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The Actin Cytoskeleton Controls the Efficiency of Killer Ig-Like Receptor Accumulation at Inhibitory NK Cell Immune Synapses

Leah J. Standeven,* Leo M. Carlin, † Peter Borszcz,* Daniel M. Davis, † and Deborah N. Burshtyn2,*

Killer cell Ig-like receptors (KIRs) are MHC class I-specific receptors expressed in NK and T lymphocytes. KIR antagonism of activation signals occurs at the immune synapse between the effector and target cells. The processes that regulate clustering of KIR are not well defined. We have expressed KIR-GFP receptor chimeras in two human NK-like lines, YTS and NK92. In this study, we show that the frequency of KIR enrichment at the synapse was decreased for a KIR that lacks a portion of the cytoplasmic tail. Strikingly, blocking actin polymerization with a high dose of cytochalasin D also substantially decreased clustering of KIR as well as KIR-induced clustering of HLA-C-GFP in target cells. However, the effect of inhibiting actin polymerization was only clearly evident at the earlier time points after cell mixing, and eventually clustering of KIR and HLA-C occurred independently of actin remodeling. Although treatment with anti-LFA-1 also decreased conjugate formation, the frequency of KIR clustering remained normal within the population of conjugates that did form, suggesting that the effect of cytochalasin D is not solely through LFA-1. Collectively, these data suggest that the actin cytoskeleton and the cytoplasmic tail of KIR regulate the efficiency by which KIR accumulates at inhibitory NK cell synapses. The Journal of Immunology, 2004, 173: 5617–5625.

Natural killer cells play a role in the early immune response to tumors, viruses, and other intracellular pathogens. NK cells are large granular lymphocytes that express a variety of activating receptors, which recognize diverse ligands on target cells (1–3). Engagement of NK cell-activating receptors and adhesion molecules initiates intracellular signaling through tyrosine kinases that lead to the generation of secondary messengers and ultimately to polarized degranulation as well as cytokine release. The constitutive killing activity of NK cells is regulated by several MHC class-I-specific inhibitory receptors. In humans, these receptors include the lectin-like CD94/NKG2 receptors and the inhibitory killer cell Ig-like receptors (KIRs) (4). Both receptor families use a similar mechanism to antagonize activating receptor signaling, whereby the cytoplasmic protein tyrosine phosphatase Src homology region 2 domain-containing phosphatase 1 (SHP-1) is recruited to the ITIM motifs in their cytoplasmic domains (5).

Inhibitory receptors are believed to act in cis, meaning that they must be in close proximity to activating receptors to block activating signals (reviewed in Ref. 6). In lymphocytes, the intracellular signaling events required for activation of effector function emanate from a region of intercellular contact between an effector and target cell that we will in this study refer to as the immune synapse. The immune synapse between T cells and APCs or target cells has been studied extensively. High resolution three-dimensional fluorescence imaging has shown that the TCR, coreceptors, costimulatory molecules, adhesion molecules, and cytoskeletal components accumulate and segregate at the immune synapse in a highly organized manner (reviewed in Ref. 7). Central to the process of T cell immune synapse formation are the actin and myosin cytoskeletal systems that are required for the formation of a scaffold for signaling components and for the transport of cell surface proteins to the region of intercellular contact (reviewed in Ref. 8).

The human NK cell immune synapse has been described for an NK cell in contact with a resistant target cell (inhibitory NK cell immune synapse) and a sensitive target cell (activating NK cell immune synapse) (reviewed in Refs. 9 and 10). The activating NK cell immune synapse is similar to the immune synapse observed for T cells interacting with APCs presenting agonist peptide/MHC (11). There are some striking differences between activating and inhibitory NK cell immune synapses. For example, ezrin, CD43, and CD45 are excluded only from inhibitory NK cell immune synapses, whereas a multimolecular stimulatory complex is assembled only at the center of the activating NK cell immune synapse (11–13). Enrichment of inhibitory receptors and ligands at the NK immune synapse has been studied using either enhanced GFP-tagged KIR (14) or GFP-tagged HLA-C molecules (12, 13, 15, 16). A recent report has also examined the inhibitory NK cell immune synapse using NK clones retrovirally transduced with GFP-tagged KIR (17). These studies have demonstrated that both KIR and HLA-C cluster at the inhibitory NK cell immune synapse. In addition, as early as 1 min after cell mixing, the location of SHP-1 distinguishes the activating NK cell immune synapse from

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3 Abbreviations used in this paper: KIR, killer cell Ig-like receptor; BADTA, bis(acetoxyethyl)methyl(2,2′-6′,2′-terpyridine-6′-dicarboxylate; DIC, differential interference contrast; EGF, enhanced GFP; PP2, 4-amino-5-(4-chlorophenyl)-7-((buty1)pyrazolo[3,4-d]pyrimidine; SHP-1, Src homology region 2 domain-containing phosphatase 1; TR-GFP, KIR without a functional ITIM domain fused to EGFP; WASP, Wiskott-Aldrich syndrome protein.

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the inhibitory NK cell immune synapse. In the activating synapse, SHP-1 is at the periphery, whereas in the inhibitory synapse SHP-1 clustering is central (18). Previous studies have also shown that HLA-C still clusters at the inhibitory NK cell immune synapse in the presence of drugs that disrupt actin polymerization and independently of the cytoplasmic tail of KIR, but that clustering does require zinc that facilitates KIR dimerization (12, 16).

In this study, we have investigated the processes that regulate KIR clustering using a mutant KIR that lacks a cytoplasmic portion, by inhibiting actin polymerization, and by blocking LFA-1-mediated adhesion. We found that the actin cytoskeleton plays a role in the stability of conjugates with either sensitive or resistant target cells and, importantly, regulates the rate at which KIR clusters. Our results also imply the cytoplasmic tail of KIR in facilitating efficient clustering.

Materials and Methods

Abs and reagents

The mAb EB6 (IgG1) specific for KIR2DL1 was purchased from Beckman Coulter (Mississauga, Canada). The AbTSI2/2.1.1.13 (IgG1) for activated LFA-1 was purified from HB202 (American Type Culture Collection (ATCC), Manassas, VA) culture supernatant by protein A-Sepharose, filtered sterilized, and stored in PBS. PE-conjugated goat anti-mouse was purchased from Cedarlane Laboratories (Hornby, Canada). Human rIL-2 (TECIN) was obtained from the Biological Resources Branch of the Division of Cancer Treatment and Diagnosis located at National Cancer Institute Frederick Cancer Research and Development Center (Frederick, MD). Cytochalasin D was purchased from Calbiochem (San Diego, CA) or from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO purchased from Calbiochem and dissolved in PBS purchased from EMD Chemicals (Gibbstown, NJ) and dissolved in distilled water. Cytofix/Cytoperm was purchased from BD PharMingen (San Diego, CA). Phalloidin/Alexafluor633 was purchased from Molecular Probes (Eugene, OR).

Construction of enhanced GFP (EGFP) chimeras

Generation of EGFP fused to the C terminus of HLA-Cw3 (Cw3-GFP) and HLA-Cw6 (Cw6-GFP) has been described (12). Generation of EGFP fused to the C terminus of the full-length cytoplasmic tail of KIR2DL1 (KIR2DL1 TR-GFP) has also been described. In this study, KIR2DL1 TR-GFP encodes the entire coding sequence of KIR2DL1 linked to GFP by the amino acids GISAT. To generate the receptor chimera KIR2DL1 TR-GFP, a Hinall and BumH fragment of KIR2DL1 was isolated such that the coding sequence is interrupted at the D276 aspartic acid residue upstream of the membrane-proximal ITIM (19). (Please note: amino acid numbering does not include the 21 aa signal peptide.) The resulting fragment was inserted into pEGFP-N1 (BD Clontech, Palo Alto, CA) using the Hinall and BumH restriction sites, creating a PPVAT linker region, followed by the GFP coding sequence. Both the KIR2DL1-GFP and KIR2DL1 TR-GFP constructs were subcloned into the vector pBSSRaeN (provided by K. Kane, University of Alberta, Edmonton, Alberta, Canada) (20).

Cells

The cell lines 221/Cw3 and 221/Cw4 were maintained in Iscove’s or RPMI 1640 medium containing 10% FBS, 2 mM t-glutamine (Invitrogen Life Technologies, Carlsbad, CA), and 0.5 mg/ml geneticin (Invitrogen Life Technologies) (21). The cell line 221/Cw6 was maintained in Iscove’s or RPMI 1640 medium containing 10% FBS, 1 mM t-glutamine, 1 mM MES nonessential amino acids, 1 mM phenylalanine/tryptophan, 1 mM sodium pyruvate, 50 μM 2-ME, and in some cases 1.6 mg/ml geneticin (12). The cell line YTS was maintained in Iscove’s or RPMI 1640 medium containing 10–15% FBS (HyClone, Logan, UT), 50 μM 2-ME, and 2 mM t-glutamine. The YTS/KIR2DL1 and YTS/KIR2DL1 TR-GFP cell lines were maintained in YTS culture medium supplemented with 0.75–1.6 μg/ml puromycin (14, 22). The cell line NK92 was purchased from ATCC (CRL-2407) and maintained in Iscove’s medium containing 10% FBS, 2 mM t-glutamine (StemCell Technologies, Vancouver, Canada), 7.5% FBS (HyClone), 25 μM 2-ME, and 1 mM t-glutamine, and supplemented with 100 U/ml human rIL-2. NK92 cells were transfected with KIR2DL1-GFP (NK92/KIR2DL1-GFP) and KIR2DL1 TR-GFP (NK92/KIR TR-GFP) using the vector pBSRaeN. NK92 cells (5 × 10^5) were incubated with 5 μg of plasmid for 30 min at room temperature in a 0.4-cm Gene Pulser Cuvette (Bio-Rad, Hercules, CA) and then pulsed at 0.25 V, 960 μF using a Gene Pulser Apparatus (Bio-Rad). The cells were allowed to recover for 10 min at room temperature, and then gently returned to a flask containing culture medium supplemented with human rIL-2. Selection was initiated 48 h later using 1 mg/ml geneticin. Drug-resistant lines were propagated, screened for expression by flow cytometry, and then subcloned using limited dilution to establish lines with stable levels of expression. Both NK92/KIR2DL1-GFP and NK92/KIR2DL1 TR-GFP were maintained in NK92 medium supplemented with 100 U/ml rIL-2 and 0.5 mg/ml geneticin.

Cytolysis assay

Target cells were labeled with Na^111CrO_4 (PerkinElmer, Wellesley, MA, and Analytical Sciences, Woodbridge, Canada) for 1 h at 37°C, 5% CO_2. Effector cells were incubated with 2.5 × 10^4 488-nm argon laser to excite GFP, and a temperature-controlled laser (PerkinElmer). Alternatively, target cells were labeled with Delfia bis(acetoxyethyl)2,2′,2′′-terpyridine-6,6′-dicarboxylate (BADTA) reagent for 30 min at 37°C, 5% CO_2. Effector cells were incubated with 3 × 10^4 BADTA-labeled target cells at various E:T ratios for 2 h at 37°C in V-bottom microtiter plates. After incubation, released BADTA was measured in Europium Atomic Absorption Standard Solution (Sigma-Aldrich) by time-resolved fluorescence using a Wallac 1420 VICTOR2 plate reader (PerkinElmer). For both assays, percent specific lysis was determined as (mean experimental release – mean spontaneous release)/(mean maximum release – mean spontaneous release) × 100. For experiments with cytochalasin D, effector cells were preincubated for 30 min at 37°C, 5% CO_2 with twice the desired final concentration of drug before dilution with the addition of target cells without drug. Assays were performed three times, unless otherwise indicated.

Conjugate assay

Two-color flow cytometry conjugate assays were performed, as previously described, except that all steps were conducted at either room temperature or 37°C unless otherwise specified. For the NK92 experiments with cytochalasin D, effector cells were preincubated for 30 min at 37°C, 5% CO_2 in the presence of 10 μM cytochalasin D or DMSO alone (no drug) before the addition of target cell suspensions containing either 10 μM cytochalasin D or DMSO. For the YTS experiments with cytochalasin D, PP2, or azide, effector cells were preincubated for 30 min at 37°C, 5% CO_2 with twice the desired final concentration of drug before dilution with the adding the target cells. For the YTS experiments with Cytochalasin D, effector cells were preincubated for 30 min at room temperature with 10 μg/ml anti-LFA-1 or control IgG. To maintain the correct final Ab concentrations, Ab was added to the target cells just before cell mixing. Percentage of conjugate formation was determined as (two-color events)/ (total effector events) × 100. Assays were performed three times, unless otherwise indicated.

Confocal imaging of living cells

Samples were prepared, imaged, and processed, as follows, unless otherwise specified. Samples were prepared by adding target cells to effector cells and then subjecting the cell mixture to a short spin. For the NK92 experiments, 2 × 10^5 NK92/KIR2DL1-GFP cells or NK92/KIR2DL1 TR-GFP cells were mixed with 2 × 10^4 target cells. For the YTS experiments, 4 × 10^6 YTS/KIR2DL1-GFP cells were mixed with 6 × 10^4 target cells. The cell mixtures were gently mixed onto a poly-L-lysine coated coverslip and then clamped onto the stage of Zeiss LSM Image Browser software. All images collected for a given data set were included in subsequent calculations. Interfaces were counted for conjugates of a GFP fluorescent effector cell in tight contact with a target cell.
Interfaces were scored positive for clustering if enrichment of the GFP signal was visible in one or more of the sections captured for the conjugate at 0.5-μm intervals. The percentage clustering (percentage with cluster) value was determined as (number of interfaces that exhibited clustering)/(total number of interfaces) × 100.

To produce images of the interface regions, snapshots of each interface region were generated using the Imaris 3D image software package (Bitplane, St. Paul, MN). Briefly, the Full 3D Viewer feature of Imaris was used to rotate a volume rendering of each conjugate at a zoom of ×1. Each conjugate was rotated to generate a view of the interface from the perspective of the target cell. A maximum intensity projection was subsequently generated for each rotated conjugate, and a snapshot of each interface region was taken. The Imaris 3D image software package (Bitplane) was also used to quantify the amount of KIR-GFP enrichment at each interface region. Subsequently, the Superscan feature was used to generate a geometric object for each region of KIR-GFP enrichment from a volume image of each conjugate. The isosurface component was then used to compute a surface enclosing all voxels (arbitrary volume unit) with intensities higher than a user-specified threshold value of 120 in the contact region, which corresponds to a ~4-fold increase over normal cell surface expression. The values included in each isosurface were then used to calculate a total intensity sum value for each region of KIR-GFP enrichment. The total intensity sum values obtained using the Imaris 3D image software package were used to calculate mean and median intensity sum values.

Imaging of cells stained for F-actin and kinetic analysis of KIR-GFP and HLA-C-GFP clustering in the presence of cytocalasin D

Effecter and target cells were preincubated separately for 1 h at 37°C, 5% CO2 with either 10 μM cytocalasin D or 0.1% DMSO. The cells (5 × 10^6 of each type) were then mixed at an E:T ratio of 1:1, centrifuged at 1100 rpm for 4 min to bring the cells into contact quickly, and then incubated at 37°C, 5% CO2 for the indicated time periods. Samples were fixed and permeabilized with Cytofix/Cytoperm for 12 min at 4°C and then washed twice in PBS containing 1% BSA, 0.1% sodium azide, and 0.025% saponin (PBS/BSA/saponin). The samples were then stained with 5 U/ml Alexafluor633-conjugated phalloidin for 45 min at 4°C, washed twice in PBS/BSA/saponin, and finally into PBS. The cells were pelleted, and a 7-μl aliquot of the concentrated cell suspension was sealed between a plain glass slide and coverslip. Optical sections 0.28 μm apart were captured by laser scanning confocal microscopy using a TCSPC2 Leica microscope (Leica Microsystems, Deerfield, IL) with a ×63 oil-immersion objective (N.A.1.32). A 488-nm argon laser and a 633-nm HeNe laser were used to excite GFP and phalloidin-Alexafluor633, respectively. At least three independent experiments were performed to give a minimum of 90 E:T cell contacts counted for each cell/drug combination. Contacts between effector and target cells were scored as demonstrating enrichment of HLA-C-GFP, KIR2DL1-GFP, or phalloidin-Alexafluor633 if the intensity at the interface was more than double that of the rest of the cell. Interfaces were reconstructed as maximum intensity projections using Volocity (Improvision, Coventry, U.K.) software. Imaged (National Institutes of Health) software was used to produce phalloidin-Alexafluor633 intensity profiles by plotting the intensity of all the pixels in the image on the z-axis.

Results

Functional characterization of the KIR-GFP receptor chimeras in NK92

To examine the dynamics of NK cell inhibitory receptor enrichment at the immune synapse in living cells, we generated GFP chimeras with KIR2DL1 both with and without a functional ITIM domain (Fig. 1A) (see Materials and Methods for details). To visualize receptor movement in living cells, we transfected the human NK-like cell line NK92 with KIR2DL1-GFP or KIR2DL1 TR-GFP. The cell surface expression profiles of two subclones that were selected for further analysis are shown in Fig. 1B. To assess the function of the chimeric receptors in NK92 cells, we performed cytolysis assays with target cells expressing either the KIR2DL1 ligand HLA-Cw4 (.221/Cw4) or the noncongeneric MHC-I protein HLA-Cw3 (.221/Cw3) (Fig. 1C). Expression of KIR2DL1-GFP in NK92 cells strongly inhibited cytolysis of the .221/Cw4 target cells. Consistent with previous studies, this result shows that the presence of GFP at the cytoplasmic tail of KIR2DL1 did not abrogate the ability of this receptor to transduce an inhibitory signal upon contact with the resistant .221/Cw4 target cells (14). Expression of the truncated receptor chimera KIR2DL1 TR-GFP in NK92 cells did not inhibit cytolysis of .221/Cw4 target cells.

KIR-GFP enrichment at the inhibitory immune synapse in living cells

To examine receptor enrichment at the immune synapse in living cells, we imaged NK92/KIR2DL1-GFP and NK92/KIR2DL1 TR-GFP cells conjugated with .221/Cw4 or .221/Cw3 target cells by confocal microscopy. The representative DIC and GFP fluorescence images of a single 0.5-μm plane are shown for conjugates between the indicated cell combinations (Fig. 2, A and B). In NK92, the KIR2DL1-GFP receptor chimera clustered at the interface with target cells bearing HLA-Cw4, but not HLA-Cw3. The KIR2DL1 TR-GFP receptor chimera also clustered at the interface with the .221/Cw4 target cells, but not with the .221/Cw3 target cells. Although varying degrees of KIR-GFP recruitment were seen for both receptor chimeras, the frequency of the truncated KIR2DL1 clustering at immune synapse was consistently less than half that of the full-length receptor chimera (Fig. 2C). Similar results were obtained 60 min after cell mixing (data not shown). The defect in truncated KIR-GFP clustering is also apparent in the YTS...
The actin cytoskeleton plays an important role in formation of NK conjugates with target cells and activation of cytolysis (23, 24). To test whether the cytoskeleton plays any role in KIR clustering, we examined the effect of cytochalasin D, an inhibitor of actin polymerization, on this process. Due to the reversibility of cytochalasin D, it was maintained throughout the experiments. We first determined the effect of 10 μM cytochalasin D on cytolysis and conjugate formation (Fig. 3). NK92 and NK92/KIR2DL1-GFP cytolysis of target cells was completely inhibited in the presence of cytochalasin D (Fig. 3A). Similarly, conjugate formation was completely inhibited in the presence of cytochalasin D (Fig. 3B). In this instance, the term conjugate refers to tight conjugates that are resistant to the vortex step in the flow cytometry conjugate assay. Notably, the engagement of KIR in NK92 did not reduce the amount of conjugates as much as has been observed in YTS cells and with a primary NK clone (11, 19). Given that previously we found that engagement of KIR2DL1 expressed at low levels in YTS resulted in only a modest but reproducible inhibition of adhesion, it is possible that the moderate reduction in NK92 conjugates is the result of relatively low receptor level expression or more potent activation (14).

FIGURE 2. KIR-GFP enrichment at inhibitory synapse in NK92 cells. A and B, KIR-GFP recruitment in living cells. Target cells and effector cells were spun together at low speed, and confocal images were collected 7–30 min after cell mixing (see Materials and Methods for details). Representative DIC and GFP fluorescence images of a single 0.5-μm plane are shown for NK92/KIR2DL1-GFP (A) or NK92/KIR2DL1 TR-GFP (B) conjugated to .221/Cw3 target cells (control) and .221/Cw4 target cells (none, low, medium, or high). In the case of .221/Cw4, the varying degrees of KIR-GFP recruitment, including no clustering (None) and increasing clustering (low, medium, high), are shown. C, Frequency of KIR-GFP clustering in NK92. Bar graph showing the percentage of interfaces that scored positive for clustering following mixing with .221/Cw3 targets (▴) or .221/Cw4 targets (▾). Results were tabulated from four independent experiments using NK92/KIR2DL1-GFP (left) or NK92/KIR2DL1 TR-GFP (right) as effector cells. The percentage with cluster value was determined as (number of interfaces that exhibited clustering)/(total number of interfaces) × 100. The n values indicate the total number of interfaces imaged. D, Pattern of KIR-GFP enrichment at the inhibitory NK cell immune synapse. Three-dimensional image software was used to generate snapshots of interfaces between .221/Cw4 and NK92/KIR2DL1-GFP (upper panels) or .221/Cw4 and NK92/KIR2DL1 TR-GFP (lower panels). The interface snapshots shown in D are from the same conjugates whose corresponding DIC and GFP fluorescence images are shown in A and B.

In determining the percentage with cluster values for KIR2DL1-GFP and KIR2DL1 TR-GFP, interfaces were scored as positive for clustering if enrichment of GFP signal was apparent in any 0.5-μm section of the Z-series captured for each conjugate. Consequently, although these values reflect whether or not a cluster was observed at a particular interface, they do not take into account the size of the cluster. Therefore, to further examine KIR enrichment at the inhibitory NK cell immune synapse, we used a three-dimensional image software package to generate snapshots of interface regions (Fig. 2D) (see Materials and Methods). Snapshots were generated for a subset of the conjugates with .221/Cw4 target cells in which clusters of either the full-length or truncated receptor were evident by visual assessment of the GFP fluorescence images. Varying patterns of KIR-GFP enrichment were evident for both receptors. To perform a comparative quantitative analysis of the KIR-GFP clustering, we used a three-dimensional image software package to calculate total intensity sum values for each region of KIR-GFP enrichment (see Materials and Methods). In this instance, the total sum intensity value is a quantification of the total amount of KIR-GFP enrichment at a particular interface region, and therefore, these values were used to compare KIR2DL1-GFP and KIR2DL1 TR-GFP clustering. Values were calculated for 30 interface regions with .221/Cw4 target cells irrespective of KIR-GFP clustering. The mean total intensity sum values as well as the deviation from the mean values were calculated for KIR2DL1-GFP (mean = 101289; SD = 141997) and KIR2DL1 TR-GFP (mean = 16957; SD = 33609) using images collected on a given day (one of four independent experiments). Application of a pooled-variance t test indicated that there was a significant difference (p < 0.05) between the mean total intensity sum of KIR2DL1-GFP and KIR2DL1 TR-GFP (calculated two-tailed p value of 0.002).

The effect of pharmacological inhibitors on KIR-GFP enrichment

Human NK cell line (P. Borszcz and D. N. Burshtyn, unpublished observations).

In determining the percentage with cluster values for KIR2DL1-GFP and KIR2DL1 TR-GFP, interfaces were scored as positive for clustering if enrichment of GFP signal was apparent in any 0.5-μm section of the Z-series captured for each conjugate. Consequently, although these values reflect whether or not a cluster was observed at a particular interface, they do not take into account the size of the cluster. Therefore, to further examine KIR enrichment at the inhibitory NK cell immune synapse, we used a three-dimensional image software package to generate snapshots of interface regions (Fig. 2D) (see Materials and Methods). Snapshots were generated for a subset of the conjugates with .221/Cw4 target cells in which clusters of either the full-length or truncated receptor were evident by visual assessment of the GFP fluorescence images. Varying patterns of KIR-GFP enrichment were evident for both receptors. To perform a comparative quantitative analysis of the KIR-GFP clustering, we used a three-dimensional image software package to calculate total intensity sum values for each region of KIR-GFP enrichment (see Materials and Methods). In this instance, the total sum intensity value is a quantification of the total amount of KIR-GFP enrichment at a particular interface region, and therefore, these values were used to compare KIR2DL1-GFP and KIR2DL1 TR-GFP clustering. Values were calculated for 30 interface regions with .221/Cw4 target cells irrespective of KIR-GFP clustering. The mean total intensity sum values as well as the deviation from the mean values were calculated for KIR2DL1-GFP (mean = 101289; SD = 141997) and KIR2DL1 TR-GFP (mean = 16957; SD = 33609) using images collected on a given day (one of four independent experiments). Application of a pooled-variance t test indicated that there was a significant difference (p < 0.05) between the mean total intensity sum of KIR2DL1-GFP and KIR2DL1 TR-GFP (calculated two-tailed p value of 0.002).
To assess the effect of cytochalasin D on inhibitory receptor enrichment in living cells, we performed confocal microscopy analysis of KIR-GFP clustering in the presence of 10 μM cytochalasin D or DMSO alone (Fig. 3C). Images were collected between 7 and 30 min following mixing of effector cells with .221/Cw4 target cells. Of note, samples for confocal microscopy were not subjected to the vortex step used in the flow cytometry conjugate assay so as to allow loose conjugates to remain intact for imaging. The clustering of full-length receptor expressed in NK92 decreased by ~50% in the presence of cytochalasin D. In contrast, cytochalasin D did not have a significant effect on the clustering of KIR2DL1 TR-GFP expressed in NK92. Similar to NK92, the frequency of KIR2DL1-GFP clustering was reduced by ~50% in YTS cells in the presence of cytochalasin D (Table I).

Given the dramatic effect of 10 μM cytochalasin D, we further examined the impacts of actin disruption with a dose response, as well as the impacts of azide, an inhibitor of intracellular ATP generation (12), and PP2, a Src family kinase inhibitor (Fig. 4 and Table I). Cytolysis of target cells and conjugate formation at 10 min were completely inhibited even at 2 μM cytochalasin D (Fig. 4, A and B). A similar effect on conjugate formation was seen 40 min after cell mixing (data not shown). In separate experiments, we determined that the effect on cytology was lessened at 1 μM cytochalasin D and no longer apparent at 0.1 μM cytochalasin D (data not shown). In contrast, the effect of cytochalasin D on KIR2DL1-GFP clustering was lessened at 5 μM and no longer apparent at 2 μM (Table I). The dose-dependent inhibition by PP2 for lysis and conjugate formation with sensitive target cells is depicted in Fig. 4, C and D. Azide also inhibited lysis and conjugate formation with sensitive target cells in a dose-dependent manner (Fig. 4, E and F). Similar effects on conjugate formation were observed 40 min after cell mixing (data not shown). Unfortunately, doses of azide that fully blocked cytology were accompanied by gross morphological changes of the target cells, as well as high spontaneous release by targets. KIR2DL1-GFP clustering was decreased in the presence of 50 mM azide (Table I). KIR2DL1-GFP clustering was unchanged in the presence of the highest dose of PP2 tested (Table I).

**Kinetics of KIR-GFP and HLA-C-GFP clustering in the presence of cytochalasin D**

We previously found that KIR induced HLA-C-GFP clustering independently of actin polymerization following a 45- to 60-min incubation of the conjugates (12). Similarly, we noticed an increase in KIR-GFP clustering in NK92 cells in the presence of cytochalasin D when the conjugates were allowed to form for 60 min (data not shown). Therefore, we performed time course analysis of KIR-GFP clustering in YTS effector cells or HLA-C-GFP clustering in .221 target cells. In this case, we used target cells

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**Table 1. Effect of cytochalasin D, azide, and PP2 on KIR2DL1-GFP clustering in YTS**

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<tr>
<th>Compound Addeda</th>
<th>Concentrationb</th>
<th>No. of Interfacesc</th>
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a Effector cells were preincubated with the indicated concentration of cytochalasin D, azide, or PP2 for 30 min prior to addition of target cells.

b Indicated concentrations were maintained in the drug-treated samples at all times during data collection. For the no-drug controls, effector cells were preincubated with the solvent used to dissolve each compound (DMSO for cytochalasin D and PP2; aqueous solution for azide).

c Total number of interfaces imaged per treatment. Tabulated results from three independent experiments using cytochalasin D and azide and two independent experiments using PP2. At least three samples were examined per treatment per experiment. Confocal images (0.5-μm sections) were captured 5–15 min after cell mixing.

d Interfaces were scored as positive for clustering if enrichment of the EGFP signal was apparent in any 0.5-μm section of the Z-series captured for the conjugate. The % with cluster value was determined as (number of interfaces that exhibited clustering)/(total number of interfaces) × 100.
expressing the KIR2DL1 ligand HLA-Cw6 (.221/Cw6) or HLA-Cw6 fused to GFP (.221/Cw6-GFP). To facilitate these experiments, the conjugates were fixed at discrete time points as well as permeabilized to verify the effect of 10 μM cytochalasin D by using phalloidin-Alexafluor633 to stain F-actin. Fig. 5 depicts conjugate images that were collected following 45-min coinoculation with target cells. Consistent with our previous findings at the 45-min time point, the degree of KIR-GFP and HLA-C-GFP clustering was similar in either the presence or the absence of cytochalasin D (Fig. 5, A–D) (12, 15). Furthermore, we verified that the block of actin polymerization was still intact at the 45-min time point by staining fixed conjugates with phallolidin-Alexafluor633 (Fig. 5, H and I). Although some variation in F-actin staining is often seen around the cell surface, accumulation of F-actin specifically at the immune synapse was frequently observed in activating synapses in the absence of cytochalasin D (as indicated by ○ in Fig. 5, E and F).

Remarkably, time-course analysis of HLA-C-GFP and KIR2DL1-GFP clustering revealed that cytochalasin D had a pronounced effect on HLA-C-GFP and KIR-GFP clustering at earlier time points after cell mixing, but not by 40 min after cell mixing (Fig. 6). In the absence of cytochalasin D, the clustering of Cw6-
of 0.1% DMSO. Although measurements were included for all indicated time points. Contacts between effector and target cells were scored as demonstrating enrichment of HLA-C-EGFP, KIR2DL1-EGFP, or Alexafluor633-conjugated phalloidin if the intensity at the interface was more than double that of the rest of the cell. Indicate enrichment in the presence of 10 μM cytochalasin D; [square] indicate enrichment in the presence of 0.1% DMSO. Although measurements were included for all indicated time points, bars representing values between 0 and 1% are not visible. HLA-C-EGFP clustering and F-actin formation at the immune synapse. Graphical representations of the confocal data are shown for Cw6-GFP and phalloidin-Alexafluor633 at the activating NK cell immune synapse (upper panels) and for Cw3-GFP and phalloidin-Alexafluor633 at the activating NK cell immune synapse (lower panels). KIR-GFP clustering and F-actin formation at the immune synapse. Graphical representations of the confocal data are shown for KIR2DL1-GFP and phalloidin-Alexafluor633 at the inhibitory NK cell immune synapse (upper panels) and the activating NK cell immune synapse (lower panels).

Cw6-GFP and KIR2DL1-GFP was substantially lower than that of the controls until the time point of 40 min. In the case of the activating NK cell immune synapse, neither Cw3-GFP nor KIR2DL1-GFP clustered at any of the time points. Staining with phalloidin-Alexafluor633 showed an increase in F-actin over time at activating NK cell immune synapses, and that this increase was blocked in the presence of cytochalasin D (Fig. 6, A and B, lower right panels). In comparison, there was no increase in accumulation of F-actin at inhibitory NK cell immune synapses at any point in time (Fig. 6, A and B, upper right panels).

**Anti-LFA-1 decreases conjugation, but not the frequency of KIR-GFP clustering**

The actin cytoskeleton is involved in several processes relevant to immune synapse formation, including integrin-mediated adhesion and transport of molecules to the synapse. LFA-1 is the principal integrin that mediates YTS cell binding to 721.221 target cells and requires actin polymerization to cluster at the activating NK cell immune synapse in ex vivo cells (19, 25). To address the possibility that the effect of cytochalasin D on KIR clustering was through inhibition of LFA-1-mediated adhesion, we determined the effect of an anti-LFA-1 Ab on adhesion and KIR clustering for YTS cells. This Ab reacts with the activated form of LFA-1 and constitutively stains YTS cells (data not shown). In the presence of anti-LFA-1, the number of effector cells able to adhere to targets was reduced to ~10% regardless of KIR engagement (Fig. 7A). In this case, the lower level of conjugation due to KIR is evident, but the number of conjugates decreased in the presence of anti-LFA-1 to a similar level whether or not KIR was engaged. Although we did not quantify the difference, the frequency of conjugates appeared lower under the conditions used for live cell imaging. However, within the population of conjugates that did form in the presence of anti-LFA-1, the frequency of KIR clustering was unchanged (Fig. 7B). These data suggest that although LFA-1-mediated adhesion is key to forming stable conjugates and therefore is most likely often engaged upstream of KIR clustering, LFA-1-mediated adhesion is not essential for KIR clustering.

**Discussion**

In the present study, we have examined the dynamic process of KIR clustering at the immune synapse in living cells using KIR-GFP fusion proteins and investigated the role of the cytoplasmic tail and actin cytoskeleton in this process. We have previously shown that the degree of clustering correlates with the strength of the inhibitory signal (14). Given that activation of cytolysis is a rapid process, the rate of KIR accumulation must be sufficient to
block activating receptors that presumably are rapidly recruited to the synapse during activation. In this study, our kinetic analysis of KIR clustering indicates KIR clustering is a relatively rapid process, reaching its maximum by 10 min. Also, we have shown that formation of new actin filaments was required for efficient clustering of KIR, because KIR enrichment took much longer in the presence of cytochalasin D. Contrary to a previous study of HLA-C-GFP clustering, we observed a decrease in KIR enrichment in the presence of azide (12). These data are reconciled in this study by the demonstration that actin polymerization affects the early accumulation of KIR, but not the accumulation of KIR following long periods of coincubation with target cells. The absence of an effect of PP2 suggests that the active process of KIR clustering is not Src family kinase dependent. In addition, we observed that a truncated KIR fused to GFP was defective in that there was a decrease in the frequency of conjugates with clusters. The mean total intensity values were statistically different, suggesting that truncated receptor behaves differently than full-length receptor, and this is what accounts for differences in the magnitude of clusters. Importantly, treatment with cytochalasin D did not substantially decrease the frequency of clustering of the truncated receptor. Thus, taken together, these results suggest that KIR clustering at the inhibitory NK cell immune synapse is modulated by both the actin cytoskeleton and the cytoplasmic tail of KIR.

Cytochalasin D disrupts the actin cytoskeleton by binding to the barbed ends of rapidly growing microfilaments, but does not interfere with basal levels of depolymerization, leading to a net reduction in F-actin. The block of lysis at low doses of cytochalasin D is consistent with recent findings by Wulfing et al. (26) that NK cell killing is particularly sensitive to interference with cytoskeletal dynamics. In their study, Wulfing et al. report that NK cell cytoskeletal polarization occurred in a stepwise fashion and a moderate interference with cytoskeletal dynamics using Jasplakinolide was sufficient to block NK cell lysis, but not cytotoxic T cell lysis. Interestingly, the low doses of cytochalasin D used in our study had little to no effect on KIR clustering. At present, we are unable to explain why receptor clustering is not as sensitive to minor interference with cytoskeletal dynamics. There is precedence for differential effects of cytochalasin D doses in lymphocytes. Low doses of cytochalasin D are thought to stimulate adhesion by the release of LFA-1 from cytoskeletal restraints, whereas high doses are thought to dramatically reduce adhesion by blocking establishment of new connections to the cytoskeleton (27, 28). A similar dichotomy could explain why, in our study, low doses are sufficient to block cytolysis, while high doses are required to block KIR clustering. Moreover, it has previously been shown that although subtle changes in the actin cytoskeleton are detectable at nanomolar concentrations, which correlates with disruption of mitosis in adherent cells, the changes in actin are much more dramatic at micromolar concentrations (29). Perhaps KIR clustering relies solely on the basal levels of F-actin, and so the levels present after brief treatment with the low doses of cytochalasin D used in our study may be sufficient for inhibitory receptor enrichment. Alternatively, cytochalasin D may act by disrupting attachments of barbed ends to membranes or other cellular structures (30). If this were true, then perhaps at the higher doses of cytochalasin D used in our study, a greater proportion of filaments would be displaced and retracted, thereby exceeding the threshold level necessary for inhibition of KIR clustering. We cannot preclude the possibility that the standard 10 μM dose of cytochalasin D is having effects on processes other than actin cytoskeleton remodeling and that these processes are key to KIR clustering. It is also possible that lower doses of cytochalasin D are having effects on processes dependent or independent of the actin cytoskeleton and that these processes are key to lysis and adhesion, but not to inhibitory receptor clustering.

It is tempting to speculate that the phenotype of the truncated receptor is related to an interaction with the actin cytoskeleton because cytochalasin D does not further decrease clustering of the truncated receptor. The truncated receptor may be impaired in its ability to accumulate at the synapse because the tail is involved in interactions with an adaptor protein. Alternatively, the events involved in formation of an activating synapse, which would occur despite engagement of the truncated receptor, could also disrupt KIR accumulation (13). For example, the coalescence of lipid rafts, recruitment of many cell surface proteins, and fusion of cytoplasmic granules at the interface might diminish the amount of KIR accumulation by competition for space. Treatment with cytochalasin D does not mimic the situation of the truncated receptor because cytochalasin D prevents activation pathways. The reason for the discrepancy between the truncated KIR-GFP receptor reported in this study and the report of another truncated KIR to induce HLA-C-GFP clustering is not obvious (16).

Although KIR was seen to accumulate at the 40-min time point in untreated conjugates, the rapid KIR clustering driven by remodeling of the cytoskeleton is most likely key to the function of KIR because taking 20–40 min to accumulate would often be too late to counteract activating signals. The role of actin polymerization in KIR clustering must be reconciled with the observation that F-actin does not increase at an inhibitory synapse (Fig. 6) (13, 31). In fact, it has been shown in YTS cells that KIR-associated SHP-1 targets Vav1 for dephosphorylation, which most likely blocks Vav1-induced actin polymerization (32). Rapid clustering of KIR driven by remodeling of the cytoskeleton similar to how receptors are translocated in other cell types (33) is an appealing idea because activation of cytolysis can occur within a few minutes (34–37). Another consideration is that the actin filament formation that is relevant for rapid KIR clustering need not be stimulated by interaction with the target cell, but instead could be part of the normal turnover of F-actin in NK cells or even in the target cells.

The actin cytoskeleton of cytolytic cells that are migrating is in constant flux with new polymerization being favored at the leading edge and depolymerization at the trailing edge (reviewed in Ref. 38).

Our results show F-actin formation is important to stabilize conjugates, and suggest this process can occur to some extent whether or not KIR will ultimately be engaged. F-actin can be regulated by different mechanisms, including those that stimulate formation of filaments such as Vav-1/Rac1 (reviewed in Ref. 39) and Wiskott-Aldrich syndrome protein (WASP)/Arp2/3 (reviewed in Ref. 40), and those that prevent actin disassembly such as RhoA/Rho-associated protein kinase 1/LIM kinase 1/ coflinin (41, 42). Vav, WASP, and RhoA have all been implicated in activation of cytotoxicity in NK cells (reviewed in Ref. 43), and pathways such as LFA-1/Vav1 or CD2/WASP could act upstream of KIR (25, 44–46). Interactions of molecules such as LFA-1 with the cytoskeleton through adaptor proteins such as talin might be required indirectly for KIR clustering. In support of this, talin is initially recruited to the interface whether or not the NK cell will become activated or inhibited by KIR (18). Talin is subsequently released from inhibitory synapses, but maintained in activating synapses. We determined that blocking the activated form of LFA-1 inhibited tight conjugate formation, but that KIR clustering is normal in conjugates that form in the absence of activated LFA-1-mediated interactions (Fig. 7). Because the results with anti-LFA-1 did not mimic treatment with cytochalasin D, it suggests that cytochalasin D exerts a more complete block on adhesion or another process that is involved in
KIR clustering. Similarly, a recent report using insect cells to express KIR ligands indicated KIR clustered and was phosphorylated independently of LFA-1/ICAM-1 interactions (47).

In summary, the observations presented in this study demonstrate that the actin cytoskeleton and the cytoplasmic tail of KIR play an important role in the efficiency of KIR accumulation at the inhibitory immune synapse. It will be interesting to dissect the underlying mechanisms and determine whether there are implications for other inhibitory receptors.

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