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Tethering of Intercellular Adhesion Molecule on Target Cells Is Required for LFA-1–Dependent NK Cell Adhesion and Granule Polarization

Catharina C. Gross,* Joseph A. Brzostowski,† Dongfang Liu,* and Eric O. Long*

αβ2 integrin (LFA-1) has an important role in the formation of T cell and NK cell cytotoxic immunological synapses and in target cell killing. Binding of LFA-1 to ICAM promotes not only adhesion but also polarization of cytolytic granules in NK cells. In this study, we tested whether LFA-1–dependent NK cell responses are regulated by the distribution and mobility of ICAM at the surface of target cells. We show that depolymerization of F-actin in NK-sensitive target cells abrogated LFA-1–dependent conjugate formation and granule polarization in primary NK cells. Degranulation, which is not controlled by LFA-1, was not impaired. Fluorescence recovery after photobleaching experiments and particle tracking by total internal reflection fluorescence microscopy revealed that ICAM-1 and ICAM-2 were distributed in largely immobile clusters. ICAM clusters were maintained and became highly mobile after actin depolymerization. Moreover, reducing ICAM-2 mobility on an NK-resistant target cell through expression of ezrin, an adapter molecule that tethers proteins to the actin cytoskeleton, enhanced LFA-1–dependent adhesion and granule polarization. Finally, although NK cells kept moving over freely diffusible ICAM-1 on a lipid bilayer, they bound and spread over solid-phase ICAM-1. We conclude that tethering, rather than clustering of ICAM, promotes proper signaling by LFA-1 in NK cells. Our findings suggest that the lateral diffusion of integrin ligands on cells may be an important determinant of susceptibility to lysis by cytotoxic lymphocytes. The Journal of Immunology, 2010, 185: 000–000.

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Abbreviations used in this paper: CDF, cumulative distribution function; FRAP, fluorescence recovery after photobleaching; Jasp, jasplakinolide; LatA, latrunculin A; TIRF, total internal reflection fluorescence.
Materials and Methods

Cells
Human NK cells were isolated from peripheral blood cells by negative selection using an NK cell isolation kit (Miltenyi Biotec, Auburn, CA). Resting NK cells (95–99% CD3+CD56+) were resuspended in IMDM (Invitrogen, Carlsbad, CA) supplemented with 10% human serum (Valley Biomedical, Winchester, VA) and used 1–2 d after isolation. Polyclonal IL-2–activated NK cells were expanded in the presence of feeder cells (0.5 × 10^8 PBLs/ml, gamma-irradiated with 4.5K) with IMDM supplemented with 10% human serum, 10 IU/ml rIL-2 (Hoffman-La Roche, Basel, Switzerland), and 10% purified human IL-2 (Hemagen Diagnostics, Columbia, MD). IL-2–activated cells were used 2–3 wk after isolation.

The B cell lymphoma line J1221.1 was cultivated in IMDM supplemented with 10% heat-inactivated FBS (Hyclone, Logan, UT). The mouse thymoma cell line BW5147 (gift from T. Kannan, National Institute of Aging, and Infectious Diseases, National Institutes of Health, Bethesda, MD) was cultured in IMDM supplemented with 10% heat-inactivated FBS.

Transfection of BW5147 cells with hEzrin-EGFP

BW5147 cells were transfected with hEzrin-EGFP (pH421) human ezrin coding sequence subcloned into pEGFP-N1 eukaryotic expression vector (a gift from J-J. Hao and S. Shaw, National Cancer Institute, National Institutes of Health [32]), using the BTX machine (ECM830, settings: 230 V, 10 ms, 1 pulse; Harvard Apparatus, Holliston, MA). Cells were recovered for 16 h at 37˚C, washed, and ezrin-EGFP-positive cells were selected by FACS. Stable transfectants were generated from the FACS-sorted cells by cultivating them in IMDM/10% FBS/1 mg/ml Geneticin (Life Technologies, Grand Island, NY) and subcloning.

Pretreatment of target cells with inhibitors

All inhibitors were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO). For latrunculin A (LatA) or jasplakinolide (Jasp) (both Calbiochem, San Diego, CA) treatment, 10 × 10^5 721.221 or K562 target cells were resuspended in 1 mL IMDM supplemented with 10% FBS containing 2% DMSO as a negative control to match the final concentration of carrier in treated cells. For the experiments, cells were treated 0.5, 3, or 20 μM LatA or 0.5, 1, or 2 μM Jasp. Staining F-actin with phallolidin revealed that 3 μM LatA was required to disrupt F-actin completely and that the effect of Jasp was evident at a concentration as low as 0.5 μM (data not shown). Cells were incubated for 40 min at 37˚C. Inhibitors were washed away prior mixing with NK cells for the different assays.

Conjugation assay

Conjugate formation between NK cells and target cells was determined as previously described (12) with minor modifications. Briefly, NK cells were labeled with 1 μg/ml Cell Tracker Green CMFDA (Invitrogen) for 30 min at 37˚C and 5% CO₂, washed, and incubated for another 30 min in 37˚C and 5% CO₂. If BW5147 cells were used as targets, NK cells were labeled with 20 μM anti-CD56 allophycocyanin (BD Biosciences, San Jose, CA) for 15 min at 37˚C and 5% CO₂. Target cells were labeled with PKH26 Red (721.221, K562) or PKH67 Green (BW5147 cells) (both Sigma-Aldrich) for 5 min at room temperature, washed extensively, and recovered for 30 min at 37˚C and 5% CO₂. NK cells and target cells that shifted into mixed at a 1:2 E:T cell ratio with 1 × 10^5 NK cells and 2 × 10^5 target-cells at 4˚C. Cells were spun down at 20 × g for 3 min, and conjugate formation was stopped by vortexing and fixation of cells using 0.5% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA) after 0-, 5-, 10-, 20-, 30-, or 60-min incubation at 37˚C. Conjugate formation was determined by flow cytometry (FACSCalibur; BD Biosciences) and is represented as the fraction of NK cells that shifted into two-color conjugates. For blocking of LFA-1 on NK cells, 1 × 10^5 Cell Tracker Green–labeled NK cells were pretreated with 20 μg/ml IgG2a (HOPC-1; Sigma-Aldrich) or anti-human CD18 (Calbiochem) for 15 min at 4˚C.

Degranulation assay

The degranulation assay was performed as described previously (14). Briefly, 2 × 10^5 NK cells were added to 4 × 10^4 721.221 or K562 cells in a total volume of 200 μl IMDM supplemented with 10% heat-inactivated FBS. Cells were mixed and incubated for 1 h at 37˚C and 5% CO₂. Afterward, the cells were spun down and stained with PE-conjugated anti-CD56 (BD Biosciences) and FITC-conjugated anti-CD107a (BD Biosciences) Ab for 45 min at 4˚C. Degranulation of CD56-positive NK cells was analyzed by flow cytometry.

Perforin polarization assay

The polarization assay was performed as described previously (15). Briefly, NK cells and target cells (721.221, K562, or BW5147) were mixed at a 1:1 E:T ratio, with 1 × 10^5 of each cell type per sample. Cells were incubated for 20 min at 37˚C and 5% CO₂. Cells were resuspended and put onto a poly-L-lysine-coated 2-well planar lipid slide (BD Biosciences). Cells were allowed to settle down for 1 h at room temperature and fixed using 4% paraformaldehyde (Electron Microscopy Sciences). Cells were permeabilized using 0.5% Triton X-100 and stained using an anti-human perforin Ab (Pierce, Rockford, IL) and an Alexa 488-conjugated secondary goat anti-mouse IgG Ab (Molecular Probes, Eugene, OR). Cells were imaged by confocal microscopy (LSM510; Zeiss, Oberkochen, Germany) with a ×40, 1.3 NA Plan Neofluar (Zeiss) oil immersion objective lens. Fifty to 200 NK cells in contact with target cells were analyzed for polarization of perforin containing granules.

Fluorescence recovery after photobleaching analysis

ICAM-1 on 721.221 cells was stained using a PE-conjugated anti–ICAM-1 Ab (BD Biosciences). Fluorescence recovery after photobleaching (FRAP) was performed using a confocal microscope (LSM510; Zeiss). A region of the cell surface was bleached using the 488-nm laser. The recovery of the bleached region was captured at one frame per 30 s for 10 frames. During the experiments, cells were maintained at 37˚C. The fluorescence intensities of the bleached region and an unbleached region of the same cell over time were analyzed using the LSM510 software (Zeiss). The medians of the relative intensities of different cells were plotted over time.

Total internal reflection fluorescence microscopy

Endogenous cell surface ICAM-1 or ICAM-2 on 721.221 or BW5147 cells was fluorescently labeled with PE-conjugated anti-human ICAM-1 (BD Biosciences), anti-human ICAM-2 (Beckman Coulter, Fullerton, CA), or anti-mouse ICAM-2 (BD Pharingen) Ab. Total internal reflection fluorescence (TIRF) imaging was performed using an Olympus IX81 TIRF microscope equipped with a 561-nm diode-pumped laser (Coibolt, Stockholm, Sweden), ×100 1.45 NA Olympus TIRF microscope lens, and a Cascade II 1024B EM-CCD camera (Photometrics, Tucson, AZ). Cells were maintained at 37˚C using a LiveCell environmental chamber (Pathology Devices, Westminster, MD). Images were captured at 32 frames/s using MetaMorph software (Molecular Devices, Sunnyvale, CA) for 250 frames. The movement of labeled ICAM molecules was automatically tracked using code developed for MatLab software (http://physics.georgetown.edu/matlab [33]). The code was modified to refine particle positioning with a two-dimensional Gaussian fit (34) and to determine intensities of individual particles. Mean square displacements were determined from positional coordinates to calculate short-range diffusion coefficients of individual particles for five time intervals. The resulting diffusion coefficients were plotted as a cumulative distribution function (CDF). A CDF plots the same information as a histogram for a given data set; however, where the histogram plots the frequency distribution (y-axis) as a function of the binned data set (x-axis), the data are not binned for a CDF plot. The CDF plot is generated as follows: the data set is sorted from the smallest to greatest value. Next, the number of data points is summed, and the value of the position of a data point on the sorted list is divided by the sum to yield the probability value (y-axis). Finally, based on its position on sorted list, the probability is plotted against the value of the data point. The CDF displays the distribution of the data set from the smallest (from left on the x-axis) to greatest value (at the right of the x-axis) and provides the probability (y-axis) of whether a particular value will occur at or less than a specified point on the x-axis. Moreover, a CDF plot allows for the rapid identification of the median value of the data set by interpolation at 50% on the y-axis, it is not prone to binning artifacts, and the separation of the data set by log scale is more apparent for visual inspection. In addition, graphical comparisons between data sets are generally more easily interpretable relative to overlaying histograms.

Imaging of NK cells on ICAM-1 bound to lipid bilayers or coverslips

Planar lipid bilayers carrying human ICAM-1 tagged with six histidines were formed between a coverslip and the microaqueduct slide of a Biotechnics parallel plate flow chamber-FCS2 (Biotechnics, Butler, PA) as described previously (10, 11). A human ICAM-1–Fc fusion protein was coated on coverslips in 100 mM sodium bicarbonate (pH 9.2) at 10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml at 4˚C overnight. Five-percent FBS-containing culture medium was used to block nonspecific binding. The ICAM-1–Fc fusion protein cloned in the C5Dsineg1 vector (35) was produced by...
transfection into 293T cells, as described previously (36). To avoid stimulusation of NK cells via CD16 by the human IgG1 Fc portion of the ICAM-1-Fc fusion protein, the CD16 binding site was mutated in the CD5lneg1 vector, as described previously (14). Briefly, aa 235 and 236 of human IgG1 within the Fc domain were mutated as Leu235 to Gly235 and Gly236 to Leu236. Whereas unmutated ICAM-1-Fc fusion protein that was attached to plates triggered degranulation in NK cells, the ICAM-1-Fc protein carrying the mutated CD16 binding site did not. The movement of NK cells was measured 20 min after injection and tracked for 25 frames with a 10-s interval between frames by Dynamic Image Analysis System (37).

Results
Disruption of actin filaments in target cells prevents conjugate formation with NK cells

Two functions of NK cells for which LFA-1 signaling is sufficient are adhesion and granule polarization. The B cell lymphoma cell line 721.221 (38), which does not express HLA-A, HLA-B, and HLA-C, is sensitive to lysis by NK cells. To test whether conjugate formation of NK cells is dependent on an intact cytoskeleton in target cells, actin filaments (F-actin) were disrupted in 721.221 cells with different concentrations of LatA (0.5, 3, and 20 μM) prior to washing and mixing those cells with NK cells in a conjugation assay. Conjugate formation of freshly isolated, primary NK cells was inhibited by LatA treatment of 721.221 cells (Fig. 1A, left panel). Moreover, conjugate formation of IL-2–activated NK cells with 721.221 cells was also prevented by pretreatment of target cells with LatA (Supplemental Fig. 1). These results suggest that either an intact actin cytoskeleton or actin cytoskeleton remodeling is required for proper conjugate formation.

To distinguish between these two possibilities, F-actin filaments in target cells were stabilized by treatment with Jasp (Fig. 1A, right panel). Jasp stabilizes existing F-actin but prevents actin cytoskeleton remodeling. Because Jasp had no effect on conjugate formation with NK cells (Fig. 1A, right panel), cytoskeleton dynamics in target cells do not seem to be important for conjugate formation of NK cells. In addition, disruption of microtubules in target cells with Nocodazole had no effect on conjugate formation with resting NK cells (data not shown). We conclude that an intact cytoskeleton, but not cytoskeleton dynamics, in target cells is important for NK cell conjugate formation.

To test whether disruption of F-actin in target cells was preventing conjugate formation specifically, or whether it had more global inhibitory effects, the ability of NK cells to degranulate in response to target cells was evaluated. Whereas conjugate formation of NK cells with ICAM-expressing target cells is strictly dependent

FIGURE 1. Disruption of actin filaments in target cells prevents conjugate formation with NK cells. A, 721.221 cells pretreated with DMSO carrier alone (●), LatA (left panel), or Jasp (right panel) at indicated concentrations for 40 min at 37°C were tested for conjugate formation with resting NK cells. Error bars indicate the SD (n = 3 individual NK cell donors in three independent experiments). B, 721.221 target cells treated as in A were mixed with resting NK cells, and degranulation was measured after 1 h at 37°C by staining for CD107a at the surface of NK cells. Each symbol indicates one of the six (left panel) or four (right panel) individual NK cell donors. Six (left panel) or four (right panel) independent experiments were performed.

FIGURE 2. Disruption of actin filaments in 721.221 target cells inhibits polarization of cytolytic granules in NK cells. 721.221 cells incubated either with DMSO carrier (A, B, filled histograms) or with 3 μM (A) or 20 μM (B) LatA were mixed with NK cells for 20 min at 37°C. The cells were transferred to poly-o-lysine–coated culture slides and incubated for 1 h at room temperature before fixation, permeabilization, and staining for perforin. Granule polarization is expressed as the fraction of cells in contact with perforin clustered at the NK-target cell interface. Error bars indicate the SD (n = 3 individual donors in three independent experiments).
on LFA-1 (Supplemental Fig. 2) (39), degranulation is LFA-1 independent (13, 14). 721.221 cells treated with lower doses of LatA induced about twice as much degranulation by NK cells as untreated 721.221 cells (Fig. 1B, left panel). Our laboratory has observed that, under certain conditions, engagement of LFA-1 by ICAM-1 reduces the amount and delays the onset of degranulation induced by activation receptors (10, 14). The enhanced degranulation seen in this study after latrunculin treatment of target cells may be due to a reversal of LFA-1–mediated inhibition of degranulation. In agreement with this hypothesis, blocking of LFA-1 with an Ab resulted in a 2-fold increase of degranulation by NK cells (Supplemental Fig. 3). At the highest dose (20 μM), however, LatA blocked degranulation by NK cells (Fig. 1B, left panel). Stabilization of F-actin in 721.221 cells by Jasp (Fig. 1B, right panel), or disruption of microtubules with Nocodazole (data not shown), had no effect on degranulation by NK cells. We conclude that LFA-1–dependent conjugate formation, but not LFA-1–independent degranulation, requires an intact cytoskeleton on target cells.

**Disruption of F-actin in target cells inhibits polarization of cytolytic granules in NK cells**

The sensitivity of conjugate formation but not degranulation of NK cells to the disruption of F-actin in target cells suggested that a functional LFA-1 interaction with ICAM is dependent on intact F-actin in target cells. To test this possibility, we evaluated the importance of an intact cytoskeleton in target cells for another LFA-1–dependent function in NK cells—granule polarization toward target cells (14, 15). Pretreatment of 721.221 cells with LatA resulted in diminished granule polarization in NK cells (Fig. 2). As expected, because of inhibition of tight conjugate formation, fewer NK cells were found in conjugates with 721.221 cells in the presence of LatA. However, the reduction of polarization was not a consequence of reduced conjugate formation as polarization was scored only in NK cells that had formed tight contact with target cells. Therefore, in addition to the LFA-1–dependent conjugate formation, another LFA-1–dependent process, polarization of cytolytic granules, is dependent on an intact cytoskeleton in target cells.

**Disruption of F-actin changes the lateral mobility of ICAM-1 and ICAM-2**

How could disruption of F-actin in target cells result in defective ICAM interactions with LFA-1 on NK cells? As ICAM is linked to the actin cytoskeleton by direct binding to α-actinin (α-actinin-1 and α-actinin-4) (40–42) and to the ezrin/radixin/moesin protein ezrin (31, 43, 44), we tested whether treatment of 721.221 cells with LatA had an effect on ICAM-1 mobility at the plasma membrane by

![FIGURE 3. Disruption of actin filaments in target cells increases ICAM-1 mobility. ICAM-1 mobility was determined by FRAP. Cells were stained with a PE-labeled anti–ICAM-1 Ab (original magnification ×80). Fluorescence recovery in the bleached area of 721.221 cells either untreated (A, upper panel; scale bar, 5 μm; B, ◦) or LatA treated (A, lower panel; B, ⊗) is displayed. Fluorescence intensity of a nonbleached area of the 721.221 cells either untreated (B, ◦) or LatA treated (B, ⊗) served as a control. Error bars indicate the SD (untreated cells, n = 7; LatA-treated cells, n = 8).](http://www.jimmunol.org/)

![FIGURE 4. Disruption of actin filaments increases the mobility of ICAM-1 on K562 cells and decreases conjugate formation with NK cells. The mobility of ICAM-1 was determined by FRAP. Cells were stained with a PE-labeled anti–ICAM-1 Ab (original magnification ×80). Fluorescence recovery in the bleached area of K562 cells either untreated (A, upper panel; scale bar, 5 μm; B, ◦) or LatA treated (A, lower panel; B, ⊗) is displayed. Fluorescence intensity of a nonbleached area of the K562 cells either untreated (B, ◦) or LatA treated (B, ⊗) served as a control. Error bars indicate the SD (untreated cells: n = 9; LatA–treated cells, n = 6). C, K562 cells pretreated with DMSO carrier alone (◦) and LatA at indicated concentrations for 40 min at 37˚C were tested for conjugate formation with resting NK cells. Error bars indicate the SD (n = 3 individual NK cell donors in three independent experiments). D, K562 target cells treated as in C were mixed with resting NK cells, and degranulation was measured after 1 h at 37˚C by staining for CD107a at the surface of NK cells. Each symbol indicates one of the four individual NK cell donors (n = 4 independent experiments).](http://www.jimmunol.org/)
FRAP (Fig. 3, Supplemental Videos 1, 2). ICAM-1 molecules on 721.221 cells were stained using a PE-conjugated anti–ICAM-1 Ab, and a region of the cell surface was bleached using a 488-nm laser (Fig. 3A, white arrow). The recovery of the bleached region of untreated (Fig. 3A, upper panel; 3B, ●, Supplemental Video 1) and LatA-treated (Fig. 3A, lower panel, 3B, ○, Supplemental Video 2) 721.221 cells was captured during 5 min. Photobleaching as a result of image acquisition was monitored in untreated and LatA-treated 721.221 cells to normalize recovery curves (Fig. 3B, ■ and □, respectively). On untreated 721.221 cells, no recovery of the bleached region was observed (Fig. 3A, upper panel, 3B, ●, Supplemental Video 1), suggesting that ICAM-1 is immobilized on 721.221 cells. However, after disruption of F-actin with 20 μM LatA, recovery of the bleached region (Fig. 3A, lower panel, 3B, ○, Supplemental Video 2) occurred during 90 s, indicating a release of ICAM-1 from the cytoskeleton. Moreover, ICAM-1, which was polarized to one side of untreated 721.221 cells (Fig. 3A, upper panel, Supplemental Video 1), was evenly distributed after disruption of F-actin with LatA (Fig. 3A, lower panel, Supplemental Video 2).

To test whether polarization of ICAM-1 to one side of target cells, rather than or in addition to ICAM-1 tethering, was important for LFA-1–dependent adhesion of NK cells, we examined the cell line K562, which does not exhibit polarity of ICAM-1 at the cell surface (Fig. 4A). Treatment of K562 cells with LatA-released ICAM-1 form the cytoskeleton and increased its mobility (Fig. 4B). In accordance with the 721.221 data, the release of ICAM-1 from the cytoskeleton of K562 target cells prevented conjugate formation in freshly isolated primary NK cells (Fig. 4C) and polarization of cytolytic granules (data not shown), indicating that ICAM-1 tethering rather than polarization is important for LFA-1–dependent signaling in NK cells. The LFA-1–dependent degranulation of NK cells was not inhibited by pretreatment of K562 target cells with different concentrations of LatA (Fig. 4D) excluding more global inhibitory effects of the drug.

We next used TIRF microscopy to visualize the distribution and mobility of ICAM molecules in the plasma membrane of target cells and to understand how the actin cytoskeleton affects ICAM mobility by treating target cells with either LatA or Jasp to disrupt or stabilize the actin cytoskeleton, respectively. TIRF microscopy is a spatially limited, high-contrast technique that eliminates interference from bulk fluorescence that may be present within cells to allow for the detection of fluorophores proximal to and within the plasma membrane of cells adhered to glass.

**FIGURE 5.** Disruption of actin filaments increases the mobility of ICAM-1 and ICAM-2. The mobility of ICAM-1 (A, upper panel; scale bar, 5 μm; B, D, left panel) and ICAM-2 (A, lower panel, B, D, right panel) in 721.221 cells treated with the indicated concentrations of LatA (A, middle panel, B) and Jasp (A, right panel, D) was determined by TIRF microscopy (original magnification ×100). The movement of ICAM particles labeled with ICAM-1 and ICAM-2 PE-conjugated Abs was tracked by capturing TIRF images at 32 frames/s for 250 frames. Diffusion coefficients of ICAM-1 (B, D, left panel) or ICAM-2 (B, D, right panel) particles are shown in CDF plots. Plots represent one of two representative experiments. The total number of ICAM-1 particles analyzed over the drug concentration range is ~40,500 for LatA-treated cells and ~15,400 for Jasp-treated cells. The p values for the latrunculin-treated cells are the following: p = 4.98 x 10^{-9} between DMSO and 0.5 μM LatA, p = 7.81 x 10^{-13} between 0.5 and 3 μM LatA, and p = 7.86 x 10^{-13} between 3 and 20 μM LatA. All are determined by Kolmogorov-Smirnov test. Similarly, ~32,700 ICAM-2 particles in latrunculin-treated cells and ~23,600 particles in Jasp-treated cells were analyzed. C. The average intensity of ICAM-1 and ICAM-2 was measured in a 5 x 5 pixel grid centered over the peak of the Gaussian distribution calculated for the particle in the first frame in which it appeared in the tracking algorithm. Average particle intensities are shown in CDF plots. Median intensities are indicated in parentheses.
coverslips (45). Endogenous, cell surface ICAM-1 or ICAM-2 on 721.221 cells were fluorescently labeled with PE-conjugated anti-ICAM Abs. Although individual ICAM proteins were labeled with a single PE-fluorophore (see Materials and Methods), photo-bleaching characteristics (the presence of multiple-step bleaching events over long track lengths; data not shown) of fluorescent PE-labeled particles suggest that ICAM proteins were mostly observed as clusters and not single molecules (data not shown).

The lateral movement of labeled ICAM-1 (Fig. 5A, upper panel, 5B, left panel, Supplemental Videos 3–5) and ICAM-2 (Fig. 5A, lower panel, 5B, right panel) particles recorded by TIRF microscopy was automatically tracked using an algorithm developed for MatLab software (33), which was further modified to refine particle positioning with a two-dimensional Gaussian fit (34). Short-range mean square displacements were determined from positional coordinates of particles tracked for five frames (>160 ms [34]) and were linearly dependent on time under all conditions measured, consistent with a simple diffusion model. Mean square displacement versus time plots clearly showed that diffusion increases, as indicated by the slope of the line, with LatA concentration (Supplemental Fig. 4). Short-range diffusion coefficients were then determined for thousands of particles in multiple cells and graphed as a cumulative distribution function (CDF) to represent the frequency of diffusion coefficients for the entire population of tracked particles (Fig. 5B) (34). Consistent with our FRAP data above, disrupting the actin cytoskeleton with LatA incurred a dose-dependent shift toward the mobile population for both ICAM-1 and ICAM-2 particles (Fig. 5B, Supplemental Videos 3, 4). The median diffusion coefficient for ICAM-1 and ICAM-2 in untreated control cells was calculated to be 0.013 and 0.030 μm²/s, respectively, and after the maximum dose of LatA treatment, mobility increased ∼10- and 5-fold, respectively (0.132 μm²/s for ICAM-1 and 0.146 μm²/s for ICAM-2) (Fig. 5B). In accordance with these results, binding of NK cells to 721.221 target cells was inhibited by disruption of F-actin filaments in

**FIGURE 6.** Expression of human ezrin in BW5417 cells reduces the mobility of mouse ICAM-2. A, Lysates of BW5417 cells and BW5417 cells transfected with human ezrin-EGFP where probed with anti-ezrin, anti-GFP, and anti–α-tubulin Ab. B and C, The mobility of ICAM-2 on BW5417 cells (C, black line) and BW5417 cells expressing human ezrin (C, gray line) was determined by TIRF microscopy as described in Fig. 5 using a PE-labeled anti-mouse ICAM-2 Ab. Scale bar, 5 μm; original magnification ×100. Plots represent the cumulative frequency of diffusion coefficients of ICAM-2 particles of one of two representative experiments. Approximately 3100 and 2700 particles in untransfected and ezrin-transfected cells, respectively, were analyzed. D, The intensity of ICAM-2 clusters on BW5417 cells (black line) and BW5417 cells expressing human ezrin (gray line) was analyzed as described in Fig. 5. Median intensities are indicated in parenthesis.

**FIGURE 7.** Expression of human ezrin in BW5417 cells restores functional interaction with LFA-1. A, Conjugate formation of IL-2–activated NK cells with untransfected BW5417 cells (▲) or with BW5417-hEzrin target cells (△) was determined by flow cytometry. B, Polarization of cytolytic granules in IL-2–activated NK cells mixed with either BW5417 (▲) or BW5417-hEzrin (△) cells was measured as described in Fig. 2. Error bars indicate the SD (n = 3 individual NK cell donors in three independent experiments).

**FIGURE 8.** Movement of human resting NK cells over mobile and immobile ICAM-1. NK cells were visualized 20 min after injection into chambers containing ICAM-1 bound to either lipid bilayers or glass surfaces, respectively. The movement of NK cells was tracked for 25 frames with a 10-s interval between frames by Dynamic Image Analysis System. The cell at the top of the stack is the cell from the last frame. A, Engagement of LFA-1 by diffusible ICAM-1 on lipid bilayer promoted active movement of NK cells. B, Engagement of LFA-1 by solid-phase ICAM-1 resulted in arrest and stable binding (left panel) or spreading and slow movement of NK cells (right panel).
721.221 cells (Fig. 1A, left panel). Although LatA reduced the intensity of the brightest ICAM-1 clusters, it did not appreciably alter the median intensity of either ICAM-1 or ICAM-2 particles (Fig. 5C), suggesting that ICAM cluster size on target cells does not affect NK cell response.

Both our FRAP and TIRF microscopy results indicate that ICAM-1 and ICAM-2 are tethered to the actin cytoskeleton, which, in turn, slows their lateral mobility in the plasma membrane; if so, then one would predict that ICAM mobility would not increase, and may be reduced even further, after treatment with the actin-stabilizing drug Jasp. Indeed, the mobility of ICAM-1 (Fig. 5D, left panel, Supplemental Video 5) and ICAM-2 (Fig. 5D, right panel) was slightly reduced with Jasp and stabilization of F-actin in target cells had little effect on LFA-1–dependent signaling (Fig. 1A, right panel). In addition, disruption of the microtubule network with Nocodazole did not affect the mobility of ICAM-1 (data not shown).

**Expression of ezrin in BW5147 cells restores ICAM-2 tethering and functional interaction with LFA-1**

Our results so far indicate that a higher mobility of ICAM in the plasma membrane hinders its interaction with LFA-1 on NK cells. However, the contribution of other changes induced by LatA in target cells that could impact on LFA-1–ICAM interactions cannot be ruled out. We therefore carried out gain-of-function experiments using a cellular system in which ICAM mobility could be reversed. A change in the distribution of ICAM-2 on the mouse BW5147 thymoma cell line from evenly distributed to polarized after transient expression of human ezrin has been reported (31). We generated BW5147 cells that stably express human ezrin tagged with GFP (Fig. 6A) and monitored the mobility of ICAM-2 by TIRF microscopy (Fig. 6B, 6C, Supplemental Videos 6, 7). As shown in Fig. 6B and Supplemental Video 6, ICAM-2 was evenly distributed and mobile on BW5147 cells. Expression of GFP-tagged ezrin (Fig. 6A, 150-kDa band) in BW5147 cells decreased the mobility of ICAM-2 (Fig. 6C, Supplemental Videos 6, 7) from 0.053 to 0.027 μm²/s. Moreover, ICAM-2 became polarized (Fig. 6B) and colocalized with the distribution of ezrin-GFP (data not shown). However, clustering of ICAM-2 molecules did not change (Fig. 6D).

To test whether the reduced mobility of ICAM-2 as a result of ezrin expression had an impact on functional interaction with LFA-1 on NK cells, conjugate assays were performed. Conjugate formation between human primary IL-2–activated NK cells and BW5147 cells was increased (Fig. 7A) when ICAM-2 mobility was reduced through ezrin expression (Fig. 6C). Moreover, expression of human ezrin in BW5147 target cells increased polarization of cytolytic granules in IL-2–activated NK cells (Fig. 7B). We conclude that tethering of ICAM-2 to the cytoskeleton of BW5147 cells by expression of human ezrin restores functional interaction with LFA-1.

**Rapid movement of NK cells over ICAM-1 inserted into lipid bilayers**

We next tested the interaction of LFA-1 on NK cells with ICAM-1 in the absence of other receptor–ligand interactions and compared interactions with mobile versus immobile ICAM-1. To visualize binding of NK cells to mobile ICAM-1, a histidine-tagged form of ICAM-1 was inserted into artificial planar lipid bilayers at the physiological concentration of 250 molecules/μm². Under these conditions, ICAM-1 exhibits high lateral mobility (10, 46). Primary resting NK cells on ICAM-1–coated lipid bilayers displayed rapid movement and failed to stop (Fig. 8A, Supplemental Video 8). A large fraction of NK cells displayed directed movement (Fig. 8A, Supplemental Video 8), whereas some moved randomly. The moving NK cells have a distinguished morphology consisting of a uropod and a dominant pseudopod at the leading edge of the cell (Fig. 8A, Supplemental Video 8). NK cells did not contact lipid bilayers in the absence of ICAM-1 (data not shown). At concentrations of 200 and 500 ICAM-1 molecules/μm², NK cells showed a similar phenotype than at 250 molecules/μm². The inability of NK cells to stop over lipid bilayers carrying ICAM-1 is not a general feature of their interaction with diffusible ligands, because the CD16 ligand IgG1 Fc inserted into lipid bilayers caused NK cell arrest and accumulation of IgG1 Fc into tight clusters (10). To monitor interaction with immobile ICAM-1, an ICAM-1-Fc fusion protein, in which the CD16 binding site had been mutated, was attached to coverslips. NK cells did not contact the coverslips in the absence of ICAM-1 or coverslips that had been coated with ICAM-1 at a concentration of 10 and 100 ng/ml (data not shown). However, ICAM-1 coating at concentrations of 1 and 10 μg/ml resulted in NK cells that were either attached and spread on the coverslips (Fig. 8B, left panel, Supplemental Video 9) or attached and spread NK cells that moved a little (Fig. 8B, right panel, Supplemental Video 10). In contrast to the persistent movement over ICAM-1–coated lipid bilayers, movement of NK cells over ICAM-1–coated coverslips was non directional and slower (Fig. 8B, Supplemental Videos 9, 10). A recent study reported that the NK cell line NKL moved over plate-bound ICAM-1-Fc (47). The difference may be due to the cells, because in our hands, NKL cells do not behave like primary NK cells when placed over lipid bilayers carrying ligands of NK cell receptors (D. Liu, unpublished observations). Taken together, our results indicate that diffusible ICAM-1 interacts with receptors on NK cells but does not promote the arrest of cell movement, whereas immobile ICAM-1 promotes stable binding of primary NK cells.

**Discussion**

Lateral mobility and clustering of transmembrane receptors and their segregation within membrane domains are often essential for proper signaling (26–28). Less is known about the importance of receptor ligand distribution at the surface of opposing cells (48). We addressed the question of how the distribution or mobility of ligands affect receptor signaling in the context of β2 integrin LFA-1 in NK cells and its functional interaction with ligands ICAM-1 and ICAM-2 on target cells. Earlier work established that the binding of LFA-1 on NK cells to ICAM-1 on target cells was sufficient for the formation of tight conjugates and polarization of cytolytic granules toward the NK-target cell interface (4, 12, 14, 15, 39). Because LFA-1 signaling for adhesion and granule polarization in human NK cells is uncoupled from signaling by other activation receptors (13, 14), it was possible to focus on NK cell functions controlled uniquely by LFA-1.

Two techniques, FRAP and TIRF microscopy, were used to visualize and quantify the distribution and movement of ICAM-1 and ICAM-2 on target cells in which the cytoskeleton was disrupted by LatA. The two-dimensional mobility of ICAM-1 and ICAM-2 at the surface of target cells was greatly enhanced after depolymerization of F-actin and correlated with reduced LFA-1–dependent NK cell responses. ICAM mobility, rather than clustering or polarization, was the key parameter for adhesion and granule polarization induced by LFA-1. LatA had minimal effect on the intensity of ICAM-1 and ICAM-2 clusters. Polarization of ICAM to one end of the cell, such as the one induced by ezrin, is not necessary for functional LFA-1–dependent responses because the target cell K562, which is highly sensitive to lysis by NK cells, does not display polarized ICAM-1 and stimulates strong LFA-1–dependent responses, which were lost upon release of ICAM-1 from cytoskeletal constraints.
lymphocytes was illustrated recently by the effect of cells. The importance of the cytoskeleton in cells that activate
important for conjugate formation and adhesion of NK cells, binding to fibronectin (55). Force contributes to conformational and
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Disclosures

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References


