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The Size of the Synaptic Cleft and Distinct Distributions of Filamentous Actin, Ezrin, CD43, and CD45 at Activating and Inhibitory Human NK Cell Immune Synapses

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In this study, we report the organization of cytoskeletal and large transmembrane proteins at the inhibitory and activating NK cell immunological or immune synapse (IS). Filamentous actin accumulates at the activating, but not the inhibitory, NK cell IS. However, surprisingly, ezrin and the associated protein CD43 are excluded from the inhibitory, but not the activating, NK cell IS. This distribution of ezrin and CD43 at the inhibitory NK cell IS is similar to that previously seen at the activating T cell IS. CD45 is also excluded from the inhibitory, but not activating, NK cell IS. In addition, electron microscopy reveals wide and narrow domains across the synaptic cleft. Target cell HLA-C, located by immunogold labeling, clusters where the synaptic cleft spans the size of HLA-C bound to the inhibitory killer Ig-like receptor. These data are consistent with assembly of the NK cell IS involving a combination of cytoskeletal-driven mechanisms and thermodynamics favoring the organization of receptor/ligand pairs according to the size of their extracellular domains. The Journal of Immunology, 2003, 170: 2862–2870.

Natural killer cells are large granular lymphocytes that are cytotoxic to some tumors and virus-infected cells (1). The outcome of NK cell immune surveillance is the result of a complex balance between inhibitory and activating signaling (2–9). Killer Ig-like receptors (KIR)* with two Ig domains, KIR2DL1 and KIR2DL2, trigger inhibitory signaling upon recognition of the class I MHC proteins HLA-Cw4 or -Cw6, and HLA-Cw3 or -Cw7, respectively (10, 11). Immunological intercellular contacts between effector and target cells or APCs can involve the organization of proteins into micrometer scale domains at intercellular contacts (12–16) creating an immunological or immune synapse (IS) (17–19). This has been most extensively studied in the T cell/APC conjugate, (12, 13, 15, 20, 21). The balance between activating and inhibitory signaling also controls the supramolecular organization of proteins at the NK cell IS (14, 22–25). Molecular mechanisms for the assembly of the IS include a role for the cytoskeleton (20, 21, 26–30), segregation of proteins according to the size of their extracellular domains (28, 31–36), and association of proteins with lipid rafts (37–41).

Previously, it has been shown that HLA-C still clusters at the inhibitory NK cell IS in the presence of drugs that deplete ATP or disrupt actin, myosin motors, or microtubules, suggesting that the cytoskeleton is not necessary for accumulation of at least some proteins at the inhibitory NK cell IS (14, 24). However, within the first minute following intercellular contact, talin clusters at both the activating and inhibitory NK cell IS, though at the inhibitory NK cell IS talin then redistributes away from the IS in the ensuing five minutes (22). The speed of the redistribution of talin compares with the speed that phosphorylated lck accumulates at the activating T cell IS just two minutes after intercellular contact, closely followed by recruitment of the cytoskeletal-associated protein of 70 kDa (42).

A role for the ezrin-radixin-moesin (ERM) proteins, linking the actin cytoskeleton to certain transmembrane proteins such as CD43, (reviewed in Refs. 43–45), has been proposed for the organization of the activating T cell IS. Ezrin was either excluded from (46), or clustered at the peripheral edges of (47), the activating T cell IS (48, 49). A dominant-negative ezrin, lacking the actin-binding domain, failed to redistribute CD43 away from the activating T cell IS (46). The phosphorylation status of moesin was also observed to regulate the exclusion of CD43 from the activating T cell IS (50). Furthermore, a mutant of CD43 unable to bind ERM proteins was no longer excluded from the activating T cell IS, resulting in reduced IL-2 production (50). In contrast, another study reported that CD43 mutated to either lack the full cytoplasmic domain or just the ERM binding motif can occupy the center of the activating T cell IS, without subsequently disrupting T cell proliferation in response to Ag (51). Significantly, the exclusion of CD43 from the activating T cell/dendritic cell IS has been demonstrated in vivo within a mouse lymph node (52). Ezrin has been shown to be capable of redistributing ICAM-2 and sensitizing target cells to NK cytotoxicity (53). In this study, the distribution of filamentous actin (f-actin), ezrin, CD43, and CD45 at the inhibitory and activating NK cell IS is reported.

In addition to cytoskeletal-mediated movement, another mechanism that may contribute to the formation of the IS involves the...
segregation of proteins according to the size of their extracellular domains (28, 31–35). Pairs of proteins with longer extracellular domains such as the integrins, spanning ~40 nm (31), would be separated from protein pairs with shorter extracellular domains, such as KIR or TCR with MHC protein which span ~15 nm (54–59). In this study, the distance across the synaptic cleft at the activating and inhibitory NK cell IS was measured and immunogold labeling was used to measure the size of the synaptic cleft where HLA-C accumulates at the inhibitory NK cell IS.

Materials and Methods

Cells and Abs

NK cell transfectants, YTS/Mock (YTS, mock-transfected), and YTS/KIR2DL1 (YTS transfected to express KIR2DL1) were previously described (60). YTS is a subclone of the human NK tumor line YT (61). An EBV-transformed B cell line 721.221 (hereafter referred to as 221) selected to lack surface expression of endogenous class I MHC protein (62), was transfected to express enhanced green fluorescent protein (GFP)-tagged HLA-Cw3, Cw4, or Cw6 (i.e., 221/Cw6-GFP, etc.), as previously described (14). Cytotoxicity assays show that YTS/KIR2DL1 is effectively inhibited from killing 211 transfected with HLA-Cw6 (i.e., 221/Cw6 or 221/Cw4, but not 221/Cw3 or 221/Cw7, target cells (60). An activating NK cell IS is created when the intercellular contact between the NK and target cell results in lysis of the target cell. In contrast, an inhibitory NK cell IS is created at the intercellular contact of noncytolytic conjugates between the NK cell and target cell. Thus, conjugates between YTS/KIR2DL1 and 221/Cw6-GFP create an inhibitory NK cell IS and conjugates of YTS/Mock and 221/Cw6-GFP or YTS/KIR2DL1 and 221/Cw3-GFP create an activating NK cell IS.

Mouse anti-human ezrin mAb (clone 18, IgG1; BD Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-human ezrin Ab (Upstate Biotechnology, Lake Placid, NY), mouse anti-human CD43 mAb (IG10, IgG1; BD PharMingen, San Diego, CA), and mouse anti-human CD45 (HI30, IgG1; BD PharMingen) were used at 10 μg/ml. Mouse anti-CD158a (EB6, IgG1; Serotec, Oxford, U.K.) was used at 10 μg/ml. Alexa Fluor 568 goat anti-mouse IgG, Alexa Fluor 633 goat anti-mouse IgG, and Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) were used at a concentration of 2 μg/ml. To phenotype cells for expression of the various CD45 isoforms, the following FITC-conjugated mAbs (Caltag Laboratories, Burlingame, CA) were used: UCHLI (IgG2a) for CD45RO, MEM56 (IgG2b) for CD45RA, and MEM55 (IgG1) for CD45RB. For double immunofluorescence labeling, both primary Abs followed the instructions. Freshly prepared rat anti-mouse IgM (R6-60.2; BD PharMingen) were used at 2 μg/ml. Alexa Fluor 568 and Alexa Fluor 633 were used for double immunofluorescence labeling. No species cross-reactivity between Alexa Fluor-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Sigma-Aldrich) and FITC-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) and FITC-conjugated rat anti-mouse IgM (R6-60.2; BD PharMingen) were used at 2 μg/ml. The cytolytic activity of NK clones against various target cells was assessed in 5-h 3H-labeled release assays as described previously (63). Assays were performed in triplicate and data values differed by ~5% of the mean. Spontaneous release of 3H was ~25% of the maximal release.

Imaging the IS

221 and YTS cell transfectants (10⁶ of each) were coincubated for 45 min at 37°C with 20,000 rad, in RPMI 1640 medium containing from human buffy coat residues that were cultured for 7 days with irradiated PBMCs. The 221 transfectants were split every 4 to 5 days in a 1:1 ratio in RPMI 1640 medium containing 4% FCS, 100 U/ml penicillin-streptomycin, (all purchased from Invitrogen), after which the cells had been diluted with culture medium. Excess medium was removed and the sedimented cells were fixed in Cytofix/Cytoperm (BD PharMingen) for 12 min at room temperature (RT). The fixed cells were then washed twice in 0.1% Tween 20/PBS before cell staining. For NK clones, the same method was used except that 2 × 10⁶ cells of each type were used and the conjugates were briefly centrifuged before fixation to ensure that the cells had sedimented before decanting excess medium. Following cell fixation, f-actin was labeled using Alexa Fluor 633-conjugated phallolidin (Molecular Probes) at 5 μM in 1% BSA/PBS, for 1 h at 4°C. Cells were washed three times in 0.1% Tween 20/PBS before imaging.

For imaging the NK cell IS at different times of coincubation, 10⁶ of both YTS and 221 transfectants were incubated in a 1:1-bottom 96-well culture plate at 37°C/5% CO₂ in RPMI 1640 medium containing 10 μM of cytochalasin D. For NK clones, the same method was used except that 2 × 10⁵ cells of each type were used and the conjugates were briefly centrifuged before fixation to ensure that the cells had sedimented before decanting excess medium. Following cell fixation, f-actin was labeled using Alexa Fluor 633-conjugated phallolidin (Molecular Probes) and 5 μM in 1% BSA/PBS, for 1 h at 4°C. Cells were washed three times in 0.1% Tween 20/PBS before imaging.

Electron microscopy

Cell conjugates were imaged using a 63X oil immersion objective using a confocal laser scanning microscope (TCS SP2; Leica, Deerfield, IL) equipped with argon/krypton and helium/neon lasers using excitation wavelengths of 488, 568, and 633 nm. Conjugates were scanned in the xy-direction every 0.3 μm throughout the z-plane. The face of the IS was then reconstructed using a maximum intensity projection (Confocal Software; Leica). To quantify the relative amounts of HLA-C, ezrin, and CD43 at the inhibitory IS within a single optical slice, their mean fluorescence intensities were obtained from a 15-μm intercellular contact. The mean intensities were then measured from the same area marked onto the unconjugated membranes of both the NK and B cell and a percentage of mean fluorescence intensity at the inhibitory IS relative to the sum of the unconjugated membranes was calculated.

Electron microscopy

Live YTS and 221 transfectants were coincubated for 45 min at 37°C/5% CO₂ as previously described for IS formation. For negative staining, cells were fixed in 2.5% glutaraldehyde/2.5% paraformaldehyde in PBS for 2 h at 4°C after which the cells were washed three times in PBS. Cells were

Phenotyping of NK clones

Abs used for flow cytometry were CyChrome-conjugated anti-human CD56 (B159), CyChrome-conjugated anti-human CD3 (UCHT1), CyChrome-conjugated IgG1 control (MOPC-21), anti-human CD94 (HP-3D9), and PE-conjugated anti-KIR2DL2 (DZ27), all purchased from BD Pharmingen. Anti-KIR2DL1 (HP3E4) was purchased from the American Type Culture Collection (Manassas, VA) and anti-Ig-like transcript-2 (HP-FI) was a kind gift from M. López-Botet (Universitat Pompeu Fabra, Barcelona, Spain). All of these Abs were used at a concentration of 1 μg/ml. FITC-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) and FITC-conjugated rat anti-mouse IgM (R6-60.2; BD Pharmingen) were used at 2 μg/ml. The cytolytic activity of NK clones against various target cells was assessed in 5-h 3H-labeled-release assays as described previously (63). Assays were performed in triplicate and data values differed by ~5% of the mean. Spontaneous release of 3H was ~25% of the maximal release.

Confocal microscopy image analysis

Cell conjugates were imaged under a 63X oil immersion objective using a confocal laser scanning microscope (TCS SP2; Leica, Deerfield, IL) equipped with argon/krypton and helium/neon lasers using excitation wavelengths of 488, 568, and 633 nm. Conjugates were scanned in the xy-direction every 0.3 μm throughout the z-plane. The face of the IS was then reconstructed using a maximum intensity projection (Confocal Software; Leica). To quantify the relative amounts of HLA-C, ezrin, and CD43 at the inhibitory IS within a single optical slice, their mean fluorescence intensities were obtained from a 15-μm intercellular contact. The mean intensities were then measured from the same area marked onto the unconjugated membranes of both the NK and B cell and a percentage of mean fluorescence intensity at the inhibitory IS relative to the sum of the unconjugated membranes was calculated.
then postfixed in 1% osmium tetroxide/0.5 M veronal acetate, pH 7.2, for 1 h at room temperature, then taken through a graded series of alcohols and embedded in Spurr’s resin. Ultrathin sections were cut using a diamond knife and stained with 5% uranyl acetate at 60°C for 30 min followed by Reynolds lead citrate for 10 min at room temperature. Sections were examined with a transmission electron microscope (Philips 410; Endoven, The Netherlands) operating at 80 kV and images were recorded on Kodak 4489 film (Rochester, NY). Distances across the synapse were measured from the negatives between the outer edges of the outer membranes at the intercellular contact between YTS and 221 transfectants, using image analysis software (Leica Q500 MC).

**Immunogold labeling**

Cell conjugates were fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBS for 10 min on ice, followed by fresh 4% paraformaldehyde/0.2% glutaraldehyde in PBS on ice for 1 h. The fixed cells were then washed three times in PBS and dehydrated through a series of alcohols as the temperature was progressively lowered to −30°C in a freeze-substitution unit (EM AFS; Leica). Next, the cells were processed in Lowicryl K4 M embedding medium (TAAB Laboratories) infiltrated overnight in 1:1 ethanol/K4 M, followed by incubation with K4 M for 24 h and finally a further incubation with fresh K4 M that was polymerized under UV light for 8 h. Ultrathin sections were cut using an ultramicrotome (UCT; Leica) and collected on Formvar support grids. The grids were blocked before labeling with 0.02 M glycine for 15 min followed by 10% FCS in PBS for 30 min at room temperature. The grids were then incubated with 20 μg/ml mouse anti-GFP (JL-8; Clontech Laboratories) in 10% FCS in PBS for 1 h at room temperature. After washing three times in PBS, the grids were incubated for 1 h in 12 μg/ml goat anti-mouse IgG conjugated with 10 nm of gold (TAAB Laboratories) in the same diluent as before. Following a further 6 washes in PBS and 10 washes in ddH₂O, the grids were contrasted with uranyl acetate and lead citrate before examination with a transmission electron microscope (Philips CM100).

**Results**

**F-actin clusters at the activating, but not the inhibitory, NK cell IS**

Conjugates of YTS/KIR2DL1 (YTS transfected to express KIR2DL1) or YTS/MOCK (mock-transfected) and 221/Cw6-GFP, 221/Cw4-GFP, or 221/Cw3-GFP were fixed and labeled with Alexa Fluor 633-conjugated phalloidin, a stain for f-actin, and were imaged by laser scanning confocal microscopy. At the intercellular contact between conjugates lacking either KIR2DL1 or its cognate MHC ligand, thus forming an activating NK cell IS, f-actin clearly clustered (Fig. 1b). Throughout this study, clustering at the IS was confirmed by projecting a single optical slice through the IS with fluorescence intensity plotted on a third axis. Where the fluorescence intensity at the IS was more than the sum of the intensity of the unconjugated membranes, that label was defined as being clustered at the IS. The fluorescence intensity of phalloidin at the activating IS (Fig. 1b) is clearly more than the sum of unconjugated membranes, whereas at the inhibitory IS (Fig. 1a) no increase in fluorescence intensity of phalloidin above the sum of unconjugated membranes was observed. At the inhibitory IS, HLA-C clustered ~70% of the time whereas f-actin accumulated only 30% of the time. In contrast, in activating synapses, f-actin was found to accumulate 70–80% of the time, while HLA-C-GFP clustered <15% of the time (Fig. 1c).

**Ezrin and CD43 are excluded from the inhibitory, but not activating, NK cell IS**

Conjugates between 221/Cw6-GFP or 221/Cw3-GFP and YTS/KIR2DL1 or YTS/Mock were fixed and either stained for ezrin and f-actin (Fig. 2) or ezrin and CD43 (Fig. 3). Consistent with analysis by flow cytometry (data not shown), the 221 transfectant often stained brighter for ezrin than the YTS cell inferring a higher level of expression in 221 cells. At the inhibitory NK cell IS, a zone devoid of ezrin and CD45 staining corresponding to the region where HLA-C clustered at the IS was evident (Figs. 2 and 3). In contrast, at the activating IS, ezrin and CD43 were evenly distributed across the membranes of apposing cells within the conjugate (Figs. 2 and 3). At the inhibitory NK cell IS, ezrin and CD43 were excluded in 50–60% of inhibitory conjugates whereas at the activating NK cell IS, exclusion of either protein occurred <10% of the time (Figs. 2d and 3f). CD43 and ezrin were equally likely to be excluded from an inhibitory NK cell IS whether each NK cell simultaneously maintains one or several synapses (data not shown). Clustering of ezrin and CD43 at either the inhibitory or activating NK cell IS was observed in <10% of all conjugates assessed. In contrast to anti-CD43 staining, an isotype-matched mAb against KIR2DL1, EB6, was observed to brightly stain the NK cell juxtaposed to where HLA-C clusters on the target cell (Fig. 3, c and e). This indicated that the staining procedure used does permit access of Abs to proteins within the inhibitory NK cell IS.

The time taken for the accumulation of HLA-C and the exclusion of ezrin and CD43 at the inhibitory NK cell IS was assessed...
by imaging cells that were fixed after different times of being incubated together (Fig. 3f). Interestingly, after 10 min of coincubation of cells, HLA-C is clustered at \(\approx 60\%\) of inhibitory NK synapses, whereas ezrin and CD43 are excluded from only \(\approx 20\%\) of these synapses. This suggests that accumulation of HLA-C precedes exclusion of ezrin and CD43 at the inhibitory NK cell IS.

The extent to which ezrin and CD43 are excluded and HLA-C-GFP is clustered at the inhibitory IS was quantified using software to analyze mean fluorescence intensities from defined regions at the inhibitory NK cell IS relative to regions on the unconjugated cell membranes, as described in Materials and Methods. From a total of 40 conjugates counted, the fluorescence intensity of ezrin and CD43 was reduced by 68% \(\pm\) 12.5 and 56.7% \(\pm\) 10.2, respectively, at the inhibitory NK cell IS relative to the amount on the two unconjugated membranes. Interestingly, there was no apparent trend between the extent of HLA-C-GFP clustering and the fluorescence intensity of ezrin or CD43 at the IS (Table I). Comparison of the amount of ezrin and CD43 staining on the NK cell and target cell with the amount at the inhibitory IS suggests that neither cell alone can account for the amount of ezrin and CD43 excluded from the IS. Thus, ezrin and CD43 from both the NK and target cell are excluded from the inhibitory NK cell IS.
Exclusion of ezrin and CD43 from inhibitory, but not activating, NK cell IS in conjugates involving human NK clones

Two human NK clones, 5H4 (KIR2DL1+/KIR2DL2−) and 6H5 (KIR2DL1−/KIR2DL2−), were selected for microscopy. By flow cytometry, both clones were found to be CD56+ CD3+ Ig-like transcript-2− CD94+. In agreement with the specificity of KIR for HLA-C alleles, lysis by NK clone 5H4, expressing KIR2DL1, was effectively inhibited by HLA-Cw6 while NK clone 6H5, expressing KIR2DL2, was inhibited by HLA-Cw3 expressed on 221 transfectants (Fig. 4a).

Conjugates of NK clones 5H4 or 6H5 with 221/Cw6-GFP or 221/Cw3-GFP targets were fixed and stained for CD43 and ezrin using mAbs. Both CD43 and ezrin were excluded from the inhibitory IS between clone 5H4 (KIR2DL1+/KIR2DL2−) and 221/Cw6-GFP (Fig. 4b). At the activating IS, ezrin and CD43 were homogeneously distributed, shown in conjugates between 5H4 and 221/Cw3-GFP (Fig. 4c). A reconstruction of the face of the inhibitory NK cell IS shows exclusion of CD43 and ezrin where HLA-Cw6-GFP clusters (Fig. 4d). For both NK clones, ezrin and CD43 were excluded from the inhibitory NK cell IS 60–80% of the time (Fig. 4e). This demonstrates that exclusion of ezrin and CD43 is a general property of the inhibitory NK cell IS rather than specific to an IS involving one type of KIR/MHC protein. At the activating NK cell IS, ezrin or CD43 was homogeneously distributed at the intercellular contact in ~80% of conjugates (Fig. 4e). Thus, the distribution of ezrin and CD43 at the inhibitory and activating IS in conjugates involving human NK clones derived from peripheral blood corresponds to that observed in conjugates of YTS and 221 transfectants (Figs. 2 and 3).

CD45 is excluded from the inhibitory, but not the activating, NK cell IS

With a view to further assessing the role of protein size in assembly of the NK cell IS, we imaged the distribution of CD45 in inhibitory and activating conjugates. Because alternative splicing produces various CD45 isoforms with extracellular domains ranging from 20 to 50 nm, we first established their respective distributions on the cells used for imaging. Abs specific for CD45RO (20–28 nm), CD45RA, and CD45RB (each ~50 nm) were used to identify the expression of the various isoforms on the 221 and YTS transfectants as well as NK clones derived from peripheral blood. By flow cytometry, it was determined that YTS/KIR2DL1 expressed only CD45RO, 221/Cw6-GFP expressed only CD45RA, and the NK clones analyzed expressed CD45RO, CD45RA, and CD45RB (data not shown). This distribution of CD45 isoforms on human NK clones is consistent with previous observations. Conjugates involving effectors YTS/KIR2DL1 or NK clone 5H4 (KIR2DL1+/KIR2DL2−) were fixed and stained with anti-CD45 mAb (HI30), which recognizes CD45RO and CD45RB. This mAb did not stain 221 cells, inferring that it does not detect CD45RA (our unpublished observations). At inhibitory conjugates, CD45RO expressed on YTS/KIR2DL1 (Fig. 5a, c and d) or CD45RO and CD45RB expressed on the NK clone 5H4 (Fig. 5d) are clearly excluded from the region where HLA-C clusters (Fig. 5c and d). At the activating IS between YTS/KIR2DL1 and HLA-Cw3-GFP (Fig. 5b), CD45RO was evenly distributed across the face of the IS in 87% of conjugates. At the inhibitory NK cell IS between YTS/KIR2DL1 and 221/Cw6-GFP, CD45RO was found to be excluded from the IS in 67% of conjugates (Fig. 5e).

The size of the synaptic cleft at the inhibitory and activating NK cell IS

If proteins are segregated at the NK cell IS according to their size, it is necessary that the size of the synaptic cleft, defined in this study as the distance between the outer membranes of apposing effector and target cells at the IS, would vary accordingly. Thus, we assessed the ultrastructure of the activating and inhibitory NK cell IS in conjugates of 221 transfectants and YTS/KIR2DL1 cells by electron microscopy. NK cells were easily distinguished from target cells by the presence of large granules within their cytoplasm. The distance across the synaptic cleft in both activating and inhibitory conjugates varied such that distinct wide and narrow domains were evident (Fig. 6a). Distances across the synaptic cleft of conjugates between 221/Cw6-GFP and YTS/KIR2DL1 and conjugates of 221/Cw3-GFP and YTS/KIR2DL1 cells were measured at 300-nm intervals along each intercellular contact. Although the size of the synaptic cleft in activating and inhibitory conjugates was found to range from 10 to 55 nm and 10 to 42 nm, respectively, the majority of regions measured between 10 and 30 nm at both the activating and inhibitory IS (Fig. 6, c and d).

To characterize the distribution of HLA-C at the inhibitory NK cell IS, immunogold labeling was used to locate HLA-C-GFP. HLA-C-GFP was detected on the surface, and to a lesser extent within the cytoplasm, of the target cell. Staining was occasionally observed on the apposing NK cell, which could reflect the intercellular transfer of HLA-C-GFP from the target cell to the NK cell (24). Strikingly, accumulation of the gold beads, marking the location of HLA-C-GFP, was observed only at the tighter intercellular contacts (Fig. 6b). Although, it may be important to note that a few gold beads were located where the distance between the cells was ~100 nm, which may or may not be part of the intercellular contact. The distance across the synaptic cleft at each gold bead that was clearly at the IS i.e., HLA-C-GFP at the IS, was measured and found to be 14.5 ± 2.2 nm (Fig. 6e), which is the size of the extracellular domains of HLA-C bound to KIR (54, 55).

Discussion

By laser scanning confocal microscopy, we show that, similar to the activating T cell IS, f-actin accumulates at the activating NK cell IS. Consistent with a role for f-actin in the activating NK cell IS, inhibitors of actin polymerization significantly reduced NK cytotoxicity (65, 66). Conversely, a homogeneous distribution of f-actin around the cell membranes of apposing cells was seen at the inhibitory NK cell IS. Unexpectedly, however, ezrin and one of its associated transmembrane proteins CD43 are excluded from the inhibitory, i.e., nonlytolytic, NK cell IS, while both proteins are homogeneously distributed across the IS of activating conjugates. Surprisingly, the exclusion of ezrin and CD43 from the inhibitory NK cell IS is reminiscent of the exclusion of these proteins from...
the activating T cell IS (46, 47, 50, 51, 67). The large size of CD43 could be responsible for its exclusion from the inhibitory NK cell IS. However, both inhibitory and activating NK cell synapses contain large synaptic clefts that could accommodate CD43 (Fig. 6), and both activating and inhibitory NK cell synapses involve interactions with smaller receptor/ligand pairs (68). Also, the size of CD43 cannot be sufficient for its exclusion from the activating T cell IS, because CD43 mutants unable to bind ERM proteins can persist at the activating T cell IS with no detriment to T cell proliferation (51).

**FIGURE 4.** Exclusion of ezrin and CD43 from the inhibitory, but not activating, NK cell IS in conjugates involving human NK clones. *a,* The cytotoxicity of two human clones, 5H4 (KIR2DL1' KIR2DL2') and 6H5 (KIR2DL1' KIR2DL2'), was assessed in a 5-h 35S release cytotoxicity assay. *b,* Inhibitory conjugates of 5H4 and 221/Cw6-GFP and *c,* activating conjugates of 5H4 and 221/Cw3-GFP were stained for ezrin and CD43. Ezrin and CD43 are excluded from the inhibitory IS where HLA-C clusters but not the activating IS. *d,* Reconstructions of the face of two inhibitory NK cell IS, stained for ezrin or CD43, are shown. A region devoid of mAb staining for ezrin and CD43 corresponds to the region where HLA-C clusters. Scale bars represent 10 μm in *a* and *c* and 5 μm in *d.* *e,* The distribution of CD43 and ezrin at the inhibitory and activating NK cell IS was assessed in *n* conjugates.

**FIGURE 5.** Exclusion of CD45 from the inhibitory, but not activating, NK cell IS. Inhibitory and activating conjugates of YTS/KIR2DL1 and 221/Cw6-GFP or 221/Cw3-GFP were fixed, stained with anti-CD45 mAb (HI30), and the distribution at the IS was determined. Inhibitory conjugates where CD45 is excluded from the IS are shown in *a* and *c.* At the activating IS, between YTS/KIR2DL1 and 221/Cw3-GFP, CD45 is evenly distributed across the intercellular contact. *c,* Reconstruction of the face of the inhibitory IS formed between YTS/KIR2DL1 and 221/Cw6-GFP demonstrates a region devoid of CD45 staining corresponding to that where HLA-C clusters. Scale bars represent 10 μm in *a* and *b,* and 5 μm in *c.* *d,* Reconstruction of the inhibitory IS formed between two human NK clones 5H4 and 221/Cw6-GFP. Exclusion of CD45 from the region where HLA-C-GFP clusters is apparent. *e,* The distribution of CD45 at the inhibitory and activating IS was assessed in numerous conjugates involving YTS/KIR2DL1. *n,* The number of conjugates analyzed.
Reasons why the exclusion of CD43 from the inhibitory, but not the activating, NK cell IS may be functionally important are not obvious. The binding of CD43 to its ligands, e.g., ICAM-1, could promote adhesion (69, 70). Therefore, inclusion of CD43 in the activating NK cell IS may be necessary to maintain effector/target contact during effector functions such as secretion. Also, CD43 has been shown to associate with the signaling molecules lyn and lck involved in T cell activation (71, 72). These molecules accumulate at the activating NK cell IS (22, 23). Consistent with a role in NK cell activation, engagement of CD43 has been shown to increase NK cell proliferation and NK cytotoxicity by redirected lysis (73, 74). Thus, the exclusion of CD43 from the inhibitory NK cell IS could assist inhibition by sequestering signaling molecules such as lyn and lck away from the site of intercellular communication. Similar to CD43, we have demonstrated that CD45 is excluded from the inhibitory, but not the activating, NK cell IS. Interestingly, some anti-CD45 mAbs selectively inhibited LFA-1-mediated NK cytotoxicity, suggesting a role for CD45 in regulating NK cell killing (64). Thus, at the inhibitory NK cell IS the redistribution of CD45 away from intercellular contact may favor inhibitory effector functions.

The proposal that proteins are segregated according to the size of their extracellular domains predicts that the distance across the synaptic cleft, i.e., extracellular space between apposing cells at the interface, is determined by the size of local receptor/ligand interactions (28, 31–36). In this study, we measured the size of the synaptic cleft between inhibitory and activating NK cell conjugates by transmission electron microscopy. We found that rather than being equidistant across the IS, the membranes of apposing cells create alternate wide and narrow regions across the synaptic cleft. Although at the activating NK cell IS the frequency of distances >30 nm remains low, perhaps these wider regions are sufficient to allow the observed prevalence of CD43 and CD45 there. Importantly, where HLA-C-GFP was localized, the synaptic cleft distance was 14.5 ± 2.2 nm, which corresponds to the size of the extracellular portions of KIR/MHC, as determined by x-ray crystallography (54, 55). These findings are consistent with the hypothesis that in addition to cytoskeletal-mediated movements, proteins at the NK cell IS can redistribute due to the size of their extracellular domains. One important next goal is to locate HLA-C/KIR and ICAM-1/LFA-1 within the synaptic cleft, using electron microscopy of peripheral blood NK cells and targets.

Finally, we note that during preparation of the revised version of this manuscript, another laboratory concluded that ezrin clustered at the activating NK cell IS (75). This different conclusion may be due to the different target and effector cells used in each study, and/or the vastly different protocol by which each analysis was made. This emphasizes that the organization of proteins at an IS is sensitive to many factors, such as environmental stimuli, that are not yet understood.

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