ly49E receptor (44, 45). Due to the lack of serological reagents for Ly49E, it is not known whether the Ly49E protein is translated and expressed on the cell surface of NK cells. In this study we generated a mAb recognizing Ly49E/C and a mAb recognizing NKG2A/C/E to examine the cellular distribution and function of Ly49E and CD94/NKG2. Our data show that a large subpopulation of fetal and neonatal NK cells expressed Ly49E on the cell surface, partially overlapping with expression of CD94/NKG2. Functional analysis demonstrated that fetal NK cells expressing Ly49E or CD94/NKG2 were able to discriminate between MHC class I-positive and MHC class I-negative tumor cells. The generation of a mAb against Ly49E also further extended the phenotypic characterization of NK cells from adult mice, NK T cells, and memory T cells.

Materials and Methods

Animals

C57BL/6J (B6) mice were originally purchased from Harlan Netherlands (Zeist, The Netherlands). (C57BL/6J × 129/Ola) β_2-microglobulin (β_2m)-deficient ($\beta_2m^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were bred in our breeding facility. To obtain dated pregnant mice, mice were mated for 15 h and the fetuses were removed at fetal day (FD)17 (plug date = day 0). Fischer 344 rats were obtained from Iffa Credo (l'Arbresle, France). Mice and rats were treated and used in agreement with the institutional guidelines.

Preparation of cell suspensions

FD17 thymuses, FD17 spleens, and lymph nodes from 11-mo-old mice were removed and disrupted using a small Potter homogenizer. Spleens from 8- to 12-wk-old mice were removed and teased apart. Erythrocytes from spleens were lysed with 0.17 M NH_4Cl, and the remaining lymphocytes were washed three times with Dulbecco's PBS. Cells were counted with trypan blue to exclude dead cells. Thymocytes and splenocytes were suspended in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.03% glutamine, and 5×10^{-5} M 2-ME (all obtained from Life Technologies, Paisley, U.K.). This medium will be further referred to as complete RPMI 1640 medium.

IL-2 stimulation

Purified human rIL-2 was provided by M. Gately (Hoffmann-LaRoche, Nutley, NJ). Spleen and thymus cell suspensions were cultured in 24-well plates (Falcon; Becton Dickinson, Mountain View, CA) at 2×10^6 cells per well in 2 ml with a final concentration of 1000 U/ml IL-2. After culture for 4 days in 5% CO_2 at 37°C, cells were harvested, washed twice, and counted with trypan blue.

Antibodies

Monoclonal Abs used for staining were anti-CD3 (FITC-, APC-, and PerCP-conjugated, clone 145-2C11; PharMingen, San Diego, CA), anti-FcγRII/III (unconjugated, clone 2.4G2, rat IgG2b; provided by J. Unkeless, Mount Sinai School of Medicine, New York, NY), anti-CD44 (PE-conjugated, clone IM7; PharMingen), anti-2B4 (unconjugated, clone 2B4; PharMingen), anti-NK1.1 (biotin- and PE-conjugated, clone PK136; PharMingen), anti-Ly49A (biotin-conjugated, clone A1; PharMingen), anti-Ly49C/I (biotin- and PE-conjugated, clone 5E6; PharMingen), and anti-Ly49C (biotin-conjugated, clone 4LO3311; provided by S. Lemieux, Institute Armand-Frappier, Quebec, Canada). The hybridoma cell line 4D11 secreting the anti-Ly49G2 was obtained from American Type Culture Collection (Manassas, VA). The Ab was biotin-conjugated after purification by ammonium sulfate precipitation. FITC-conjugated polyclonal goat anti-rat Ig was obtained from PharMingen. Monoclonal Abs 4D12 and 3S9 were biotin- and FITC-conjugated after purification by adsorption column chromatography.

Flow cytometric analysis (FCA) and sorting

Where indicated, freshly isolated adult splenocytes were depleted of B cells using sheep anti-mouse IgG Dynabeads (Dynal, Hamburg, Germany). To avoid aspecific binding, FcγR was blocked by preincubation of cells with saturating amounts of anti-FcγRII/III mAb. Cells were incubated with the indicated mAbs for 45 min at 4°C. After washing, biotin-conjugated mAbs were revealed with second step streptavidin-APC (Becton Dickinson). To perform double-staining of NK cells, mAbs 5E6 and 4LO3311 were added 30 min before mAb 4D12. Cells were analyzed for fluorescence using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) equipped with an argon laser (488 nm) and a helium-neon laser (540 nm) with the CellQuest software program (Becton Dickinson) for data acquisition and analysis. Propidium iodide was added to the cells (2 μg/ml) just before FCA. Gating was performed on propidium iodide-negative cells to exclude dead cells. Sorting was performed on a FACSVantage (Becton Dickinson) equipped with an argon laser.

Immunization and screening

For the generation of mAb 4D12 (anti-Ly49E/C), $5–8 \times 10^6$ IL-2-cultured FD17 thymocytes in PBS were injected twice i.p into Fischer rats with an interval of 3 wk. For the generation of mAb 3S9 (anti-NKG2A/C/E), $5–10 \times 10^6$ IL-2-cultured splenocytes in PBS from adult mice were injected i.p. three times into a second group of Fischer rats. For both groups of rats, a final boost of $5–10 \times 10^6$ cells was given i.v. 3 days before fusion of rat splenocytes and SP2/0 myeloma cells. Supernatants of growing hybridomas were screened as follows: human embryonic kidney T cells (HEK-T cells) transiently transfected with Ly49E cDNA in the expression vector pcDNA1.1 or HEK-T cells transiently cotransfected with the expression vectors BSRαEN encoding CD94 cDNA and NKG2A cDNA were incubated with the different supernatants. The presence of Abs to these receptors was analyzed by flow cytometry through binding of FITC-conjugated anti-rat Ig polyclonal Ab. The 4D12 (anti-Ly49E/C) and 3S9 (anti-NKG2A/C/E) hybridomas were selected and cloned by limiting dilution. Monoclonal Abs 4D12 and 3S9 are of the rat IgG2a(κ) and IgG2b(λ) isotype, respectively.

Immunoprecipitation, deglycosylation, and Western blotting

Where indicated, cells were surface biotinylated with 0.1 M D-biotin-N-hydroxysuccinimidester (Pierce, Rockford, IL) and lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 1 mM EDTA, 50 mM Tris-HCl, 200 mM NaCl, 1% BSA, protease inhibitors). Nuclei were removed by centrifugation at 14,000 rpm at 4°C for 30 min. Lysates were precleared with protein G-Sepharose 4 Fast Flow (Pharmacia Biotech, Uppsala, Sweden), incubated with specific mAb, and followed by incubation with protein G-Sepharose. Immunoprecipitates were washed with 0.5% Nonidet P-40 lysis buffer and separated on SDS-PAGE. For removal of *N*-linked sugars, immunoprecipitates were treated with *N*-glycosidase F using a deglycosylation kit according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany) and analyzed on SDS-PAGE. Blotting was performed on polyvinylidene difluoride membranes (Novex, San Diego, CA), and blots were blocked with 10% Western blocking reagent (Novex). Blots were incubated with streptavidin-conjugated HRP, and biotin-labeled proteins were visualized with precipitating HRP substrate (both obtained from Boehringer Mannheim).

Stimulation and anti-phosphotyrosine detection

IL-2-cultured FD17 thymocytes ($10–20 \times 10^6$) containing ~20% NK cells were harvested and resuspended at 6×10^5 cells/ml in complete RPMI 1640 medium. Cells were stimulated with 0.01% H_2O_2 and 0.1 mM sodium orthovanadate (pervanadate) at 37°C for 20 min. Pervanadate is an inhibitor of protein tyrosine phosphatases, and treatment of cells with pervanadate induces protein tyrosine phosphorylation as described (48). Cells were lysed in 1% Nonidet P-40 lysis buffer containing 1 mM orthovanadate, and lysates were precipitated with mAb 4D12 (anti-Ly49E/C). Immunoprecipitates were separated on SDS-PAGE followed by Western blotting. Blots were incubated with 0.4 μg/ml HRP-conjugated anti-phosphotyrosine mAb (clone PY99) (Santa Cruz Biotechnology, Santa Cruz, CA) and revealed with precipitating HRP substrate.

RT-PCR

Trizol (Life Technologies) was added to sorted cells, and RNA was extracted according to the manufacturer's instructions. Before reverse transcription, digestion of DNA was performed with deoxyribonuclease I (Life Technologies). cDNA was synthesized with oligo(dT) as primer using the Superscript kit (Life Technologies). For HPRT, a housekeeping enzyme, oligonucleotides were GTA ATG ATC AGT CAA CGG GGG AC (sense primer) and CCA GCA AGC TTG CAA CCT TAA CCA (antisense primer). For CD94, oligonucleotides were GTG CAA TTG TTA CTT TAT TTC C (sense primer) and CTG AGA ATT CTG GAA ATA AAT C (antisense primer). For NKG2A, primers were GGT TGA CTC GAG CCA TGA GTA ATG AAC GCG TCA C (sense primer) and CGT GAA TCT AGA TTA TCA GAT GGG GAA TTT ACA CT (antisense primer). PCR amplification was performed using a 96-well thermocycler (Omnigene, Hybaid Teddington, U.K.) with 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min (HPRT and CD94), with 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min (NKG2A). In each PCR, water and 50 ng mouse genomic DNA were included as negative controls.

cDNA constructs and transfection of HEK-T cells

We received plasmids encoding Ly-49A, Ly-49B, Ly-49C, Ly-49D, and Ly-49H from F. Takei (Terry Fox Laboratory, Vancouver, Canada) (11, 12, 49) and plasmids encoding Ly-49E, Ly-49F, and Ly-49G1 from W. M. Yokoyama (Mount Sinai Medical Center, New York, NY) (13). The constructs for eukaryotic expression of Ly49 were prepared by subcloning the Ly49 cDNAs into the pcDNA1.1 expression vector (Invitrogen BV, Leek, The Netherlands). In addition, the Ly49E cDNA was subcloned into the BSRαEN expression vector (provided by J.C. Ryan, University of California, San Francisco, CA). The sense primers for cloning Ly49E in pcDNA1.1 and BSRαEN contained the ATG start codon present at position 99 of the Ly49E cDNA (13). The Ly49I cDNA in the eukaryotic expression vector pTS was provided by M. Bennett (University of Texas Southwestern Medical Center, Dallas, TX) (50). For cloning CD94 and NKG2A into the BSRαEN expression vector, a PCR was performed on cDNA generated from FD17 thymocytes. For CD94, oligonucleotides were GGT TGA CTC GAG ATA CCA TGG CAG TTT CTA GGA TCA CTC GG (sense primer) and CGT GAA TCT AGA GAA ACA TTT AAA TAG GCA GTT TC (antisense primer). For NKG2A, primers were GGT TGA CTC GAG CCA TGA GTA ATG AAC GCG TCA C (sense primer) and CGT GAA TCT AGA TTA TCA GAT GGG GAA TTT ACA CT (antisense primer). pME18S plasmids encoding NKG2C, NKG2E-hemaglutinin (HA), and DAP12 cDNAs were provided by D. Raulet (University of California, Berkeley, CA).

HEK-T cells were transiently transfected using the calcium phosphate-mediated transfection (51). HEK-T cells transfected with the empty expression vector or with the human IL-2Rα cDNA encoded in the pcDNA1.1 expression vector were used as a negative control. After 2 days, HEK-T cells were harvested and analyzed by flow cytometry or used for immunoprecipitation.

Cell mediated cytotoxicity

Tumor targets used were the NK-sensitive cell line, RMA (H-2^b; obtained from A. Kruisbeek, Amsterdam, The Netherlands) and the TAP-2 mutant derivative of RMA, RMA-S (provided by A. Geldhof, Vrije Universiteit Brussel, Brussels, Belgium). Target cells (1×10^6) were labeled with 100 μCi ^{51}Cr (Amersham International, Buckinghamshire, U.K.) for 60 min at 37°C. Cells were washed three times. Effector cells were incubated with 5 μg/ml mAb 2B4 at 4°C. As shown previously, the cytotoxicity of fetal NK cells can be triggered by preincubation of NK cells with mAb 2B4 (41, 44, 45). After 1 h, the unbound mAb was removed by washing the cells. Graded effector cell numbers were added in duplicate to 10^3 tumor cells in V-bottom wells of a 96-well plate in a final volume of 100 μl/well. After incubation for 4 h at 37°C, 75 μl of supernatant was removed from each well. Optiphase Supermix (225 μl; Wallac, Turku, Finland) was added to the supernatants, and radioactivity was measured using a 96-well scintillation counter (Microbeta; Wallac). The spontaneous release of radioactivity was determined in wells without effector cells, and the maximal release in wells in which target cells were lysed by addition of 1% Triton X-100 at the start of incubation. Percent specific lysis was calculated as 100 $\times$ (experimental $-$ spontaneous release)/(maximal $-$ spontaneous release).

Results

Generation of a mAb recognizing Ly49E/C and a mAb recognizing NKG2A/C/E

To produce an anti-Ly49E mAb, Fischer rats were immunized with IL-2-cultured murine FD17 thymocytes. One hybridoma, secreting mAb 4D12, was selected by flow cytometry for its binding to Ly49E-transfected cells. The specificity of mAb 4D12 was analyzed on HEK-T cells transiently transfected with plasmids encoding different Ly49 cDNAs. As shown in Fig. 1*A*, mAb 4D12 binds to Ly49E and Ly49C but not to Ly49A, B, D, F, G1, H, or I. As expected, mAbs A1 (anti-Ly49A), 12A8 (anti-Ly49D), 4D11 (anti-Ly49G), and 5E6 (anti-Ly49C/I) stained HEK-T cells transfected with Ly49A, D, G1, and I, respectively. Expression of Ly49F and Ly49H was shown by using a rat antiserum generated against B6 adult splenocytes. We could not demonstrate expression of Ly49B using the rat antiserum (data not shown).

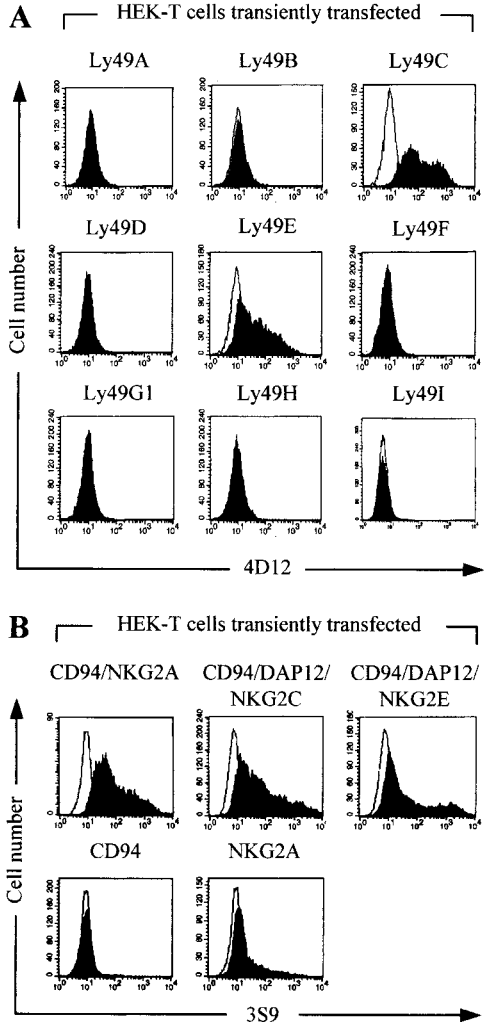

FIGURE 1. Specificity of mAbs 4D12 and 3S9. *A*, HEK-T cells were transiently transfected with different Ly49 cDNAs, stained with FITC-conjugated mAb 4D12, and analyzed by flow cytometry (filled histograms). HEK-T cells transfected with the human IL-2Rα cDNA in pcDNA1.1 were used as a negative control (open histograms). *B*, HEK-T cells transiently transfected with CD94, NKG2A, CD94/NKG2A, CD94/DAP12/NKG2C, or CD94/DAP12/NKG2E-HA cDNAs were stained with FITC-conjugated mAb 3S9 (filled histograms). HEK-T cells transfected with the empty BSRαEN vector was used as a negative control (open histograms).

We also generated a mAb against NKG2 using IL-2-cultured adult splenocytes as an immunogen. The mAb 3S9 specifically recognizes HEK-T cells cotransfected with NKG2A and CD94 cDNAs as well as HEK-T cells transfected with NKG2A cDNA alone. It did not bind HEK-T cells transfected with CD94 cDNA alone (Fig. 1*B*). We observed low expression of NKG2A alone on the surface of transfected HEK-T cells (Fig. 1*B*). The extracellular domains of NKG2C and NKG2E are >90% identical with the carbohydrate recognition domain of NKG2A (9). Therefore, the specificity of mAb 3S9 to NKG2C and NKG2E was also tested (Fig. 1*B*). Although it has not been shown that murine NKG2C and NKG2E associate with DAP12, cotransfection of DAP12 with CD94/NKG2C and CD94/NKG2E enhances receptor expression (9). Fig. 1*B* shows that mAb 3S9 binds to HEK-T cells transfected with CD94/DAP12/NKG2C and CD94/DAP12/NKG2E.

Biochemical analysis of Ly49E and NKG2A

To analyze the biochemical characteristics of Ly49E, the protein was immunoprecipitated with mAb 4D12 both from lysates of biotin-labeled HEK-T cells transfected with the plasmid BSRαEN encoding Ly49E cDNA and from lysates of IL-2-cultured FD17 thymocytes. SDS-PAGE under nonreducing conditions showed that mAb 4D12 immunoprecipitates a ~90-kDa protein from FD17 thymocytes and Ly49E-transfected HEK-T cells. HEK-T cells transfected with the empty vector were used as a negative control (Fig. 2*A*). Immunoprecipitation of lysates from Ly49E transiently transfected HEK-T cells with mAb 4D12 followed by SDS-PAGE under reducing conditions identified a protein with a molecular mass of ~46 kDa. Removal of *N*-linked sugars revealed a protein backbone of ~31 kDa (Fig. 2*B*), which is in agreement with the predicted molecular mass of the monomer (13). These data show that Ly49E is expressed as a homodimer with ~46-kDa subunits, each containing ~15-kDa *N*-linked carbohydrates.

Lysates from HEK-T cells expressing CD94/NKG2A were immunoprecipitated with mAb 3S9. SDS-PAGE under reducing conditions and Western blot analysis showed that NKG2A migrates as a ~38-kDa protein (Fig. 2*C*). The extracellular domain of NKG2A contains five potential *N-linked glycosylation sites (8). This property could explain the difference between the observed molecular mass and the predicted molecular mass (27 kDa) (8).*

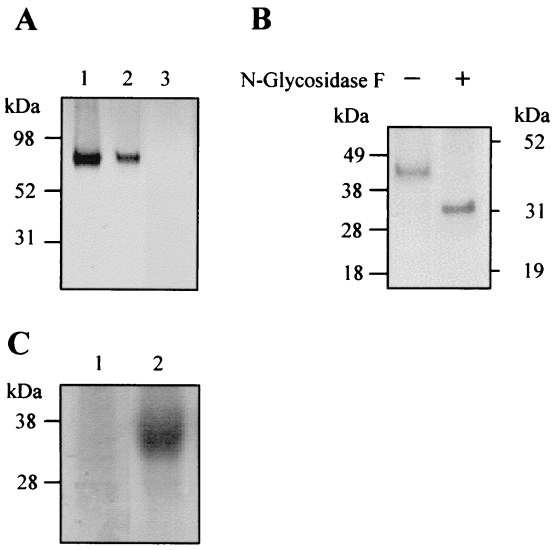

FIGURE 2. Biochemical analysis of Ly49E and NKG2A. *A*, IL-2-cultured FD17 thymocytes (*lane 1*), HEK-T cells transiently transfected with Ly49E cDNA in the expression vector BSRαEN (*lane 2*), and HEK-T cells transfected with the empty vector (*lane 3*) were surface biotinylated and lysed as described in *Materials and Methods.* Lysates were immunoprecipitated with mAb 4D12 (anti-Ly49E/C), separated by SDS-PAGE under nonreducing conditions, blotted, and incubated with streptavidin-conjugated HRP (revealed with HRP-precipitating substrate). *B*, Ly49E-transfected HEK-T cells were surface biotinylated, lysed, and immunoprecipitated with mAb 4D12 (anti-Ly49E/C). Immunoprecipitations were treated under reducing conditions in the absence (−) or presence (+) of N-Glycosidase F, separated by SDS-PAGE, and blotted as described in *A*. *C*, HEK-T cells transiently cotransfected with the expression vectors BSRαEN encoding CD94 cDNA and NKG2A cDNA (*lane 2*) and HEK-T cells transfected with the empty vector BSRαEN (*lane 1*) were surface biotinylated, lysed, and immunoprecipitated with 3S9 mAb (anti-NKG2). Immunoprecipitations were separated by SDS-PAGE under reducing conditions, and blots were analyzed as described in *A*.

Stimulation of Ly49E results in tyrosine phosphorylation

The presence of one ITIM consensus sequence within the cytoplasmic domain of Ly49E suggests an inhibitory role for this receptor. To investigate whether the tyrosine residue within the ITIM could be phosphorylated, IL-2-cultured FD17 thymocytes were stimulated with pervanadate or were left untreated. Cells were lysed and immunoprecipitated with mAb 4D12. The immunoprecipitates were separated by SDS-PAGE under nonreducing conditions, and blots were incubated with anti-phosphotyrosine mAb. Fig. 3 shows a protein band at ~90 kDa, demonstrating that Ly49E can be phosphorylated upon pervanadate stimulation.

Ly49E is expressed on a subpopulation of fetal and adult NK cells

The above data showed that mAb 4D12 recognizes the Ly49E receptor, with cross-reactivity for Ly49C. To outline further the phenotype of fetal and adult NK cells, we analyzed the expression of Ly49E by FCA. Therefore, freshly isolated FD17 splenocytes, FD17 thymocytes, and adult splenocytes were analyzed for their ability to bind mAb 4D12 by gating on $CD3^-NK1.1^+$ cells. Monoclonal Ab 4D12 recognized 50 ± 4%, 30 ± 2%, and 36 ± 4% (average % ± SD from more than three independent experiments) of NK cells from FD17 thymocytes, FD17 splenocytes, and adult splenocytes, respectively (Fig. 4*A*). Analysis of 4-day IL-2-cultured FD17 thymocytes, splenocytes, and adult splenocytes revealed that 68 ± 6%, 50 ± 5%, and 41 ± 4% (average % ± SD from more than three independent experiments), respectively, of $CD3^-NK1.1^+$ cells were stained by mAb 4D12 (data not shown). Fig. 4*A* also shows that mAb 4LO3311, specifically recognizing Ly49C (35), does not stain fetal NK cells. Therefore, the present data demonstrate that Ly49E is expressed on a considerable subpopulation of fetal NK cells. Colabeling of adult NK cells with mAb 4D12 (anti-Ly49E/C) and mAb 4LO3311 (anti-Ly49C) revealed that 6% of freshly isolated adult NK cells were single positive for 4D12 (Fig. 4*B, lower right quadrant of the left dot plot*).

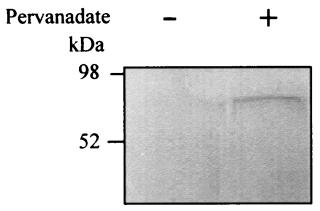

FIGURE 3. Tyrosine phosphorylation of Ly49E. IL-2-cultured FD17 thymocytes ($10–20 \times 10^6$) were either unstimulated (−) or stimulated (+) with pervanadate, lysed, and immunoprecipitated with mAb 4D12. Immunoprecipitations were separated by SDS-PAGE under nonreducing conditions, and blots were incubated with HRP-conjugated PY99 mAb (anti-phosphotyrosine) and revealed with HRP-precipitating substrate.

However, as the staining intensity of mAb 4LO3311 on B6 NK cells is low (Fig. 4*B* and S. Lemieux, unpublished observation) it is reasonable to assume that only the bright 4D12 single positive NK cells, of which the frequency is 1% (Fig. 4*B, oval area in the left dot plot*), are really negative for 4LO3311 and resemble Ly49C$^-$E$^+$ NK cells. As mAbs 4LO3311 (anti-Ly49C) and 5E6 (anti-Ly49C/I) have overlapping binding specificity for Ly49C, we expected a similar percentage of 4D12 single positive cells after colabeling of adult NK cells with mAb 4D12 plus mAb 5E6. However, in this case we observed 22% of 4D12 single positive NK cells (Fig. 4*B*). As mAb 4LO3311 does not recognize Ly49E (Ref. 35 and data not shown), a possible explanation for this discrepancy could be that mAbs 4LO3311 and 4D12 also recognize an unknown Ly49 molecule, which is not or only weakly recognized by mAb 5E6. Costaining of B cell-depleted splenocytes with mAbs 4LO3311 and 5E6 revealed a 4LO3311 single positive population when gated on CD3$^-$ cells (Fig. 4*C*). FCA showed that 28% of CD3$^-$ cells were NK1.1$^+$ (data not shown).

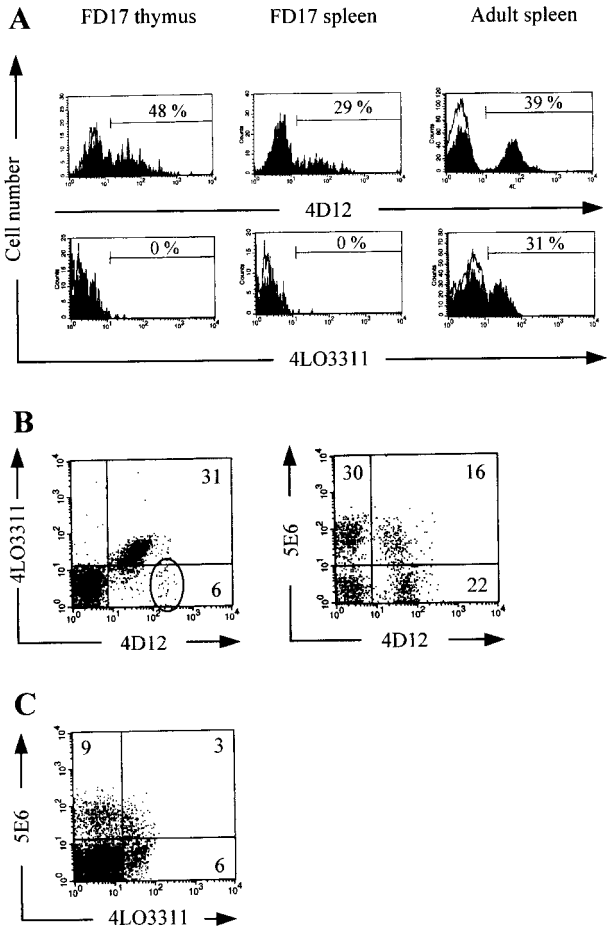

FIGURE 4. Expression of Ly49E on fetal and adult NK cells. *A*, FD17 thymocytes, FD17 splenocytes, and adult splenocytes were freshly isolated and labeled with APC-conjugated anti-CD3, PE-conjugated anti-NK1.1, and FITC-conjugated mAb 4D12 (anti-Ly49E/C) or with FITC-conjugated anti-CD3, PE-conjugated anti-NK1.1, and biotin-conjugated mAb 4LO3311 (anti-Ly49C), revealed with streptavidin-APC, and analyzed by flow cytometry. Filled histograms show staining of mAbs 4D12 or 4LO3311 on CD3$^-$NK1.1$^+$ gated cells. Open histograms represent staining with control mAbs. *B*, Freshly isolated splenocytes from adult mice were stained with PerCP-conjugated anti-CD3, PE-conjugated anti-NK1.1, FITC-conjugated 4D12, and biotin-conjugated 4LO3311, revealed with streptavidin-APC or with PerCP-conjugated anti-CD3, biotin-conjugated anti-NK1.1, revealed with streptavidin-APC, FITC-conjugated 4D12, and PE-conjugated 5E6 (anti-Ly49C/I), and analyzed by gating on CD3$^-$NK1.1$^+$ cells. *C*, Freshly isolated splenocytes from adult mice were depleted of B cells. Cells were stained with FITC-conjugated anti-CD3, PE-conjugated 5E6, and biotin-conjugated 4LO3311, revealed with streptavidin-APC, and analyzed by gating on CD3$^-$ cells. Results shown are representative of more than three experiments.

Expression of CD94/NKG2A/C/E on fetal and adult NK cells

FCA using mAb 3S9 was performed on freshly isolated thymocytes and splenocytes from FD17 mice and on adult splenocytes. Fig. 5*A* shows that ~90% of uncultured fetal thymic as well as splenic NK cells expressed high levels of CD94/NKG2, whereas <50% of NK cells from adult spleen were CD94/NKG2^{high}. The remaining fetal and adult NK cells were not completely negative for 3S9 binding and showed low fluorescence intensity. Fig. 5*A*

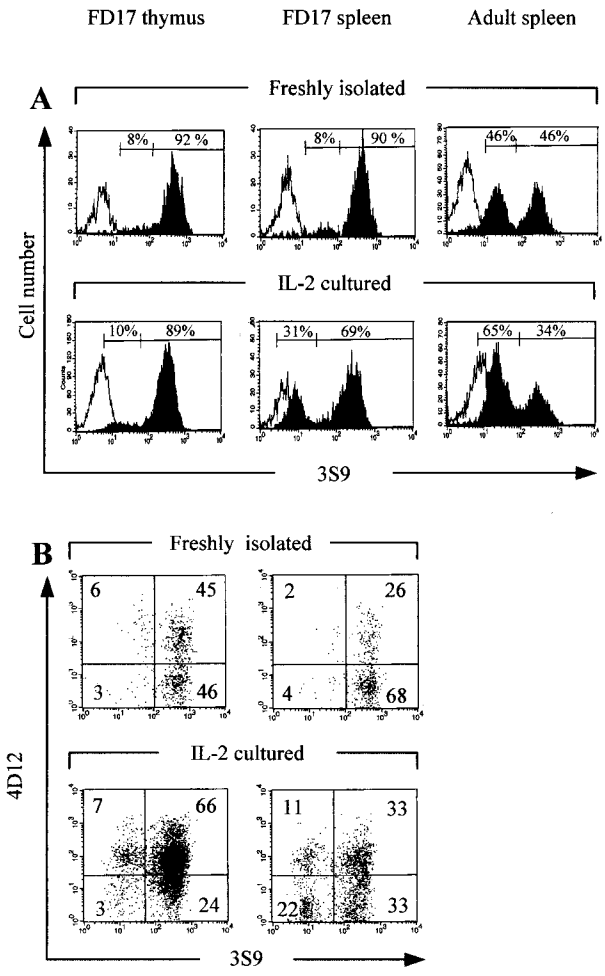

FIGURE 5. Expression of CD94/NKG2 and coexpression with Ly49E on NK cells from FD17 thymocytes, FD17 splenocytes, and adult splenocytes. *A*, Freshly isolated and IL-2-cultured cells were labeled with APC-conjugated anti-CD3, PE-conjugated anti-NK1.1, and FITC-conjugated anti-NKG2A/C/E (mAb 3S9) and analyzed by flow cytometry. Filled histograms show the expression of NKG2 on CD3$^-$NK1.1$^+$ gated cells. Open histograms represent staining with control rat mAb (IgG2b). *B*, Freshly isolated and IL-2-cultured fetal thymocytes (*left dot plots*) and fetal splenocytes (*right dot plots*) were labeled with PerCP-conjugated anti-CD3, PE-conjugated anti-NK1.1, FITC-conjugated anti-NKG2 (mAb 3S9), and biotin-conjugated anti-Ly49E/C (mAb 4D12) and revealed with streptavidin-APC. FCA shows coexpression of Ly49E/C and CD94/NKG2 on CD3$^-$NK1.1$^+$ gated cells. Results shown are representative of more than three experiments.

further reveals that after 4 days of IL-2 culture, the percentage of the CD94/NKG2^{low} population increased in both fetal and adult splenic CD3$^-$NK1.1$^+$ cells as compared with uncultured NK cells. Fig. 5*B* shows that binding of mAbs 4D12 and 3S9 on freshly isolated and IL-2-cultured fetal CD3$^-$NK1.1$^+$ cells revealed four distinct subpopulations.

To obtain additional evidence that the low staining with mAb 3S9 (anti-NKG2) of a subpopulation of NK cells is not due to aspecific binding, but correlates with low expression of the CD94/NKG2 receptor, semiquantitative RT-PCR for NKG2A and CD94 was performed on sorted 3S9^{low} and 3S9^{high} subpopulations of uncultured splenic NK cells. As a negative control, semiquantitative RT-PCR for NKG2A and CD94 was performed on naive T cells (CD3$^+$CD44$^-$3S9$^-$) sorted from lymph nodes (Fig. 6). The results show that both NKG2A and CD94 transcripts were clearly present in 3S9^{low} cells, but at lower levels as compared with 3S9^{high} cells.

Expression of Ly49E and CD94/NKG2 on developing NK cells

Expression of Ly49E during NK cell ontogeny was analyzed by determining the percentage of 4D12$^+$ cells by gating on 4LO3311-negative NK cells. Fig. 7 shows that 27–29% of splenic NK cells from FD17 and 1 day postnatal mice were 4D12 single positive. From the second week after birth, the percentage of 4D12 single positive cells decreased to 5–10%. As discussed previously, if only the bright 4D12 single positive NK cells, which are negative for 4LO3311, resemble Ly49C$^-$E$^+$ NK cells, the percentage of Ly49E$^+$ NK cells decreased to 1–3% from the second week after birth (data not shown). In contrast, the frequency of NK cells positive for Ly49A, C/I, and/or G2 increased after birth. Consistent with a previous study (30), the frequency of NK cells expressing high levels of CD94/NKG2 decreased from ~90% in FD17 mice to ~50% in adult mice (Fig. 7). Also the median fluorescence intensity of the CD94/NKG2^{high} subpopulation decreased during NK cell development (data not shown).

Expression of Ly49E and CD94/NKG2 on memory T cells and NK T cells

A significant population of memory CD8$^+$ T lymphocytes express the inhibitory receptors Ly49A, C/I, F, and G2, and this percentage increases with age (52). In 11-mo-old mice 12% of memory T cells (phenotypically defined as CD3$^+$CD44^{high}) were positive for staining with mAb 4D12 (data not shown), but only 3% of memory T cells were 4D12$^+$4LO3311$^-$ (Fig. 8), showing that the putative inhibitory receptor Ly49E is expressed on a minor population of peripheral memory T cells. Interestingly, ~30% of memory T lymphocytes expressed CD94/NKG2 as shown by binding of mAb 3S9 (Fig. 8). We also examined NK T cells. These cells have been shown to coexpress CD3 and NK1.1, to be CD4$^+$ or CD4$^-$CD8$^-$,

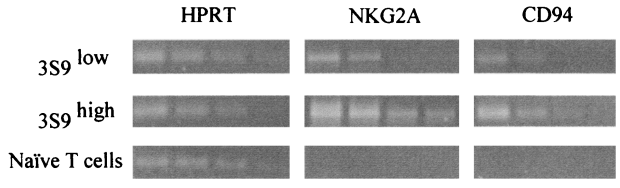

FIGURE 6. Semiquantitative analysis of NKG2A and CD94 in 3S9^{low} and 3S9^{high} NK cell populations. CD3$^-$NK1.1$^+$ cells from freshly isolated adult splenocytes were sorted into 3S9^{low} and 3S9^{high} populations using mAb 3S9 (anti-NKG2). As a negative control, naive T cells (CD3$^+$CD44$^-$3S9$^-$) were sorted from freshly isolated B6 lymph nodes. The purity of the sorted cells was ≥99.7%. cDNA was prepared and semiquantitative RT-PCR for HPRT, NKG2A, and CD94 was performed. For semiquantitative RT-PCR, four 3-fold dilutions of each cDNA were amplified. H_2O and genomic DNA were used as negative controls (data not shown).

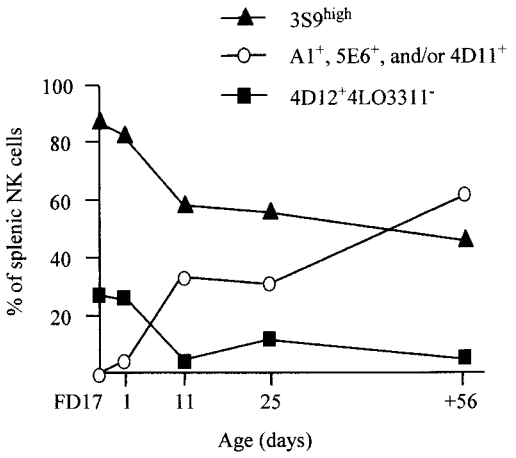

FIGURE 7. Frequency of NK cells expressing Ly49E and CD94/NKG2 during ontogeny. Splenocytes from mice of different ages were freshly isolated and incubated with APC-conjugated anti-CD3, PE-conjugated anti-NK1.1, and FITC-conjugated 3S9 or with FITC-conjugated CD3, PE-conjugated anti-NK1.1, biotin-conjugated A1, biotin-conjugated 5E6, and biotin-conjugated 4D11, revealed with streptavidin-APC or with PerCP-conjugated anti-CD3, PE-conjugated anti-NK1.1, FITC-conjugated 4D12, and biotin-conjugated 4LO3311, revealed with streptavidin-APC, and analyzed on CD3$^-$NK1.1$^+$ cells. To analyze the percentage of NK cells expressing Ly49E but not Ly49C, CD3$^-$NK1.1$^+$ cells were gated on the 4LO3311$^-$ population.

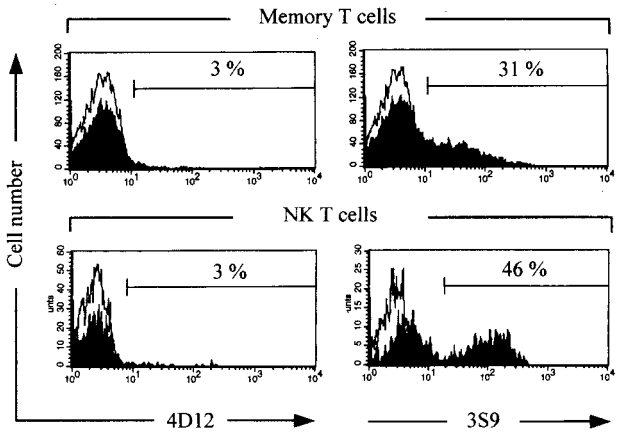

FIGURE 8. Expression of Ly49E and CD94/NKG2 on memory T cells and NK T cells. For analysis of memory T cells (*upper histograms*), lymph nodes from 11-mo-old mice were freshly isolated. Cells were incubated with PerCP-conjugated anti-CD3, PE-conjugated anti-CD44, FITC-conjugated 3S9 (anti-NKG2), or with FITC-conjugated 4D12 (anti-Ly49E/C) and biotin-conjugated mAb 4LO3311 (anti-Ly49C), and revealed with streptavidin-APC. Expression of Ly49E and CD94/NKG2 on memory T cells was analyzed by gating on, respectively, 4LO3311$^-$CD3$^+$CD44^{high} and CD3$^+$CD44^{high} cells. For analysis of NK T cells (*lower histograms*), splenocytes from 2-mo-old mice were freshly isolated. Cells were incubated with PerCP-conjugated anti-CD3, PE-conjugated anti-NK1.1, FITC-conjugated 4D12, and biotin-conjugated 4LO3311, revealed with streptavidin-APC or with APC-conjugated anti-CD3, PE-conjugated anti-NK1.1, and FITC-conjugated 3S9. Expression of Ly49E and CD94/NKG2 on NK T cells was analyzed by gating on, respectively, CD3^{int}NK1.1$^+$4LO3311$^-$ and CD3^{int}NK1.1$^+$ splenocytes.

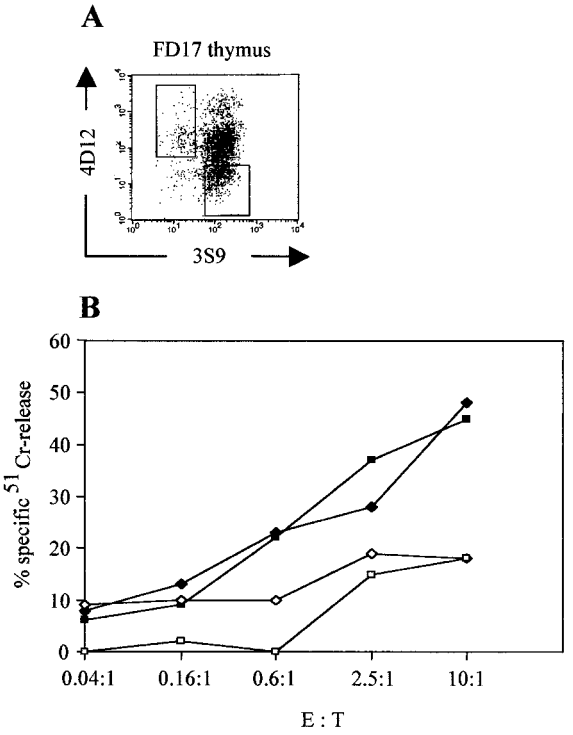

FIGURE 9. Cytotoxicity of IL-2-cultured Ly49E$^+$CD94/NKG2^{low} and Ly49E$^-$CD94/NKG2^{high} fetal NK cells. *A*, After culture of FD17 thymocytes with IL-2 for 4 days, CD3$^-$NK1.1$^+$ cells were sorted into either Ly49E$^+$CD94/NKG2^{low} and Ly49E$^-$CD94/NKG2^{high} subsets using mAbs 4D12 (anti-Ly49E/C) and 3S9 (anti-NKG2) as shown in the regions of the dot plot. *B*, After 3 days of additional IL-2 culture, the cytotoxicity of Ly49E$^+$CD94/NKG2^{low} (diamonds) and Ly49E$^-$CD94/NKG2^{high} (squares) fetal NK cells was analyzed in a ^{51}Cr release assay against RMA (open symbols) and RMA-S (filled symbols) tumor cells.

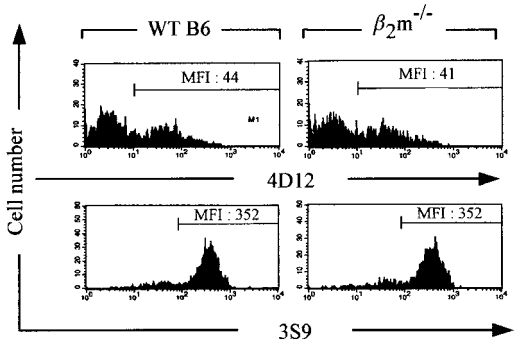

FIGURE 10. Expression levels of Ly49E and CD94/NKG2 on NK cells from B6 WT and β_2m$^{-/-}$ mice. FD17 thymocytes were freshly isolated and stained with APC-conjugated anti-CD3, PE-conjugated anti-NK1.1, and FITC-conjugated 4D12 or with FITC-conjugated 3S9. Histograms show the median fluorescence intensity of Ly49E (*upper histograms*) and CD94/NKG2 (*lower histograms*) by gating on CD3$^-$NK1.1$^+$ cells.

and to express inhibitory Ly49 receptors (53–56). Approximately 10% of NK T cells (phenotypically defined as CD3^{int}NK1.1$^+$) stained with mAb 4D12 (data not shown), but only 3% were 4D12$^+$4LO3311$^-$ (Fig. 8). So, in contrast to Ly49A and Ly49G2, which are expressed on, respectively, 11 and 17% of NK T cells (data not shown), Ly49E is expressed on a very small population of NK T cells. Staining with mAb 3S9 showed that ~50% of NK T cells express high levels of CD94/NKG2. The remaining NK T cells have low expression levels of CD94/NKG2 (Fig. 8).

Lysis of RMA-S target cells by fetal NK cells expressing either Ly49E or CD94/NKG2

In search of a functional role of the Ly49E receptor on fetal NK cells, we first sorted Ly49E$^-$ and Ly49E$^+$ subpopulations from IL-2-cultured FD17 thymocytes and assessed their lytic capacity against RMA and the TAP-deficient RMA-S target cells. The Ly49E$^-$ and Ly49E$^+$ subpopulations were equally capable to differentially lyse RMA-S cells (data not shown). This might be explained by the fact that a major part of the cells of both the Ly49E$^-$ and Ly49E$^+$ subpopulations express CD94/NKG2 (Fig. 5*B*). Therefore, we sorted Ly49E$^+$CD94/NKG2^{low} NK cells from 4-day IL-2-cultured FD17 thymocytes. As a control, also Ly49E$^-$CD94/NKG2^{high} NK cells were sorted (Fig. 9*A*). Sorted cells were cultured in the presence of IL-2 for an additional period of 3 days to remove the mAbs from the cell surface. RMA and RMA-S target cells were still differentially lysed by the sorted Ly49E$^+$CD94/NKG2^{low} cells, and the differential killing was comparable to that of sorted Ly49E$^-$CD94/NKG2^{high} cells (Fig. 9*B*). This differential lysing of RMA and RMA-S cells by the Ly49E$^+$CD94/NKG2^{low} cells could be due either to the expression of the Ly49E receptor or to the low expression of CD94/NKG2. To further assess whether Ly49E is involved in the resistance of RMA target cells, blocking studies by incubating effector cells with F(ab′)$_2$ of anti-Ly49E mAb (mAb 4D12) were performed. This did not reverse the resistance of RMA target cells to lysis (data not shown). It is possible that mAb 4D12 is not able to functionally block the interaction of Ly49E with its ligand or, alternatively, that RMA cells do not express the ligand for the Ly49E receptor.

Expression levels of Ly49E and CD94/NKG2 on NK cells from WT and $\beta_2m^{-/-}$ mice

The ligand for Ly49E is still unknown. Because the expression level of several other Ly49 receptors on NK cells is down-regulated in vivo by the presence of the corresponding MHC class I ligands (37, 38, 57–61), we investigated whether H-2^b MHC class I molecules are able to influence the expression levels of Ly49E and CD94/NKG2A receptors. Because Ly49E is expressed as the only member of the Ly49 family on fetal NK cells, the expression level of Ly49E was compared between fetal thymic NK cells from WT B6 mice and β_2m$^{-/-}$ mice. Fig. 10 shows that the expression level of Ly49E was not down-regulated on NK cells from WT B6 mice as compared with β_2m$^{-/-}$ mice, suggesting that the ligand for Ly49E is not expressed in WT B6 mice.

Vance et al. demonstrated that CD94/NKG2 heterodimers recognize the nonclassical MHC Qa-1^b molecules (9, 27). Because the expression of Qa-1^b is β_2m-dependent (62), this would suggest a higher expression level of CD94/NKG2 in β_2m$^{-/-}$ mice compared with WT. Interestingly, we found that the relative fluorescence intensity of CD94/NKG2 was the same in both strains of mice (Fig. 10). Also the percentages of CD94/NKG2^{low} and CD94/NKG2^{high} cells were not significantly different (data not shown).

Discussion

In this study, we generated mAb 4D12, which is the first reported mAb recognizing Ly49E, with cross-reactivity to Ly49C. Using this mAb we showed that the expression of Ly49E is different from the expression of the other Ly49 receptors at two levels: Ly49E is expressed on a considerable part of NK cells during fetal life, and the frequency of NK cells expressing Ly49E decreases after birth. In adult spleen, only 1% of the NK cells express Ly49E. The low

frequency of Ly49E$^+$ NK cells in adult spleen is in agreement with a study described by Kubota et al. (63). They could not detect Ly49E mRNA by single-cell RT-PCR in 62 adult NK cells examined, whereas Ly49E mRNA could be detected in a pool of 1000 NK cells. Also, we previously reported that Ly49E mRNA is present at a 30-fold lower level in adult NK cells compared with fetal splenic NK cells (45).

The FCA of adult NK cells with the 4LO3311, 5E6, and 4D12 Abs gave unexpected results (Fig. 4, *B* and *C*). As we have shown here that mAb 4D12 detects Ly49C and Ly49E (Fig. 1*A*), colabeling with mAbs 4D12 plus 5E6 (recognizes Ly49C and Ly49I; Ref. 35) should yield a similar percentage of 4D12 single positive NK cells as obtained after colabeling with mAbs 4D12 plus 4LO3311 (recognizes only Ly49C; Ref. 35). However, the percentage of 4D12 single positive NK cells was much higher in the case of mAbs 4D12 plus 5E6 colabeling (Fig. 4*B*). As mAb 4LO3311 does not recognize Ly49E (Ref. 35 and data not shown), and as both mAbs 4LO3311 (Ref. 35 and 4D12, Fig. 1*A*) do not recognize Ly49A, B, D, F, G, H, and I, this indicates that mAbs 4LO3311 and 4D12 also bind another, possibly unknown, Ly49 member, which is not or only weakly recognized by mAb 5E6. Further evidence for this is provided by our finding that a 4LO3311 single positive population can be identified after double-staining with mAbs 4LO3311 plus 5E6 (Fig. 4*C*). Brennan et al. could not detect a 4LO3311 single positive population after similar double-staining of B6 spleen cells (35). However, the 4LO3311$^+$5E6$^+$ double positive population they observed seemed to contain two subpopulations, of which one subpopulation was bright positive for 5E6 staining whereas the other subpopulation only weakly bound 5E6. They also argued that this might indicate that 4LO3311 mAb detects other Ly49 molecules. These data show evidence for the presence of a novel Ly49 receptor and require further investigation.

In this study, we also generated mAb 3S9, recognizing NKG2A, NKG2C, and NKG2E, but not CD94. Vance et al. reported the generation of mAb 20d5 (anti-NKG2A/C/E) (9). They demonstrated that ~50% of NK1.1$^+$ splenocytes from adult mice stained with mAb 20d5, whereas the other part of NK cells were completely negative. This seems to be in contradiction with the staining profile of our mAb 3S9 that shows two different expression levels: 3S9^{low} and 3S9^{high}. An acceptable explanation is that mAbs 20d5 and 3S9 differ in the affinity for NKG2 and/or the epitope recognized on NKG2. We showed with RT-PCR on sorted 3S9^{low} NK cells that these cells clearly express mRNA for NKG2 and CD94, demonstrating that 3S9^{low} cells indeed express CD94/NKG2 receptors. This is in agreement with a recent study by Sivakumar et al. using Qa-1^b tetramers, which also inferred the presence of low and high expression levels of CD94/NKG2 receptors (46).

A major issue in NK cell maturation and education is the expression of NK cell receptors. Expression of Ly49A, C/I, D, G2, and H is almost not detectable during fetal life and after the first days of birth. Thereafter, the expression does not occur at once at a given time but gradually increases, reaching adult levels at the age of 6–8 wk (30, 41–43). In this study we demonstrate that Ly49E is frequently expressed on fetal NK cells, and that the frequency of NK cells expressing Ly49E decreases drastically after birth. The observation that the expression of Ly49A, C/I, and G2 lags behind the expression of Ly49E, suggests a model in which the initiation and regulation of Ly49E expression during NK cell ontogeny is completely different from that of other Ly49 receptors. Therefore, it can be predicted that the initiation of Ly49E expression on developing NK cells is limited to an early phase during ontogeny. After this point, it is possible that the percentage of NK cells expressing the other Ly49 molecules starts to increase resulting in a minor population of Ly49E$^+$ NK cells. Others have shown that the initiation of expression of Ly49A, C/I, and G2 receptors occurs successively. In vivo transfer of NK cells suggested that the initiation for expression of Ly49A is an early event and that the expression of Ly49C/I and Ly49G2 receptors occurs thereafter (42). So it can be hypothesized that Ly49E is the first Ly49 receptor within this specific sequence of Ly49 expression.

The acquisition of CD94/NKG2 receptors during NK cell ontogeny was evaluated by analyzing its expression on NK cells from mice at different ages using mAb 3S9. Our results further extend previous studies where soluble Qa-1^b tetramers were used (30). Thus, in contrast to the gradual increase of Ly49A, C/I, and G2 receptors on developing NK cells, the frequency of NK cells expressing high levels of CD94/NKG2 decreases from ~90% on fetal NK cells to ~50% on adult NK cells. Because the expression of Ly49 receptors, with the exception of Ly49E, is absent on fetal NK cells and almost not detectable on neonatal NK cells, it is possible that high surface levels of CD94/NKG2 are necessary to receive sufficient inhibitory signals for the maintenance of self-tolerance. We speculate that accumulation of inhibitory Ly49 receptor results in a decreased expression of CD94/NKG2. This may be required to avoid that developing NK cells receive too many inhibitory signals and, therefore, would be insensitive for small changes in MHC class I expression. It is also interesting to note that the CD94/NKG2^{low} and CD94/NKG2^{high} subpopulations of adult NK cells equally coexpress Ly49A, C/I, or E/C as detected with the mAbs A1, 5E6, or 4D12, respectively (data not shown). Consistent with previous studies, the expression of Ly49G2 is diminished on the CD94/NKG2^{high} subpopulation compared with CD94/NKG2^{low} (27, 43).

We demonstrated that mAb 3S9 (anti-NKG2A/C/E) is abundantly expressed on memory T lymphocytes and NK T cells. As activating Ly49 receptors, Ly49D and Ly49H, are not expressed on T lymphocytes (43, 52, 56), it can be suggested that the CD94/NKG2$^+$ memory T lymphocytes and NK T cells express the inhibitory CD94/NKG2A receptor rather than the activating CD94/NKG2C and CD94/NKG2E receptors. Expression of Ly49A, C/I, and G2 inhibitory receptors on T lymphocytes has been shown to confer inhibition of T cell activation and effector functions (31, 52, 56, 64, 65), suggesting a similar regulatory role for CD94/NKG2A on T lymphocytes. In contrast to CD94/NKG2 and Ly49 receptors, which are expressed on a considerable part of memory T cells and NK T cells, we found that only 3% of memory T and NK T cells expressed Ly49E.

Functional analysis revealed that Ly49E$^-$CD94/NKG2^{high} NK cells from FD17 thymocytes differentially lysed the TAP2-deficient RMA-S target cells. Similar results were found in a recent report demonstrating that fetal NK cell clones, positive for staining with Qa-1^b tetramers, are able to discriminate between TAP2-deficient RMA-S and WT RMA tumor cells (47). Although we were not able to reverse inhibition of RMA cells using 3S9 F(ab′)$_2$ (data not shown), the inhibitory role of CD94/NKG2 receptors has been well reported by others (46, 47). So, our finding that >90% of fetal NK cells express the CD94/NKG2 receptor suggests that this receptor plays an important role to maintain self-tolerance during fetal life.

In search of a functional role of the Ly49E receptor, we analyzed the lytic activity of sorted Ly49E$^+$CD94/NKG2^{low} fetal NK cells. Also these cells differentially lysed RMA-S target cells. Two possible explanations for this phenomenon can be offered. First, Ly49E could be the receptor for a TAP2-dependent ligand expressed on RMA target cells, and second, the low expression level of CD94/NKG2 may be responsible for the discrimination between

RMA and RMA-S target cells. A counter argument for the first possibility is that blocking studies with $F(ab')_2$ of mAb 4D12 did not reverse the inhibition of $Ly49E^+CD94/NKG2^{low}$ NK cells by RMA tumor cells (data not shown), but this could be due to the fact that mAb 4D12 does not functionally block the interaction of Ly49E with its ligand. Therefore, a functional role of Ly49E still has to be demonstrated. In this regard, identification of the ligand for Ly49E would be important. As Ly49E is most similar to Ly49F and Ly49C (13), and as these receptors have been shown to bind MHC class I molecules (12, 31, 32, 66), it can be suggested that Ly49E is also a receptor for MHC class I molecules. Our data demonstrate that the surface expression of Ly49E is not up-regulated on NK cells from $\beta_2m^{-/-}$ mice as compared with WT B6 mice, indicating that H-2^b is not the ligand for Ly49E. This is supported by cell-cell adhesion assays, including the use of Con A blasts from B6 mice and by studies using soluble MHC class I tetramers, including H-2^b tetramers, because no efficient binding to Ly49E has been detected in these models (31, 67). Therefore, it seems likely that the killing of RMA-S tumor cells by $Ly49E^+CD94/NKG2^{low}$ NK cells is due to the low expression level of CD94/NKG2. In analogy to Ly49E, the low and high surface expression levels of CD94/NKG2 were not different in $\beta_2m^{-/-}$ mice as compared with B6 mice. Also Salcedo et al. described similar expression levels of Qa-1b receptors in $NK1.1^+$ cells from B6 and $\beta_2m^{-/-}$ mice (29, 30).

In conclusion, this study analyzed the cellular distribution of Ly49E on NK cells and demonstrated that the expression of Ly49E is different from the expression of other Ly49 receptors. Ly49E is expressed on a considerable part of fetal NK cells and the number of $Ly49E^+$ NK cells decreased after birth. We also showed expression of CD94/NKG2 on fetal NK cells using a novel mAb generated against NKG2. Expression of Ly49E and/or CD94/NKG2A on fetal NK cells might be important to maintain self-tolerance during fetal life. We reported that the expression level of Ly49E is not influenced by the presence of β_2m-dependent molecules expressed in B6 mice. Identification of the ligand for Ly49E will gain further insight in the functional regulation of $Ly49E^+$ NK cells.

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