Exclusion of Lipid Rafts and Decreased Mobility of CD94/NKG2A Receptors at the Inhibitory NK Cell Synapse^V

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CD94/NKG2A is an inhibitory receptor expressed by most human natural killer (NK) cells and a subset of T cells that recognizes human leukocyte antigen E (HLA-E) on potential target cells. To elucidate the cell surface dynamics of CD94/NKG2A receptors, we have expressed CD94/NKG2A-EGFP receptors in the rat basophilic leukemia (RBL) cell line. Photobleaching experiments revealed that CD94/NKG2A-EGFP receptors move freely within the plasma membrane and accumulate at the site of contact with ligand. The enriched CD94/NKG2A-EGFP is markedly less mobile than the nonligated receptor. We observed that not only are lipid rafts not required for receptor polarization, they are excluded from the site of receptor contact with the ligand. Furthermore, the lipid raft patches normally observed at the sites where FcεR1 activation receptors are cross-linked were not observed when CD94/NKG2A was coengaged along with the activation receptor. These results suggest that immobilization of the CD94/NKG2A receptors at ligation sites not only promote sustenance of the inhibitory signal, but by lipid rafts exclusion prevent formation of activation signaling complexes.

INTRODUCTION

NK cells are one of the three lymphoid lineages, the others being B and T lymphocytes, and are characterized by their ability to inherently lyse a large variety of cell types. NK cell lysis of target cells does not require prior sensitization of the host and is not restricted by major histocompatibility complex (MHC) encoded molecules (Moretta *et al.*, 2002). However, lysis of target cells does inversely correlate with the level of cell surface expression of MHC class I molecules expressed by the target. This observation led to the “missing self” hypothesis of NK cell recognition, whereby the postulated role of NK cells is to destroy cells that express decreased levels of self-MHC class I molecules, a common

feature of virally-infected and transformed cells (Ljunggren and Karre, 1990; Yokoyama, 2002).

Because the ligands recognized by receptors that activate NK cell lytic activity can also be expressed by “normal” cells, a mechanism must exist to override activation signals generated after NK cells encounter normal cells. To accomplish this, NK cells express inhibitory receptors that recognize MHC class I molecules on target cells (Long *et al.*, 2001; Natarajan *et al.*, 2002). MHC class I molecules are expressed on virtually all normal cells, whereas, as mentioned above, their expression on viral-infected and transformed cells tends to be downregulated (Ploegh, 1998; Algarra *et al.*, 2000). On encountering a potential target cell, one or several of the myriad of NK cell-activating receptors will be engaged (Lanier, 2001; Colucci *et al.*, 2002). NK inhibitory receptors will also be engaged if appropriate MHC class I molecules are expressed on the target cell. The cross-linking of the NK inhibitory receptors results in the phosphorylation of their immunoreceptor tyrosine-based inhibitory motifs (ITIMs). These phosphorylated motifs act as docking sites for the recruitment of the phosphatases SHP-1 and SHP-2, thereby activating them, and as a consequence of this, the activation signals generated by ligation of the activating receptors are suppressed (Long *et al.*, 2001; Kabat *et al.*, 2002; Yusa and Campbell, 2003).

In humans, three groups of such inhibitory receptors have been described (Borrego *et al.*, 2002a). Two of them belong to the immunoglobulin superfamily. They are the killer Ig-like receptors (KIR) and Ig-like transcripts (ILT). Although KIRs are expressed on NK cells and a subset of T cells, ILTs are expressed mainly on B, T, and myeloid cells, but some members of this group are also expressed on NK cells. KIRs interact with HLA-A, -B and -C molecules, and ILTs react

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[†] Corresponding author. E-mail address: Fborrego@niaid.nih.gov. Abbreviations used: APC, antigen presenting cells; BCR, B-cell receptor; Ctx-B, cholera toxin B; D_{eff} , diffusion coefficient; DIC, differential interference contrast; FcεR1α, Fc receptor for IgE alpha chain; HLA, human leukocyte antigen; ILT, Ig like transcript; IS, immunological synapse; ITIM, immunoreceptor tyrosine-based inhibitory motif; KIR, killer Ig-like receptor; MCD, methyl-β-cyclodextrin; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; NK, natural killer; NKIS, NK immunological synapse; RBL, rat basophilic leukemia; RMA-SE, HLA-E-transfected RMA-S cells; ROI, region of interest; SHP, SH2 domain-bearing tyrosine phosphatase; TCR, T-cell receptor.

with a variety of HLA class I molecules, including the non-classical HLA-G, and the CMV-encoded class I-like UL18 molecules (Lanier, 1998; Colonna *et al.*, 1999). Interestingly, in both groups there are also activating receptors characterized by the absence of ITIMs in the intracytoplasmic tail and the association with adaptor proteins for the transmission of the activating signal (Bianconi *et al.*, 2000). The third group of NK receptors is composed of heterodimers that belong to the C-type lectin family of proteins. They are composed of CD94 plus a member of the NKG2 family (Lanier, 1998; Borrego *et al.*, 2002a). The inhibitory member of this family is CD94/NKG2A (and the alternatively spliced form NKG2B), whereas the activating receptor members are CD94/NKG2C and CD94/NKG2E (and the alternatively spliced form NKG2H). The ligand for the CD94/NKG2 receptors is the nonclassical MHC class I molecule HLA-E that is expressed by virtually all cells (Borrego *et al.*, 1998; Braud *et al.*, 1998; Lee *et al.*, 1998).

The accumulation of receptor molecules to the site of the antigen-presenting cell (APC) or target cell contact appears to be very important for T-cell signaling (Grakoui *et al.*, 1999; Bromley *et al.*, 2001). Ordered macromolecular complexes that form within the plasma membrane of the effector T cells are referred to as the immunological synapse (IS). NK cells also appear to organize receptors into macromolecular complexes termed the NK immunological synapse (NKIS) at the points of target cell contact (Davis *et al.*, 1999; Vyas *et al.*, 2001; McCann *et al.*, 2002; Vyas *et al.*, 2002b, a). Although most T cells may never or rarely encounter specific ligand leading to the formation of the IS, NK cells are constantly exposed to "activation" ligands that are present on most cells that they encounter that can lead to target cell destruction and/or production of inflammatory cytokines. As mentioned above, inhibitory receptors negatively regulate this self-destructive potential. It has been demonstrated that NK cells can simultaneously bind susceptible and MHC class I protected target cells with selective killing of the susceptible target cell. This indicates that inhibitory signals do not globally inhibit cell function, but rather are spatially restricted toward resistant target cells (Eriksson *et al.*, 1999). It is clear that the NKIS formed by activation receptors is a complex structure requiring ATP for formation, lipid raft polarization, and involvement of the cytoskeleton (Lou *et al.*, 2000; Vyas *et al.*, 2001, 2002b; McCann *et al.*, 2002). KIR inhibitory receptors do not appear to disrupt activation by intertwining within this NKIS, but instead function as a distinct unit, outside lipid rafts, not requiring ATP or active involvement of the cytoskeleton (Fassett *et al.*, 2001). Nonetheless, because they function spatially within the cell, one would expect the ability of inhibitory receptors to enrich at the point of cell contact to be critical for their function.

Despite performing similar biological functions, inhibitory KIR and CD94/NKG2A receptors clearly represent distinct families of molecules that are differentially regulated (Borrego *et al.*, 2002a), yet essentially nothing is known about the membrane dynamics of this receptor. In this initial study, we examined the lateral mobility of the CD94/NKG2A inhibitory receptor with FRAP technique and show that surface-expressed CD94/NKG2A exhibits mobility that fits a free diffusion model. On ligation with polystyrene beads coated with anti-NKG2A or anti-CD94 mAb or with target cells expressing HLA-E, CD94/NKG2A polarized to the site of contact with a consequent decrease in the receptor mobility and exclusion of lipid rafts, features that most likely promote the predominance of inhibitory over activat-

ing signals. In support of this, we show that ligated activation receptors, in the presence of ligated CD94/NKG2A, fail to associate with lipid rafts that are prerequisite for the generation of activation signals.

MATERIALS AND METHODS

Antibodies and Reagents

Sources for each antibody are as follows: purified and PE-conjugated anti-human NKG2A mAb (Z199, mouse IgG2b), purified and PE-conjugated anti-human CD94 mAb (HP-3B1, mouse IgG2a), purified anti-CD8 and isotype controls from Beckman Coulter (Fullerton, CA); F(ab')₂ goat anti-mouse-PE from Jackson ImmunoResearch Laboratories (West Grove, PA); anti-SHP-1 rabbit polyclonal IgG, antiphosphotyrosine mAb 4G10 (IgG2b) and anti-rat FcεR1α from Upstate Biotechnology (Lake Placid, NY); anti-SHP-2 mAb (PTP1D/SHP2) from BD Transduction Laboratories (San Diego, CA). The NKG2A-specific mAb 8E4 (mouse IgG) was derived by Dr. J. P. Houchins (Houchins *et al.*, 1997). Mouse anti-DNP IgE mAb was purchased from Sigma-Aldrich (St. Louis, MO). Immunoblots were developed with HRP-conjugated anti-mouse and anti-rabbit IgG from Amersham Pharmacia Biotech (Piscataway, NJ). Protein A-coated beads were purchased from Bangs Laboratories (Fishers, IN), Alexa 594 Ctx-B and DiI-C₁₈ were from Molecular Probes (Eugene, OR) and MCD from Sigma-Aldrich.

Cell Lines, Immunoblot, and Functional Analysis

The CD94/NKG2A expressing rat basophilic leukemia (RBL) cell line (Kabat *et al.*, 2002) and the TAP (transporter-associated peptide) deficient HLA-E-transfected RMA-S cells (Borrego *et al.*, 1998) have been previously described. Generation of the CD94/NKG2A-EGFP expressing RBL cell line was previously described (Borrego *et al.*, 2002b). Examination of the expressed receptor for biochemical integrity by immunoprecipitation analysis and functional capabilities by its ability to inhibit serotonin release were performed in this study using previously described methods (Kabat *et al.*, 2002).

Conjugation Assays

Approximately 0.1 mg of protein A-coated microspheres were washed two times with antibody-binding buffer (50 mM sodium borate, pH 8.0), resuspended in 1 ml of antibody-binding buffer with 0.02 mg of anti-CD94 mAb, 0.02 mg of anti-NKG2A mAb, 0.02 mg of anti-CD8, or 0.05 mg of mouse anti-rat FcεR1α, and incubated at room temperature for 45 min on a mechanical rocking apparatus, followed by incubation at 4°C for 2–3 additional hours on the same rocking apparatus. For beads coated with both anti-FcεR1α and anti-NKG2A mAb, we used 0.005 mg of anti-NKG2A mAb and 0.025 mg of anti-FcεR1α mAb. The mixtures were then washed two times in PBS and stained with PE-conjugated goat anti-mouse Ig for 30 min, washed and mixed with 1×10^6 CD94/NKG2A-EGFP RBL cells, and centrifuged at $500 \times g$ for 5 min at 4°C. The mixtures were divided into samples of equal amount and placed on ice for 10 min and then transferred to 37°C for different time periods. After the incubation period, 100 μl of 0.5% paraformaldehyde in PBS was added to the samples followed immediately by flow cytometric analysis. For conjugates between HLA-E-transfected RMA-S cells and CD94/NKG2A-EGFP RBL cells, RMA-S cells were cultured without or with 300 mM of peptide overnight. Approximately 2×10^6 RMA-S cells were labeled with the PKH26 red fluorescent cell linker kit (Sigma-Aldrich) following the manufacturer's instructions. Labeled RMA-S cells were then washed and mixed with CD94/NKG2A-EGFP RBL cells at a 1:1 ratio. The mixture was centrifuged at $500 \times g$ for 5 min and placed on ice for 10 min. After that, conjugates were allowed to form at 37°C for different time periods and then fixed and analyzed by flow cytometry.

Confocal Microscopy

Image Acquisition All images were collected using a Leica TCS SP2 AOBS microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany) at the Biological Imaging Facility (NIAID, RTB). All images were acquired with a 63× oil immersion objective, NA 1.32. EGFP was excited at 488 nm and Alexa Fluor 594 at 568 nm. Emission wavelengths collected were 530–560 nm for EGFP and 600–660 nm for Alexa Fluor 594. Huygens Essential version 2.5.6a0 (Scientific Volume Imaging b.v., Hilversum, Netherlands) was used for deconvolution of microscopic images using an MLE (Maximum Likelihood Estimation) algorithm. Image analysis was done using Imaris version 3.2.2 (Bitplane AG, Zurich, Switzerland), Leica Confocal Software version 2.5 build 1104 (Leica Microsystems, Mannheim, Germany), and Adobe Photoshop version 7.0 (Adobe Systems, San Jose, CA). 3D reconstructions were prepared using Imaris 3.2.2.

FRAP Measurements Experiments were conducted by first defining a region of interest (ROI) on the membrane. Bleaching was done by illuminating the ROI with 488 nm for 4 s (AOTF for 488 nm set to 100% transmission;

previously determined by using fixed CD94/NKG2A-EGFP RBL cells). Recovery in the bleached region was recorded by acquisition of an image every 1.6 s (AOTF for 488 nm set to 3%) and quantifying pixel intensities within the ROI. The percent of recovery and mobility was calculated with the following equation: % recovery = (bleached ROI – background ROI)/(membrane ROI – background ROI), where bleached ROI is the MFI (mean fluorescence intensity) of the bleached ROI in the plasma membrane, background ROI is the MFI of a noncell region, and membrane ROI is the MFI of a nonbleached region of the plasma membrane. The MFI of membrane ROI represents the 100% values of the membrane fluorescence. D_{eff} was determined by using a simulation program kindly provided by Dr. Eric Siggia (Rockefeller University, New York). The mobile fraction and recovery rates were normalized by setting up the MFI of the first recovery to 0% and the MFI of the prebleached membrane ROI to 100%. Recovery values were graphically generated using Microsoft Excel spreadsheet software (Redmond, WA).

Polarization Studies CD94/NKG2A-EGFP RBL cells were cultured in four-well coverslip chambers (Nalge Nunc International, Naperville, IL) until they reached 90% confluence. Cells were then washed two times with PBS, and 1.0 ml of prewarmed media (RPMI 1640 without phenol red) was added to the wells, with or without 10 mM MCD supplemented with 1% FCS. The chambers were placed at 37°C for 25 min, and the cells were washed again. For observation, the chambers were transferred to a temperature controlled stage (37°C) on the confocal microscope, with the cells in RPMI 1640 without phenol red. Cells were stained with Ctx-B to label lipid rafts. Antibody-coated beads (40 μ l) were added to the cells before analysis for lipid rafts and EGFP polarization. In other experiments, HLA-E-transfected RMA-S cells were cultured in the chambers with VMAPRTVLL peptide and CD94/NKG2A-EGFP RBL cells were added.

Online Supplementary Material Z-stacks were collected with a 63 $\times$ oil immersion objective, NA 1.32, with z-step thickness of 0.2 μ m. The movie was exported as a rotation of the image stack using the 3D reconstruction feature in Imaris v.4.0.1.

RESULTS

Characterization of CD94/NKG2A-EGFP Heterodimer

To investigate the membrane dynamics of CD94/NKG2A receptors, RBL-2H3 cells were transfected with plasmids encoding CD94 and NKG2A with EGFP fused to its intracellular tail. RBL-2H3 cells were selected because they do not express the ligand of CD94/NKG2A, allowing us to study CD94/NKG2A lateral mobility with or without receptor cross-linking. Stable clones were shown to express the CD94/NKG2A-EGFP heterodimer as shown by immunoprecipitation and immunoblot analyses (Figure 1A). Confocal microscopy showed that CD94/NKG2A-EGFP was expressed in the plasma membrane and in unidentified intracellular compartments (Borrego *et al.*, 2002b; Figure 1B).

We then determined whether or not CD94/NKG2A-EGFP could function equivalently to wild-type CD94/NKG2A in the RBL cell transfectants (Kabat *et al.*, 2002). To determine if NKG2A-EGFP can be tyrosine phosphorylated, cells were lysed after being treated with pervanadate to inhibit phosphatases. Immunoblot analysis showed that NKG2A-EGFP is tyrosine phosphorylated comparably to the wild-type NKG2A (Figure 1C). In addition, we showed through coimmunoprecipitation and immunoblot analyses that the attachment of EGFP to NKG2A does not interfere with the association of the SHP-1 phosphatase to phosphorylated NKG2A-EGFP (Figure 1D). As with the native CD94/NKG2A (Kabat *et al.*, 2002), the SHP-2 phosphatase was also coimmunoprecipitated with NKG2A-EGFP (unpublished data). These results indicated that CD94/NKG2A-EGFP expressed in RBL-2H3 cells retains its functional capabilities.

We verified that this functional capacity was viable by demonstrating that CD94/NKG2A-EGFP can inhibit activation of this mast cell line. To do this, we utilized a serotonin release assay to determine whether or not signaling events generated by cross-linking Fc ϵ R1 expressed by RBL-2H3 cells could be inhibited by coligating Fc ϵ R1 and CD94/NKG2A-EGFP. Figure 1E shows that when Fc ϵ R1 and

CD94/NKG2A-EGFP are coligated, serotonin release is reduced by ~40% compared with Fc ϵ R1 cross-linking alone. We also observed that the conjugation of CD94/NKG2A-EGFP RBL-2H3 cells with anti-Fc ϵ R1 α antibody-coated beads induces Ca²⁺ influx that is significantly reduced when cells are conjugated with beads coated with both anti-Fc ϵ R1 α and anti-NKG2A antibodies (unpublished data). Altogether, these results confirm that CD94/NKG2A-EGFP expressed on RBL-2H3 cells functions comparably to that previously shown for wild-type CD94/NKG2A (Kabat *et al.*, 2002).

CD94/NKG2A Mobility in the Absence of Cross-linking

We next examined by FRAP analysis (Lippincott-Schwartz *et al.*, 2001) the diffusion properties of CD94/NKG2A-EGFP receptors within the plasma membrane. To do this, an ROI in the plasma membrane is selected and the CD94/NKG2A-EGFP molecules in this region are photobleached by exposure to a laser beam (see MATERIALS AND METHODS). The diffusion properties of CD94/NKG2A-EGFP molecules can then be determined by measuring the rate of fluorescence recovery in this region. As shown in Figure 2, A and B, fluorescence is recovered in the bleached area, indicating that unbleached CD94/NKG2A-EGFP molecules are free to migrate into this region and the intensity of the recovered fluorescence in the bleached region almost reaches the same value as the membrane fluorescence intensity outside of the bleached region. This indicates that the majority, 65%, of the CD94/NKG2A-EGFP molecules exist as a membrane mobile fraction. The treatment of cells with the protein synthesis inhibitor cyclohexamide (CHX) had no significant effect on the fluorescence recovery in the bleached region (Figure 2, C and D), indicating that the lateral mobility within the plasma membrane is not dependent on protein synthesis. This indicates that newly synthesized molecules, either CD94/NKG2A-EGFP or NKG2A-EGFP, are not directly contributing to the fluorescence recovery. From the rate of recovery, the diffusion coefficient (D_{eff}) can be calculated, which reflects the mean squared displacement that the mobile fraction of a protein explores through a random walk over time (Lippincott-Schwartz *et al.*, 2001). Key factors that affect the diffusion of membrane proteins are the viscosity of the environment and the extent of their association with other molecules or cellular processes such as the cytoskeleton. The D_{eff} of CD94/NKG2A-EGFP is 0.067 μ m² s⁻¹, which is comparable to values obtained for other plasma membrane receptors such as E-cadherin, lutenizing hormone receptor, and TCR (Table 1). These data indicate that in the absence of ligation the CD94/NKG2A receptor can move relatively freely within the plasma membrane.

CD94/NKG2A Lateral Mobility and Receptor Clustering after Cross-linking

After demonstrating that the majority of CD94/NKG2A-EGFP receptors can move freely within the plasma membrane, we examined how this lateral mobility is impacted by receptor ligation. For these experiments, we used two different types of ligands. In the first, we made use of mAb-coated beads as surrogate target cells. In this experimental approach, only CD94/NKG2A-EGFP receptors on the cell surface of RBL-2H3 cells are ligated. In the second case, target cells, RMA-S, expressing the natural ligand, HLA-E (Borrego *et al.*, 1998; Brooks *et al.*, 1999), were used to ligate the CD94/NKG2A-EGFP receptor. Clearly the latter system is more physiological, but has the potential for complication by other interactions between the effector and target cells. Comparison of results obtained with the two ligands al-

Figure 1. Biochemical and functional characterization of CD94/NKG2A-EGFP RBL cells. (A) Whole cell lysates of RBL cells transfected with CD94/NKG2A (lanes 1 and 3) or CD94/NKG2A-EGFP (lanes 2 and 4) were run on SDS-PAGE, transferred to nitrocellulose, and detected with anti-NKG2A (clone 8E4) and anti-EGFP mAb. (B) Confocal microscopic images of CD94/NKG2A-EGFP RBL cells. (C) CD94/NKG2A RBL cells (lanes 1, 2, 5, and 6) and CD94/NKG2A-EGFP RBL cells (lanes 3, 4, 7, and 8) were treated or not treated with pervanadate (PV), and whole cell lysates were subjected to immunoprecipitation with anti-CD94 mAb. Immunoprecipitates were electrophoresed by SDS-PAGE and transferred to nitrocellulose membranes for reaction with anti-NKG2A (clone 8E4) and antiphosphotyrosine mAb. (D) From CD94/NKG2A-EGFP RBL cells treated with pervanadate, anti-CD94 mAb immunoprecipitates were fractionated by SDS-PAGE, transferred to nitrocellulose, and developed with anti-SHP-1 and anti-NKG2A (clone 8E4) mAb. (E) Serotonin release after ligation of FcεRI or coligation of FcεRI (20 μg/ml IgE anti-DNP mAb + DNP-BSA) and CD94/NKG2A (20 μg/ml anti-NKG2A mAb). As control, coligation IgG of the appropriate isotype was substituted for CD94/NKG2A mAb (IgE+IgG).

lowed us to assess whether or not CD94/NKG2A mobility after ligation with a "pure" cross-linking reagent led to results that differed in any way from those obtained with the natural ligand.

Figure 3, A and B, shows that CD94/NKG2A-EGFP RBL-2H3 transfected cells can form conjugates with anti-NKG2A or anti-CD94 mAb-coated beads. As controls, we show that cells are also able to form conjugates with anti-FcεRIα mAb-coated beads, but not with anti-CD8 mAb-coated beads. Data presented in Figure 3C show that CD94/NKG2A-EGFP enriches at the site of contact with the beads. This accumulation observed with mAb to CD94 or NKG2A is specific as indicated by the fact that anti-FcεRIα mAb-coated beads formed conjugates but did not polarize CD94/NKG2A-EGFP (see Figures 5C and 6A). Sometimes, as for example in Figure 5C, it is possible to see an accumulation of signal in the GFP channel in the center of the mAb-coated beads. We were concerned that this spurious fluorescence could affect

the accuracy of our GFP signal readings at the contact sites. We ruled out this possibility by showing that the GFP signal at the contact sites can be completely suppressed by photobleaching, whereas it is impossible to photobleach the signal emanating from the beads themselves (unpublished data). Quantification of the enrichment of CD94/NKG2A-EGFP was done by taking sections in the z-plane of the cells and analyzing the fluorescence intensity in each plane. Figure 3D shows the mean fluorescence intensity (MFI) for each section at the region in contact with the bead, along with the corresponding values for a remote membrane region of the same cell (see also Figure 6A).

We then analyzed the lateral mobility by FRAP analysis of the CD94/NKG2A-EGFP ligated by anti-CD94 or anti-NKG2A mAb-coated beads. Figure 3, E and F, depicts prebleached and postbleached images of the CD94/NKG2A-EGFP enriched at the site of contact between cells and beads, showing that there is recovery in the bleached region. How-

Figure 2. Fluorescence recovery after photobleaching. (A) Areas in the plasma membrane of CD94/NKG2A-EGFP RBL cells were selected for photobleaching. Images are of prebleached (right panel) and 9 s (middle panel) and 187 s (left panel) after bleaching. (B) Fluorescence intensity at various time points for a bleached and non-bleached region is represented as the percentage of MFI for CD94/NKG2A-EGFP in the plasma membrane. (C and D) Analogous data for cells treated with CHX.

Table 1. Diffusion coefficients of plasma membrane GFP fusion proteins

Molecule	D ($\mu\text{m}^2 \text{s}^{-1}$)
E-cadherin-GFP (Adams <i>et al.</i> , 1998)	0.03–0.04
TCR-GFP (Favier <i>et al.</i> , 2001)	0.12
GFP-aquaporin (Umenishi <i>et al.</i> , 2000)	0.009
Luteinizing hormone receptor-GFP (Horvat <i>et al.</i> , 1999)	0.16
CD94/NKG2A-GFP	0.067

ever, the recovery was dramatically reduced in the areas of conjugation compared with nonconjugated CD94/NKG2A-EGFP, indicating that receptor migration is retarded by engagement with the immobilized mAb (Table 2). Nevertheless, the fact that there is some recovery in the bleached area indicates that some CD94/NKG2A-EGFP receptor outside the engaged area could migrate to the inhibitory signaling domain. These results indicate that ligated CD94/NKG2A-EGFP receptors, as might be expected, are less mobile than unligated receptors. The reduced mobility is a direct consequence of the interaction with its ligand because when the cells are in contact with anti-Fc ϵ R1 α mAb-coated beads, the mobility of CD94/NKG2A-EGFP is the same as in membranes not interacting with beads (Figure 3, G and H, and Table 2).

The use of mAb-coated beads allowed us to assess membrane fluidity of ligated CD94/NKG2A receptors in the absence of secondary effector/target cell interactions, but, on the other hand, it is possible that the observed dramatic decrease in the CD94/NKG2A-EGFP receptor mobile fraction after ligation is a consequence of high affinity between immobilized mAb and the receptor (Vales-Gomez *et al.*, 1999). Therefore, for comparison, we examined CD94/NKG2A enrichment and mobility after ligation with target cells expressing the natural ligand, HLA-E. Stable cell surface HLA-E expression was achieved by preincubating HLA-E-transfected RMA-S cells, RMA-SE, with the HLA-B7–derived signal sequence peptide as previously described (Borrego *et al.*, 1998; Brooks *et al.*, 1999). CD94/NKG2A-EGFP RBL cells only form conjugates when HLA-E is stabilized on the cell surface with appropriate peptides. RMA-SE cells incubated with irrelevant peptide or no peptide did not stably express HLA-E and failed to form conjugates (Figure 4A). Interaction of CD94/NKG2A-EGFP RBL cells with cell surface stabilized HLA-E expressing RMA-S cells induced a strong accumulation of CD94/NKG2A-EGFP to the contact site between these cells (Figure 4B). Photobleaching experiments showed a dramatic decrease in the receptor mobile fraction at the contact site similar to the decrease obtained with mAb antibody-coated beads (Figures 4C, 4D and Table 2). These data clearly indicate that contact with potential target cells bearing the ligand of CD94/NKG2A inhibitory receptors results in enrichment at the sites of contact, along with a consequent marked reduction in the lateral mobility.

Role of Lipid Rafts

Finally, we examined if lipid rafts are involved in the accumulation of ligated CD94/NKG2A-EGFP. To do this, we used cholera toxin B (Ctx-B) to label lipid raft-associated ganglioside GM1 on the receptor-bearing RBL-2H3 cells and anti-CD94 mAb or anti-NKG2A mAb-coated beads as surrogate targets. Some proportion of membrane-bound Ctx-B may not concentrate in lipid rafts (Kenworthy *et al.*, 2000;

Nichols, 2003), but because of our results with the Fc ϵ R1-activating receptor (see below) and supporting results with KIR inhibitory receptors published by others (Lou *et al.*, 2000; Fasset *et al.*, 2001), we are confident that the majority of the Ctx-B staining is in membrane lipid rafts. As it is shown in Figure 5A (top and middle), clustering of CD94/NKG2A-EGFP occurs, but lipid rafts are not polarized. Not only are they not polarized but they are excluded from the area where CD94/NKG2A-EGFP accumulates, as indicated by the failure to observe colocalization in the Figure 5 overlays. To further emphasize this point, the Supplementary Video to Figure 5A shows an enlarged rotating view of this contact site. Quantification of CD94/NKG2A-EGFP and of Ctx-B at the site of contact with mAb-coated beads is presented in Figure 6A. In addition to that, we have also used DiI-C₁₈ to label the plasma membrane. DiI-C₁₈ preferentially partitions into ordered lipid domains and can be used to localize raft formations in living cells (Gousset *et al.*, 2002). Results in Figure 5A (bottom) show that DiI-C₁₈ is also excluded at the site of contact with anti-NKG2A mAb-coated beads. When HLA-E-transfected RMA-S cells are used as targets, a similar deficiency in lipid rafts is usually observed at the contact sites containing polarized CD94/NKG2A-EGFP (Figures 5B and 6A). These results indicate that ligated CD94/NKG2A-EGFP accumulates in the cell membrane to sites external of lipid rafts. As a control, we showed that lipid rafts accumulate at the site of cell contact with anti-Fc ϵ R1 α mAb-coated beads, whereas CD94/NKG2A-EGFP does not (Figures 5C and 6A). To confirm that lipid rafts play little or no role in CD94/NKG2A-EGFP enrichment, we treated the cells with the cholesterol-depleting reagent methyl- β -cyclodextrin (MCD). CD94/NKG2A-EGFP polarization was unimpeded in MCD-treated cells, confirming that disruption of cholesterol-dependent lipid rafts has no effect on the lateral mobility of CD94/NKG2A-EGFP receptors for the formation of an inhibitory signaling domain (Figure 5A, bottom).

Ligation of NK cell activation receptors has been shown to result in the polarization of lipid rafts at the NKIS (Lou *et al.*, 2000; Vyas *et al.*, 2001, 2002b; McCann *et al.*, 2002). Therefore, we examined the consequence of coligating an activating receptor with the CD94/NKG2A inhibitory receptor. Ligation of Fc ϵ R1 by anti-Fc ϵ R1 α mAb-coated beads, which mimic the cross-linking of Fc ϵ R1 receptor by IgE, showed that, as expected, lipid rafts are enriched as indicated by patches formed at the site of cell contact with anti-Fc ϵ R1 α mAb-coated beads (Figures 5C, top, and 6A). CD94/NKG2A-EGFP is not polarized and does not form patches at these sites. As shown in Figure 5C, anti-Fc ϵ R1 α mAb-coated beads are often localized inside the cell indicating that Fc ϵ R1 is rapidly internalized after cross-linking in agreement with previously reported results (Ra *et al.*, 1989; Mao *et al.*, 1993; Barker *et al.*, 1995). Fc ϵ R1 receptor-mediated signals are known to induce phagocytosis (Massol *et al.*, 1998). The internalization of anti-Fc ϵ R1 α mAb-coated beads is observed in 20–30% of the cells (unpublished data). In those situations where the bead was not internalized, CD94/NKG2A-EGFP also did not enrich at the site of contact with the bead (Figure 5C, second panel). In addition, we have examined the formation of lipid rafts patches at the site of contact with anti-Fc ϵ R1 α mAb-coated beads for cells treated with MCD. As expected, the anti-Fc ϵ R1 α mAb-coated beads never internalized and we no longer observed formation of lipid rafts patches at the site of contact with the beads (Figure 5C, third row). To determine if engagement of CD94/NKG2A receptor would interfere with Fc ϵ R1-induced lipid raft polarization, we incubated CD94/NKG2A-EGFP RBL-2H3-transfected cells with beads coated with both anti-NKG2A and

Figure 3. CD94/NKG2A-EGFP polarizes toward the site of contact with mAb-coated beads and exhibits a decreased lateral mobility. (A) CD94/NKG2A-EGFP RBL cells were mixed with mAb-coated beads for 10 min, and flow cytometric analysis was done to show the binding between cells and beads. mAb on the beads are as indicated. (B) Percentage of mAb-coated beads bound to CD94/NKG2A-EGFP RBL cells at different time points. (C) DIC and GFP image showing CD94/NKG2A-EGFP polarized toward the site of contact with anti-NKG2A-coated beads. (D) MFI for z sections through the site of contact with beads and a nonligated area of the plasma membrane.

anti-FcεRIα mAb. In this case CD94/NKG2A-EGFP is enriched at the site of contact with the beads (Figure 5C, bottom), but lipid rafts are excluded, indicating that CD94/NKG2A-EGFP-mediated inhibitory signal is able to inhibit FcεRI-mediated lipid raft polarization. Figure 6B illustrates this quanti-

tatively by showing the percentage of contact site synapses that exclude lipid rafts when CD94/NKG2A, FcεRI or both are ligated. This result is in agreement with our observation that inhibition of IgE-mediated serotonin release is inhibited by CD94/NKG2A-EGFP (Figure 1E). In addition, internalization

Figure 3 (cont). (E) DIC and GFP images of the site of interaction between anti-NKG2A mAb-coated beads and CD94/NKG2A-EGFP RBL cells. Left panel, before photobleaching; middle panel, 9 s after photobleaching; right panel, 180 s after photobleaching. (F) CD94/NKG2A-EGFP fluorescence after photobleaching at the site of contact of cells with mAb-coated beads compared with a noncontact site. (G) DIC and GFP images at the site of interaction between anti-FcεR1α mAb-coated beads and CD94/NKG2A-EGFP RBL cells. Left panel, before photobleaching; middle panel, 9 s after photobleaching; right panel, 180 s after photobleaching. (H) CD94/NKG2A-EGFP fluorescence after photobleaching at the site of contact of cells with mAb-coated beads compared with a noncontact site.

Figure 4. CD94/NKG2A-EGFP interacting with its natural ligand shows decreased lateral mobility. (A) Percentage of RMA-SE cells forming conjugates with CD94/NKG2A-EGFP RBL cells. HLA-E-transfected RMA-S cells were incubated overnight with or without peptides as indicated, after washing away the unbound peptide, they were mixed with CD94/NKG2A-EGFP RBL cells and conjugates were measured by flow cytometry. (B) HLA-E-transfected RMA-S cells preincubated with VMAPRTVLL peptide were grown in glass coverslips wells for 2 days, and then CD94/NKG2A-EGFP RBL cells were added and the mixture was analyzed by confocal microscopy. (C) DIC and GFP images of the site of interaction between RMA-S cells and CD94/NKG2A-EGFP RBL cells. Left panel, before photobleaching; middle panel, 9 s after photobleaching; right panel, 180 s after photobleaching. (D) Fluorescence recovery at the site of interaction between CD94/NKG2A-EGFP RBL cells and HLA-E-transfected RMA-S cells compared with fluorescence at a noncontact site.

of mAb-coated beads was significantly decreased when activation and inhibitory receptors were simultaneously engaged (unpublished data), suggesting that inhibitory signals controls FcεR1 receptor-mediated internalization.

DISCUSSION

Our analysis using CD94/NKG2A-EGFP transfectants revealed that CD94/NKG2A receptors are homogeneously distributed throughout the plasma membrane and within as yet unidentified intracellular compartments (Borrego *et al.*, 2002b). In the absence of cross-linking, the majority of CD94/NKG2A receptors are free to diffuse within the plasma membrane. However, 25–30% of the receptors are immobile. This immobility might be due to receptors that are irreversibly bound to a fixed/anchored substrate or non-

diffusional factors such as diffusion barriers or discontinuities within the plasma membrane, which could explain the reduced mobility (Lippincott-Schwartz *et al.*, 2001). We plan to investigate these possibilities. Comparison to the data in the literature indicates that nonligated CD94/NKG2A diffusion properties are within the range of other cell surface receptors (see Table 1). When CD94/NKG2A-EGFP-expressing RBL cells are coincubated with cell surface stabilized HLA-E-expressing cells or with mAb-coated beads, receptors accumulated at the contact sites. These results are consistent with those obtained for inhibitory KIR-expressing cells after coincubation with HLA-C positive target cells (Davis *et al.*, 1999; Borszcz *et al.*, 2003; Faure *et al.*, 2003). There is the possibility that some of the receptor enrichment at the site of contact with the ligand is due to submicroscopical folds and ruffles in the membrane (van Rhee and

Figure 5. Lipid rafts are not required and are excluded from contact sites where CD94/NKG2A-EGFP is ligated. (A) CD94/NKG2A-EGFP RBL cells were labeled with Alexa 594 Ctx-B or Alexa 594 DiI-C₁₈ (lower panel) and mixed with anti-CD94 or anti-NKG2A mAb-coated beads in the absence or presence of MCD. Left panels, CD94/NKG2A-EGFP (green); middle panels, Ctx-B or DiI-C₁₈ staining (red); right panels, overlay of both images. (B) CD94/NKG2A-EGFP RBL cells were labeled with Alexa 594 Ctx-B and mixed with HLA-E-transfected RMA-S incubated with VMAPRTVLL peptide. Left panel, CD94/NKG2A-EGFP (green); middle panel, Ctx-B staining (red); right panel, overlay of both images. (C) CD94/NKG2A-EGFP RBL cells were labeled with Alexa 594 Ctx-B and mixed with anti-Fc ϵ R1 α mAb-coated beads or anti-Fc ϵ R1 α mAb and anti-NKG2A mAb-coated beads in the absence or presence of MCD. Left panels, CD94/NKG2A-EGFP (green); middle panels, Ctx-B staining (red); right panels, overlay of both images. A star shows where the internalized bead is located. Figures are representative of two to five experiments in each condition. Five to 25 cells were analyzed in each experiment.

A **B**

Figure 6. Relative CD94/NKG2A-EGFP and raft concentrations at contact sites, and the percentage of contact sites synapses that exclude lipid rafts. (A) MFI of CD94/NKG2A-EGFP and Ctx-B-labeled lipid rafts from CD94/NKG2A-EGFP-transfected cells measured at the site of contact with mAb-coated beads or HLA-transfected RMA-S cells with VMAPRTVLL peptide. % of polarity = fluorescence at the contact site/fluorescence at noncontact site $\times$ 100. (B) Percentage of contact sites synapses that exclude lipid rafts.

Jalink, 2002). This possibility requires further analyses by electron microscopy. FRAP analysis clearly demonstrated that the mobility of ligated CD94/NKG2A-EGFP is markedly reduced. Although not previously investigated for other inhibitory receptors, dramatically retarded mobility after receptor cross-linking has been observed for other receptors. Other cell surface receptors, such as bombesin/

gastrin releasing peptide receptor, are similarly immobilized after binding ligand (Young *et al.*, 2001). Yet it is not clear that receptor ligation always results in decreased lateral mobility; some investigators have shown that TCR-CD3 complexes exhibit a relatively fast lateral mobility that resembles measurements for other cell surface receptors (Favier *et al.*, 2001). After receptor cross-linking with mAb-coated beads, the authors found little or no difference in the D_{eff} and only a small decrease in the mobile fraction (Favier *et al.*, 2001). However, another study (Grakoui *et al.*, 1999) showed that fluorescein-labeled MHC-peptide complexes within the mature synapse are kept immobile by engagement with TCR, suggesting that TCR itself is immobilized. The discrepancy between those studies could be related to the different methods and/or cell types used to study the dynamics of TCR. Other possible explanations for the discrepancy in the results obtained by these investigators could be the nature of the ligand used: anti-TCR mAb vs. MHC class I ligands. In our study, we obtained nearly identical results when CD94/NKG2A is ligated by either type of ligand, mAb-coated beads or the natural ligand HLA-E. Ligation of CD94/NKG2A inhibitory receptors results in the polarization and subsequent immobilization of the receptor at

Table 2. Recovery of CD94/NKG2A-EGFP fluorescence after photobleaching

Conditions	% recovery $\pm$ SD
None	67 $\pm$ 9
CHX ^a	66 $\pm$ 15
Anti-CD94 or anti-NKG2A mAb coated beads ^b	20 $\pm$ 1
Anti-FcεR1α mAb coated beads ^b	63 $\pm$ 11
RMA-SE cells ^b	16 $\pm$ 9

^a Cells grown in the presence of cyclohexamide.

^b Measured at the site of contact with these entities. Calculation for each condition was done for 8 to 12 times in 3 to 5 independent experiments.

the contact site between the receptor bearing cell and the ligand bearing entity.

Although polarization of the inhibitory receptors does not involve protein synthesis (Figure 2, C and D), the cytoskeleton, energy processes, or a signaling competent receptor (Fassett *et al.*, 2001), it is not known if these receptors polarize strictly by diffusion and coalescence at the synaptic contact site. The interaction between CD94/NKG2A and HLA-E has a very fast association and dissociation rate constant when measured in solution (Vales-Gomez *et al.*, 1999). Although it is difficult to directly translate this first-order kinetics to interactions occurring at membrane interfaces, it opens the possibility that the reduced mobility at the site of enrichment need not just be a matter of passive restraint due to ligation by HLA-E, but it may be a more complex process. Nonetheless, we suggest that not only enrichment but also immobilization of NK cell inhibitory receptors at the site of cell contact is a requirement to sustain a localized inhibitory signal within cells that are continuously poised to kill cells that they encounter. Recent evidence indicates that dominance of the inhibitory signal is achieved once the concentration of inhibitory receptor, KIR in this case, exceeds a threshold at the contact site (Borszcz *et al.*, 2003), suggesting that polarization alone of the receptor at the contact site with target cell is not enough for a sustained inhibitory signal and that other factors are playing a role in signal transmission. Our data support the notion that immobilization at the contact site with the target cell is also probably required for sustaining a fully competent inhibitory signal.

Ligation of TCR on CD8⁺ T cells by stimulatory antigens leads to the formation of a complex structure with distinct signaling and secretory domains termed the IS (Stinchcombe *et al.*, 2001). When NK cells encounter a sensitive target cell, they form a similar structure that has been termed the activating NKIS (Davis *et al.*, 1999; McCann *et al.*, 2002; Vyas *et al.*, 2002b). The formation of these structures along with the spatial organization of intracellular signaling molecules requires an intact cytoskeleton and ATP (Lou *et al.*, 2000; Vyas *et al.*, 2001, 2002b; McCann *et al.*, 2002). In these activating IS formed by immune cells, lipid rafts are also polarized at the site of contact with target cells, which appears to be a necessary platform for the enrichment of molecules involved in transmitting activating signals (Dykstra *et al.*, 2003). Inhibitory NK cell receptors localize to sites of contact with potential target cells to a region termed the inhibitory NKIS that differs markedly from the activating IS described above. Inhibitory KIR receptors polarize independent of the cytoskeleton and do not require an energy source (Fassett *et al.*, 2001). Moreover, it has been shown for inhibitory KIR (Fassett *et al.*, 2001) and for CD94/NKG2A (Figures 5 and 6) that lipid rafts are apparently excluded from the cellular contact points, indicating that they are not involved in receptor polarization and immobilization.

A vast number of transmembrane proteins are excluded from lipid rafts and they are not translocated to lipid rafts upon ligation or oligomerization (Simons and Toomre, 2000; Dykstra *et al.*, 2003). A small group of proteins reside constitutively in lipid rafts, and S-palmitoylation is required for this residency (Dykstra *et al.*, 2003). Finally, some integral membrane proteins normally exist outside lipid rafts but when ligated they become raft associated (Simons and Toomre, 2000; Dykstra *et al.*, 2003). Multichain immune recognition receptors, which include the T-cell receptor (TCR), B-cell receptor (BCR), and the FcεR1 receptor, are examples of this group (Simons and Toomre, 2000; Dykstra *et al.*, 2003). Cross-linking alone does not

necessarily lead to association with lipid rafts, as has been demonstrated for CD45 and the type I IL-1 receptor (Field *et al.*, 1999; Dykstra *et al.*, 2001). We have shown here that lipid rafts are excluded from the site of contact with the ligand, suggesting that CD94/NKG2A receptors do not associate with lipid rafts after ligation. This is in contrast with the FcγRIIB1 inhibitory receptor, which requires association with lipid rafts for the transmission of the inhibitory signal (Aman *et al.*, 2001). Similar to CD94/NKG2A receptors, this receptor requires the ITIMs present in the intracellular tail for the signal transmission, but, unlike CD94/NKG2A receptors, it binds the SHIP phosphatase instead of the SHP-1/2 phosphatases. The CTLA-4 inhibitory receptor expressed by T cells is translocated to lipid rafts after T cells encounter antigen, and this partition into lipid rafts is required for transmission of the inhibitory signal (Darlington *et al.*, 2002). CTLA-4 does not have ITIMs in the intracellular tail and SHP-1 is not involved in the signaling cascade generated from this receptor (Darlington *et al.*, 2002). Thus the CD94/NKG2A and KIR NK inhibitory receptors are so far unique in that they transmit dominant inhibitory signals from outside the lipid rafts.

Lymphocyte activation receptors seem to require lipid rafts for transmission of signals, and CD94/NKG2A NK cell inhibitory receptors are prominent for their exclusion of lipid rafts from the NKIS (see Figures 5 and 6). When NK cells encounter susceptible target cells, there is a polarization of lipid rafts as a result of the cross-linking of NK activation receptors by ligand-bearing target cells (Lou *et al.*, 2000; Vyas *et al.*, 2001, 2002b; McCann *et al.*, 2002). This enrichment of lipid rafts is directly correlated with the susceptibility of target cells to NK cell-mediated killing (Lou *et al.*, 2000; Vyas *et al.*, 2001, 2002b; McCann *et al.*, 2002). It has been shown that lipid rafts do not enrich when target cells express ligands for KIR inhibitory receptors (Lou *et al.*, 2000; Vyas *et al.*, 2001, 2002b; McCann *et al.*, 2002). Our results clearly show that when both activating receptors (FcεR1) and inhibitory receptors (CD94/NKG2A-EGFP) are coengaged, CD94/NKG2A-EGFP is enriched and lipid rafts are excluded at the site of cell contact (Figures 5 and 6).

From these results it is tempting to speculate that active exclusion of lipid rafts from the inhibitory NKIS plays a role in the predominance of inhibitory signals over activation signals. In support of this finding, it has been described that KIR-mediated inhibitory signals control the access of activating receptors to lipid rafts (Watzl and Long, 2003). CD244 (or 2B4), an activating receptor expressed by NK cells, is translocated to lipid rafts after engagement by its ligand. Evidence indicates that after translocation to the lipid rafts 2B4 is phosphorylated and initiates the signaling cascade. It has been shown that coengagement of 2B4 and KIR blocks actin cytoskeleton-dependent translocation of 2B4 to the lipid rafts and, as a consequence, 2B4 is not phosphorylated and is not able to initiate the signaling cascade. Despite this preliminary data, it is not clear whether exclusion of lipid rafts from NK cell/target cell synaptic contact points is an active mechanism involved in inhibitory signal transmission or, it is a secondary effect of inhibiting the translocation of activating receptors to lipid rafts, and thereby preventing assembly of lipid rafts capable of targeting to synaptic contact points. In any case, the response of inhibitory receptors must be rapid and predominant.

In summary, our data with CD94/NKG2A receptors suggest that the polarization and stabilization of the in-

hibitory receptors and exclusion of lipid rafts at the inhibitory signaling domain in the inhibitory NKIS act to sustain an inhibitory signal spatially localized in NK cells and also impede the development of activation signals. This would serve to prevent the killing of normal cells engaged at the contact site, while at the same time allowing the recognition and lysis of susceptible target cells at distant contact sites.

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