Spatial Organization of Signal Transduction Molecules in the NK Cell Immune Synapses During MHC Class I-Regulated Noncytolytic and Cytolytic Interactions

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Spatial Organization of Signal Transduction Molecules in the NK Cell Immune Synapses During MHC Class I-Regulated Noncytolytic and Cytolytic Interactions

Yatin M. Vyas,* Kamini M. Mehta,* Margaret Morgan,† Hina Maniar,* Linda Butros,* Steffen Jung,‡ Janis K. Burkhardt,§ and Bo Dupont*§

The cytolytic activity of NK cells is tightly regulated by inhibitory receptors specific for MHC class I Ags. We have investigated the composition of signal transduction molecules in the supramolecular activation clusters in the MHC class I-regulated cytolytic and noncytolytic NK cell immune synapses. KIR2DL3-positive NK clones that are specifically inhibited in their cytotoxicity by HLA-Cw*0304 and polyclonal human NK cells were used for conjugate formation with target cells that are either protected or are susceptible to NK cell-mediated cytotoxicity. Polarization of talin, microtubule-organizing center, and lysosomes occurred only during cytolytic interactions. The NK immune synapses were analyzed by three-dimensional immunofluorescence microscopy, which showed two distinctly different synaptic organizations in NK cells during cytolytic and noncytolytic interactions. The center of a cytolytic synapse with MHC class I-deficient target is comprised of a complex of signaling molecules including Src homology (SH)2-containing protein tyrosine phosphatase-1 (SHP-1). Closely related molecules with overlapping functions, such as the Syk kinases, SYK, and ZAP-70, and adaptor molecules, SH2 domain-containing leukocyte protein of 76 kDa and B cell linker protein, are expressed in activated NK cells and are all recruited to the center of the cytolytic synapse. In contrast, the noncytolytic synapse contains SHP-1, but it is lacking other components of the central supramolecular activation cluster. These findings indicate a functional role for SHP-1 in both the cytolytic and noncytolytic interactions. We also demonstrate, in three-cell conjugates, that a single NK cell forms a cytolytic synapse with a susceptible target cell in the presence of both susceptible and nonsusceptible target cells. The Journal of Immunology, 2001, 167: 4358–4367.
cell-cell contact site as well as reorientation of microtubule-organizing center (MTOC) (20–24). In recent studies Ras-independent mitogen-activated protein kinase signals and phosphoinositide-3 kinase were shown to critically control lytic function and perforin/granzyme B polarization in NK-92 cells during tumor cell lysis (25, 26).

Direct analysis of signaling events in single NK cell-target cell conjugates regulated by MHC class I is limited to date. It has been shown that lipid rafts, enriched in signaling molecules, become polarized to the cell-cell contact area in NK cell conjugates with sensitive tumor cells, and this redistribution requires activation of Src and Syk kinases (27). It has also been shown that translocation of the MTOC is associated with activation of the tyrosine kinase, PYK-2 (28). In two other recent reports the inhibitory NK receptors and their corresponding MHC class I ligands were shown to colocalize in the contact area between NK cells and target cells (29, 30).

More information has been obtained from analysis of TCR-mediated signaling events in single Th cell conjugates interacting with peptide-MHC specific APCs (31, 32). It has been demonstrated that a highly structured intercellular interface, termed the supramolecular activation cluster (SMAC) (33, 34) or immunological synapse (35, 36) is formed, in which the adhesion molecule LFA-1 and the actin binding protein talin accumulate in the peripheral SMAC (pSMAC), while the TCR and the signaling molecule protein kinase C-θ (PKC-θ) were shown to cluster in the central SMAC (cSMAC) (33, 34). This structure is thought to be critical for optimizing the interactions between signaling molecules and their substrates. Although the distribution of only a small number of molecules has been reported to date, it is highly characteristic that LFA-1 and talin form a ring, which encloses TCR/MHC complexes and PKC-θ. The SMAC forms only under conditions where T cells receive a productive signal. When APCs lack peptide or when antagonistic peptide is used, no molecular segregation has been observed (34–38). Recently, in Jurkat T cell-APC conjugates, PKC-θ was shown to translocate to the membrane lipid rafts in the immune synapse (39). Furthermore, two recent studies in T cell-B cell conjugates and T cell-dendritic cell conjugates have shown an active role of the target cell in formation of the T cell immune synapse (40, 41).

In the present study we have performed an analysis of the NK cell immune synapse (NKIS) in cytolytic and noncytolytic interactions with target cells. Two different in vitro systems have been investigated. In the first system polyclonal human NK cells were used as effector cells, and an autologous EBV-transformed B lymphoblastoid cell line (BLCL) was considered the nonsusceptible target and compared with the MHC class I-deficient B cell line 721.221, which is susceptible to NK cell-mediated cytotoxicity. In the second system NK cell-target cell conjugates were analyzed using KIR2DL3-positive NK clones that in the in vitro cytotoxicity assay were noncytolytic against 721.221 transfected with HLA-Cw*0304 and were cytolytic against untransfected 721.221 targets. In the first model system all the inhibitory interactions between NK cells and MHC class I ligands are assessed simultaneously in conjugates with autologous BLCL, while none of these interactions can occur with the 721.221 target. In the second system the target cells differ for only a single HLA class I allele, which is the ligand for the inhibitory KIR molecule, KIR2DL3, present on the NK clone. In both systems the inhibition of NK cytotoxicity is lifted in the presence of anti-HLA class I mAb (42–44).

A detailed immunofluorescence analysis of the spatial recruitment of early signal transduction molecules, cytoskeletal elements, and secretory organelles in conjugates formed between NK cells and target cells was assessed. Selection of Abs for the characterization of NKIS was based on information gained from biochemical analysis of signal transduction in NK cells (12–19, 27, 45–47) and previous studies of the immune synapses formed in Ag-specific T cell interactions with APC (33, 36–38). We anticipated that the cytolytic NKIS would have features in common with the Ag-specific T cell immune synapse. Therefore, Abs detecting Src kinases, Syk kinases, adaptor molecules, PKC-θ, and Itk were included in this analysis and expected to be present in the cytolytic cSMAC. In contrast, activation and recruitment of the cytoplasmic tyrosine phosphatase SHP-1 were expected to dominate the cSMAC in the noncytolytic NKIS. Redistribution of LFA-1 and talin into the pSMAC of the Ag-specific T cell immune synapse has been observed (33–38); therefore, Abs to these molecules were also included in the present study. Our analysis establishes that two distinctly different reorganizations occur in cytolytic and noncytolytic NKIS.

Materials and Methods

Cells

Polyclonal NK cells (i.e., NK cell lines) were generated and maintained as previously described (48). Briefly, FACS-sorted NK cells (CD56+1, CD3−) were cocultured with irradiated allogeneic PBMC and EBV-transformed allogeneic BLCL (JY) and activated with 300 IU/ml IL-2 (provided by the National Cancer Institute/Biological Response Modifiers Program, Frederick, MD). NK clones were generated after FACS sorting for CD56+ CD3− subpopulation of lymphocytes from freshly isolated PBMC of a donor homozygous for HLA-Cw*0304. The postsort purity of NK cells was confirmed. NK cells were then plated in limiting dilution at 0.3 cells/well in 40-μl tissue culture plates (Robbins Scientific, Sunnyvale, CA). NK cell clones were cultured in IMDM (Life Technologies, Gaithersburg, MD) with 10% FCS containing heat-inactivated human AB serum (Pel-Freez Biologicals, Rogers, AR) and 300 IU/ml IL-2. The cells were cultured at 37°C in 7.5% CO2. At the start of the culture and weekly thereafter until expanded into 48-well culture plates, the clones were cocultured with 1 × 105/ml irradiated allogeneic PBMC and 1 × 106/ml irradiated EBV-BLCL (JY). Coculturing with PBMC and BLCL-JY was discontinued once cultures were expanded into 24-well plates. All clones were then allowed to grow in 24-well plates until an adequate number of cells was reached, usually within 5–7 days. Clones were then characterized for receptor phenotype (DX9, GL138, EB6, and CD94) and used for 51Cr release cytotoxicity and conjugation assays. NK clones selected for further studies were CD5+ CD56−, and GL183+ and mediated NK cytolic function against 721.221 (class I-negative EBV-BLCL), but protected autologous BLCL and 221-Cw*0304 cells (self-allele transfected). The HLA class I- negative, 221-Cw*0304 cells was used as an NK-sensitive target, while autologous BLCL and 221-Cw*0304 cells were used as an NK-protected target as previously described (42). The 221-Cw*0304 cell line was a gift from Dr. P. Parham (Stanford University, Stanford, CA). For conjugation assays, the target cells were preincubated with CellTracker Blue CMAC (Molecular Probes, Eugene, OR) for 30 min at 37°C and washed in serum-free medium before use. This allowed easy identification of target cell in an NK-target conjugate. In three cell conjugates only autologous BLCLs were prelabeled blue, and 721.221 cells were unlabeled. The 721.221 cells were identified by their large size and lack of LFA-1 staining, whereas NK cells stained for CD11a.

Antibodies

Primary. NK cells were phenotyped with anti-KIR3DL1 (DX9), anti-KIR2DL3/2DS2/2DS3 (GL183), anti-KIR2DL1/2DS1 (EB6), and anti-human CD94 mAbs, which were purchased from Immunotech (Marseille, France). Anti-CD56 and anti-CD3 were purchased from BD Biosciences (San Jose, CA). Mouse monoclonal anti-human talin and α-tubulin (identifies tubules and MTOC) were purchased from Chemicon International (Temecula, CA) and Amersham Pharmacia Biotech (Piscataway, NJ), respectively. Goat polyclonal anti-human Itk, PKC-θ, ZAP-70, mouse monoclonal anti-human phospholipase C-γ (PLC-γ), rabbit polyclonal anti-human SHP-1, Fyn, Lck, and SYK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-B cell linker protein (goat-anti-BLKNK) affinity-purified antisera raised against the C-terminal 19 aa of human BLNK and a mouse mAb detecting BLNK (anti-BLNK) were both used in the experiments (Santa Cruz Biotechnology). The mouse anti-BLKNK Ab identifies the epitope corresponding to aa 4–205 mapping at the N terminus of BLNK of human origin. A sheep anti-human SH2 domain-containing leucocyte protein of

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Fluorescent microscopy

In all experiments an Intelligent Imaging Innovations imaging system (Intelligent Imaging Innovations, Denver, CO) with a Zeiss Axioplan 2 microscope and AttoArc mercury light source (Zeiss, New York, NY), which includes motorized 0.1-μm linear encoders on x- and y-axes, harmonic drive z-focusing, Nomarski optics, motorized filter turret, a Sensicam SX2 high performance camera to record fluorescence, and different interference contrast images, was used. Based on the Nomarski imaging, cells that were clearly conjugated were examined for their fluorescence and selected for scoring analysis using SlideBook analysis software (Intelligent Imaging Innovations). Images were obtained in both two-dimensional (2D) (x-y-axis) and in three-dimensional (3D) (x-z-axis) (34). Sixty to 70 serial optical sections of 0.1-μm thickness were acquired for each label. The axial recorded data were then deconvolved using Nearest Neighbor Deconvolution, giving the fluorescent image representative of the label in the entire cell and not just in a single plane. The contact areas (synapses) were acquired using both the mask function and the three-view function of the SlideBook software, which displays all three (x, y, z) orthogonal planes.

Analysis of immune synapses

Based on the Nomarski images, cells that were clearly conjugated were selected for fluorescent analysis. Fifty to 70 conjugates were randomly selected for each label and target combination from two to four independent experiments. It is specifically stated in Results when <50 conjugates were analyzed. Two- and three-dimensional images of all such conjugates were acquired and analyzed. The scoring of the synaptic regions was made following acquisition, deconvolution, and rendering of 60–70 z-stack images. Such projections in the z-axis of the contact areas yielded three distinct patterns: 1) formation of ordered SMAC structures with peripheral and central clusters of molecules, 2) absence of the ordered SMAC structures resulting in homogenous distribution of the molecules, and 3) uninterpretable SMACs. SMAC was considered uninterpretable when the x-y-axis of the deconvolved fluorescent image was not congruous with the plane of the three-view function, thus allowing only partial visualization of the projections in the z-axis. Uninterpretable SMACs were observed in 14% (range, 8–20%) of the randomly selected conjugates.

Analysis of polarization event

Localization of MTOC, talin, and lysosomes in the NK-target cell conjugate was evaluated to determine the presence or absence of polarization of these organelles toward the contact site. NK cell was divided into three zones: 1) proximal one-third, as an area in the cell closest to the cell-cell contact with the target; 2) distal one-third, as an area most distant from the cell-cell contact; and 3) middle one-third, as an area in between the two zones. MTOC and lysosomes were considered polarized to the cell-cell contact when they were located in the proximal one-third of the NK cell. Any other observations, i.e., localization in the middle or distal one-third of the cell from the contact area, were considered nonpolarized events. Talin was considered polarized when it was seen to cluster at the cell-cell contact, as determined by the fluorescent intensity of the molecule being highest in the cell-cell contact area compared with the rest of the cell. Clustering of talin in any other site except the contact zone and equal distribution of talin around the cell were both considered nonpolarized events. Scoring analysis of the polarization events was performed after conducting three independent experiments with each target using either polyclonal NK cells or NK clones in conjugates with .221, .221-Cw*0304, and autologous BLCL target cells. Fifty conjugates were scored in each of the three experiments.

Results

Functional characterization of cytolytic and noncytolytic conjugates

Two experimental models were applied to analyze differences in the immune synapses during the cytolytic and noncytolytic interactions of human NK cells. In the first model polyclonal NK cells (i.e., NK cell lines) generated from healthy donors were used as effectors. The targets in this assay included the human major histocompatibility Ag (HLA) class I-negative cell line 721.221 (.221) as a susceptible target and in vitro established autologous EBV-transformed BLCL as a nonsusceptible target. As shown in Fig. 1A, NK cells do not mediate cytotoxicity against the autologous BLCL, while the HLA class I-deficient target .221 is susceptible to

Western blot analysis

The presence of BLNK was assessed in NK, T, and B cells. NK cells, Jurkat T cells, and EBV-transformed B cells were washed and lysed with the lysis buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 1 μg/ml leupeptin). Lysates were run on 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Bio-Rad, Richmond, CA), and the blots were blocked with 5% milk. Membranes probed with a 1/200 dilution of mouse anti-human BLNK mAb (Santa Cruz Biotechnology) were then washed and incubated with a 1/10,000 dilution of goat anti-mouse HRP secondary Ab (Bio-Rad). Following multiple washes the membranes were developed with ECL (NEL Life Sciences, Boston, MA). Cell lysates stained only with goat anti-mouse HRP secondary Ab were used as controls.

Flow cytometry

Analysis of NK cell conjugates using polyclonal NK cell lines and NK clones was performed on FACSscan (BD Biosciences) as previously described (49). Target cells were labeled with CellTracker Blue CMAC or Orange CMTMR, and NK cells with FITC-labeled anti-CD56. Polynuclear NK cells were used to form conjugates with .221 and autologous BLCL, and NK clones were used to form conjugates with .221 and .221-Cw*0304. Aliquots of effectortarget cell suspensions were removed after incubation at 37°C for 1, 3, 5, 10, 15, and 30 min and were placed on ice for immediate FACS analysis. Effectors and targets mixed in the presence of EDTA, which prevents conjugation, served as a control sample. The percentage of conjugated effector cells was determined after dividing the number of dual-labeled particles by the total number of effector lymphocytes and multiplying the result by 100 (49).

Cytotoxicity assay

Cell-killing assays were performed using polyclonal NK cells and NK clones as effectors and .Cr-labeled autologous BLCL, .221, and .221-Cw*0304 as targets. Assays were performed in triplicate for 4 h at an E:T ratio of 7:1. The highest specific lysis was calculated as previously described (48). Auteologous BLCL and .221-Cw*0304 targets were tested in the presence (10 μg/ml) or the absence of anti-HLA class I mAb, DX17, and its isotype control Ab IgG1. DX17 is known not to induce ADCC (42–44). For cold target cell inhibition, either unlabeled autologous BLCL or .221 were added at increasing E:T cell ratios to determine if the target was considered polarized when it was seen to cluster at the cell-cell contact when they were located in the proximal one-third of the NK cell. Any other observations, i.e., localization in the middle or distal one-third of the cell from the contact area, were considered nonpolarized events. Talin was considered polarized when it was seen to cluster at the cell-cell contact, as determined by the fluorescent intensity of the molecule being highest in the cell-cell contact area compared with the rest of the cell. Clustering of talin in any other site except the contact zone and equal distribution of talin around the cell were both considered nonpolarized events. Scoring analysis of the polarization events was performed after conducting three independent experiments with each target using either polyclonal NK cells or NK clones in conjugates with .221, .221-Cw*0304, and autologous BLCL target cells. Fifty conjugates were scored in each of the three experiments.

Conjugation assay

NK cells and targets were prepared for conjugation after washing in serum-free medium and adjusting the concentration to 1 × 10^6/ml. Effector and target cells were mixed at an E:T cell ratio of 3:2 and spun at 500 rpm for 5 min at room temperature. For triple-cell conjugates equal numbers of autologous BLCL and .221 were mixed with NK cells. Before mixing, autologous BLCL cells were prelabeled with blue cell tracker dye while the HLA class I-deficient target .221 were prelabeled with blue cell tracker dye while the HLA class I-negative cell line 721.221 (.221) as a susceptible target and in vitro established autologous EBV-transformed BLCL as a nonsusceptible target. As shown in Fig. 1A, NK cells do not mediate cytotoxicity against the autologous BLCL, while the HLA class I-deficient target .221 is susceptible to

Without further mention, the document appears to detail various experimental procedures and analyses related to cellular interactions, particularly involving NK cells and the analysis of immune synapses. The text includes descriptions of methods for labeling and analyzing cells, as well as the results of experiments conducted with various cell types and conjugates. It also discusses the analysis of polarization events and the functional characterization of cytolytic and noncytolytic conjugates.
NK cell-mediated lysis. When BLCL target is tested in the presence of the isotype control Ab IgG1 (Fig. 1A, left panel) or anti-CD56 (data not shown). Protection of .221-Cw*0304 was also lifted with mAb GL 183 (data not shown), consistent with previous studies (42). Therefore, the ligand-receptor pair of HLA-Cw*0304 and KIR2DL3 that controls the inhibition of NK cytotoxicity is directly tested in this model system. The number of conjugates formed with the nonsusceptible target, .221-Cw*0304, was fewer than the number obtained with the susceptible target, .221 (Fig. 1B, right panel), which is similar to the findings made in the first model (Fig. 1A, right panel). A higher number of conjugates was formed at each time point using NK clones compared with polyclonal NK cells (Fig. 1, right panels).

**Cytoskeletal remodeling and polarization events in the cytolytic and noncytolytic conjugates**

Conjugates with the susceptible and nonsusceptible target cells using polyclonal NK cell lines and NK clones were analyzed for localization of MTOC and lysosomes (cytolytic granules) (22, 23) as well as the clustering of actin and talin. The data in Fig. 2 are representative of 150 conjugates analyzed for each label and target combination. Conjugates formed using the polyclonal NK cell line that is cytotoxic for .221 and noncytotoxic for autologous BLCL were initially analyzed. Polarization toward .221 cells was observed for the MTOC in 80% (n = 78–82), lysosomes in 78% (n = 76–80), and talin in 75% (n = 70–78) of the conjugates (Fig. 2, second row). The results for redistribution of actin and talin were similar (data not shown). In contrast, <25% (n = 18–30) of NK cell conjugates showed polarization of any of these elements toward autologous BLCL targets (Fig. 2, first row). In the second dataset conjugates formed using GL1837 NK clones that are cytotoxic to .221 and noncytotoxic to .221-Cw*0304 were analyzed. Here, polarization toward .221 cell was observed for the MTOC in 84% (n = 82–86), lysosomes in 80% (n = 78–82), and talin in 78% (n = 75–80) of the conjugates (Fig. 2, third row). In contrast, <20% (14–25) of NK cell conjugates showed polarization of any of these elements toward .221-Cw*0304 target cells (Fig. 2, fourth row). Therefore, conjugates with autologous BLCL and .221-Cw*0304 expressing a single self HLA-Cw allele gave similar results in cortical cytoskeletal remodeling and polarization events in both polyclonal NK cells and NK clones. These results demonstrate that inhibitory signaling does not require large scale remodeling of the cortical cytoskeleton, nor does it involve changes in cytoplasmic structures normally associated with activation of the cytolytic machinery.

**Distribution of signaling molecules in NK cells**

To address whether the signaling molecules do or do not passively move along with the MTOC in the conjugates, the IL-2-activated NK cells were dual-labeled with Abs against a variety of signal transduction molecules and α-tubulin. Dual-labeling with closely related molecules such as the two Syk kinases, SYK and ZAP-70, and two adaptor molecules, SLP-76 and BLNK, was also evaluated to study the possible redundancy of such molecules in NK signaling. Digital immunofluorescence microscopy was used to acquire fluorescent images keeping MTOC and the labeled molecule in the same focal plane. The data in Fig. 3 are representative of 20 NK cells analyzed for each Ab combination. The distribution of signaling molecules in relation to MTOC was different for each molecule. The distribution of SHP-1 and SLP-76 was distinct from the location of MTOC (in green, Fig. 3, rows 3 and 4), Lck, ZAP-70, BLNK, Itk, and PKC-θ have some portion of their molecules that colocalize with MTOC (in yellow, Fig. 3, panels 1, 2, 5, 6, and 7, overlay). These results indicate that at least a subset of some of the signal transduction molecules colocalizes with MTOC. Therefore, they may be translocated with MTOC to the contact site with
susceptible targets similar to recent findings observed for the tyrosine kinase PYK2 (28). It is also possible that the molecules colocalized with MTOC exist as preformed complexes in IL-2-activated NK cells. Furthermore, NK cells have closely related signaling molecules that otherwise are predominantly expressed in either B cells (e.g., BLNK and SYK) or T cells (e.g., SLP-76 and ZAP-70). The presence of BLNK was confirmed by Western blot analysis, in which an appropriate size band (68 kDa) was detected in human NK cells and EBV-transformed B cells, but not in Jurkat T cells (Fig. 4). Cellular localization of the signaling molecules was also determined. In activated NK cells the distributions of Lck, ZAP-70, and SYK were similar, having both cytoplasmic and perimembraneous localizations (in red, Fig. 3, rows 1, 2, and 8). SHP-1 and SLP-76 have predominantly perimembraneous localization (in red, Fig. 3, rows 3 and 4), whereas BLNK, Itk, and
PKC-θ have predominantly cytoplasmic localization (in red, Fig. 3, rows 5–7). Although, SYK and ZAP-70 have mostly overlapping distribution (in yellow, Fig. 3, row 8, overlay), there are areas where the two molecules are not colocalized.

Cytolytic and noncytolytic NKIS

NKIS visualized as projections in the z-axis were analyzed using the 3D digital immunofluorescence microscopy. The word synapse is used to describe structures visualized in 3D (x-y-axis) and not in 2D (x-y-axis) fluorescent images (34). In the figure the fluorescent distributions of the signaling molecules are shown to be discrete and clumped, as the panels represent only the most intense signals within the NK cell obtained following deconvolution of 60–70 images. In one set of experiments analysis of the immune synapses was made in conjugates between polyclonal NK cells and either .221 or autologous BLCL (Fig. 5, a–c), and in the other set of experiments conjugates between NK clones and either .221 or .221-Cw*0304 (self allele) were analyzed (Fig. 5, d–f). The results of synaptic molecular redistribution obtained in both experimental models were similar. Data in the figure represent 75–80% of 50–70 conjugates analyzed for each Ab and target combination obtained in at least two independent experiments (see Materials and Methods). Recruitment to the immune synapse of the signaling molecules, PKC-θ, PLC-γ1, Itk, and SLP-76, which are expected to be involved in the granule exocytosis activation pathways, was evaluated. BLNK was selected to determine the involvement of other adaptor molecules in NK signaling (50, 51). LFA-1 and components of the cortical cytoskeleton, actin and talin, are known to mediate essential functions during T cell interactions with target cells and APCs and to accumulate at the contact sites (35). Here we demonstrate that talin is clustered at the contact site with the susceptible target (i.e., .221) as seen in 2D fluorescent images (Fig. 5, panels 2 and 4, b and e) and is also redistributed in the cytolytic synapses (3D images) of both NK cell lines (Fig. 5, panels 2 and 4c) and NK clones (Fig. 5, panels 2 and 4f). LFA-1 did not cluster at the cell-cell contact of the cytolytic conjugates when analyzed as 2D images (Fig. 5, panels 6, 8, and 10, b and e); however, there was a distinct redistribution of LFA-1 in the cytolytic synapses (3D images), similar to that observed for talin (Fig. 5, panels 6, 8, and 10, c and f). Both LFA-1 and talin are localized in the periphery of the synapse (pSMAC), while PKC-θ (Fig. 5, panel 2, c and f), PLC-γ1 (Fig. 5, panel 4, c and f), Itk (Fig. 5, panel 6, c and f), SLP-76 (Fig. 5, panel 8, c and f) and BLNK (Fig. 5, panel 10, c and f) are localized in the center (cSMAC). In contrast, redistribution in the noncytolytic immune synapse of these molecules was not observed with either autologous BLCL (Fig. 5, panels 1, 3, 5, 7, and 9c) or .221-Cw*0304 (Fig. 5, panels 1, 3, 5, 7, and 9f). This demonstrates that cytolytic effector function is associated with dramatic changes in the plasma membrane at the contact sites between NK cells and susceptible targets and can be visualized in 3D images of the projections in the x-z-axis.

Protein tyrosine kinases (PTKs) in cytolytic and noncytolytic conjugates

The early recruitment of PTKs, such as the Src kinases Lck and Fyn, and the Syk kinases SYK and ZAP-70, was visualized in cytolytic and noncytolytic NK cell conjugates formed between polyclonal NK cells and either .221 or autologous BLCL and analyzed after 10 min of NK cell-target cell mixing. The data in Fig. 6 represent 75–80% of 50–70 conjugates analyzed collectively from two to four independent experiments for each Ab and target combination. Analysis of the immune synapses in cytolytic interactions with .221 showed all four PTKs as well as BLNK to be clustered in the center of the synapse (cSMAC) surrounded by LFA-1, which is localized in the pSMAC (Fig. 6, panels 1–3C). It was also demonstrated by independent visualization of signaling molecules obtained after removing either red or blue colors that there was some compartmentalization within the cytolytic cSMAC. Further studies including fluorescence resonance energy transfer will be needed to address issues regarding colocalization and coassociation of molecules in the immune synapses. Recruitment to the synapse of the PTKs or adaptor proteins is not identified in NK cell conjugates with autologous cells or with .221-Cw*0304 (data not shown).

SHP-1 in cytolytic and noncytolytic conjugates

Biochemical analysis of signal transduction events mediated by inhibitory NK receptors for MHC class I Ags have clearly demonstrated that SHP-1 is recruited to these receptors and activated by a tyrosine phosphorylation-dependent mechanism (12–15, 52). Therefore, localization of SHP-1 in cytolytic and noncytolytic conjugates was determined. Experiments were performed using both model systems. In one experimental system .221 and autologous BLCL were used as targets for polyclonal NK cells, and in the other .221 and .221-Cw*0304 were used as targets for GL183 NK clones. Fifty conjugates were collectively analyzed from two to four independent experiments for each Ab and target combination. The results obtained from the analysis of cytolytic conjugates with either .221 targets and NK cell lines or .221 targets and NK clones are representative of 85–90% of the conjugates (Fig. 7A, top row). It is demonstrated that SHP-1 and ZAP-70 are recruited to the cell-cell contact site with .221 (Fig. 7A, b and e, top row) and are clustered in the cytolytic cSMAC (Fig. 7A, c and f, top row). This is further demonstrated in a detailed four-color analysis of NK cell conjugates, where SHP-1 is recruited to the contact area with .221 target cells in the cytolytic conjugate (Fig. 7B, a–e). Here, SHP-1 is seen to occupy a central position along with ZAP-70 and SLP-76 at the cell-cell contact site (Fig. 7B, a–e).

Recruitment of SHP-1 to the NK cell-target cell synapse could not be identified in the majority of noncytolytic conjugates with either autologous BLCL or .221-Cw*0304 target cells analyzed 10 min after NK cell-target cell mixing. However, there is a well-defined rearrangement at the synapse (x-z-axis, 3D image) in 32% of conjugates with BLCL (16 of 50) and 40% of conjugates with .221-Cw*0304 (20 of 50 conjugates) analyzed at 10 min. Here, SHP-1 alone is recruited to the cell-cell contact site of either autologous BLCL or .221-Cw*0304 (Fig. 7A, b and e, bottom row, and Fig. 7Bg) and is clustered in the cSMAC, while LFA-1 is redistributed in the pSMAC of the noncytolytic NKIS (Fig. 7A, c and f, bottom row, and Fig. 7Bg, inset). Although only 32–40% of the noncytolytic conjugates demonstrated cSMAC with SHP-1, the other conjugates did not demonstrate any redistribution of LFA-1 in the synapse when analyzed at 10 min of conjugate formation. In contrast to cytolytic conjugates formed with HLA class I-deficient target cells, SHP-1 was not associated with ZAP-70 or SLP-76 in the noncytolytic conjugates formed with either autologous BLCL or .221-Cw*0304, as demonstrated by absence of any other molecules in the cSMAC after removing the red color of SHP-1 (Fig. 7A, c and f, bottom row). This is also illustrated in the four-color immunofluorescence image of a noncytolytic conjugate, where SLP-76 and ZAP-70 are located in the distal one-third of the NK cell (Fig. 7B, f–h). This analysis demonstrates that noncytolytic NK cell interactions with autologous target cells involve the generation of an inhibitory immune synapse, which is dominated by SHP-1, while downstream signaling molecules are excluded from the contact region. Our results also implicate a role for SHP-1 in cytolytic NK interactions.

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Recruitment of SHP-1 to the immune synapses.

The regulated processes that mediate such unidirectional induction of cytolytic granule exocytosis were investigated in NK cell conjugates in which one NK cell interacts with .221 and autologous BLCL simultaneously. Thirty triple-cell conjugates were analyzed after triple labeling with blue tracker dye, LFA-1 and SLP-76 (see Materials and Methods). The data in the figure were observed in 25 of 30 three-cell conjugates analyzed. NK cells in such conjugates demonstrate unidirectional polarization of SLP-76.
only toward .221 (Fig. 8B, lower cell in differential interference contrast overlay and three-color image). Here, SLP-76 clusters in the cSMAC of the NK cell-contacting membrane with .221, while this was not observed in the contacting membrane with autologous BLCL (Fig. 8B). Triple-cell conjugates formed between a single NK cell and two susceptible target cells demonstrate different patterns dependent upon the indicator used for assessment of recognition and the induction of cytotoxicity. SLP-76 and PKC-δ were polarized toward only one of the two targets; in contrast, talin was polarized toward both target cells (data not shown).

Discussion
We have analyzed, at the single-cell level, the composition of signal transduction molecules in the SMACs in the MHC regulated cytolytic and noncytolytic NKIS. The NKIS were analyzed by 3D immunofluorescence microscopy for the presence or absence of several signal transduction molecules, including PTKs, a PTP, adaptor molecules, PKC-δ and PLC-γ1, in the immune synapse, and for synaptic redistribution of LFA-1 and the actin-associated molecule, talin. Two different in vitro models yielded similar results. It is demonstrated that two distinctly different NKIS can be defined: the inhibitory NKIS and the cytolytic NKIS.

The inhibitory NKIS is formed when polyclonal IL-2-activated NK cells form conjugates with autologous BLCL or when KIR2DL3-positive NK clones interact with .221 cells expressing the cognate ligand HLA-Cw*0304. When the target cell lacks self MHC, a cytolytic NKIS is formed at the contact site with the target cell. Here, the NK-target cell contact area assemblies into topologically and spatially distinct regions characterized by redistribution of LFA-1 and talin in the pSMAC, and a multimolecular signaling complex including SHP-1 is assembled in the cSMAC. In contrast, the inhibitory NKIS is characterized by redistribution of LFA-1 in the pSMAC and clustering of only SHP-1 in the cSMAC. The cytolytic NKIS has several features in common with the TCR-specific immune synapse observed for Th lymphocytes interacting with their Ag-specific APCs (34–38).

Visualization of the signaling molecules included in the cytolytic cSMAC provides further insight into the spatial organization of enzymes and adaptor molecules needed for initiation of cytolytic effector function. Translocation of signaling molecules from the cytosol to the NK cell plasma membrane occurs within a limited region of contact with the target cell. The complex consists of Src kinases, Syk kinases, the Tec kinase Itk, adapter molecules, PKC-δ, and PLC-γ1. These findings are consistent with expectations derived from biochemical analysis of receptor-mediated signaling pathways in lymphocytes (12–19, 27, 45–47, 50–55). Although the molecular basis for NKIS formation and its function remains to be defined, recent studies have suggested that the compartmentalization of the plasma membrane imposed by glycolipid-enriched microdomains (GEMs) is essential for TCR-mediated signaling (56). GEMs are enriched in molecules involved in signal transduction, such as the PTKs Lck and Fyn and the transmembrane adaptor linker of activation in T cells (57). In contrast, the PTPs CD45, SHP-1, and SHP-2 are excluded from the GEMs (58), and such restricted distribution of PTPs may play a critical role in the initiation of signaling.

Our results on clustering of the GEM-resident PTKs in the cSMAC of the cytolytic conjugates are consistent with a model in which aggregated GEMs get reorganized during cell-mediated cytotoxicity (27). We also demonstrate that SLP-76 in the cytolytic interaction is brought to the contact area in such a way that it could provide the scaffold where Itk (54), PKC-θ (55), and PLC-γ1 (17–19) are brought in contact with their substrates (Fig. 7, A–E). Therefore, clustering of this activation signaling complex to the cytolytic NKIS could occur by recruitment to the GEM-resident anchoring molecule, linker of activation in T cells (51), which is tyrosine phosphorylated in cytolytically active NK cells (19). Given that the majority of SHP-1 in T cell membranes is present outside the lipid rafts (58), it was surprising to find SHP-1 in the cytolytic NKIS as a component of the multimolecular signaling complex in the cSMAC. However, it has been shown in Jurkat T cell lines that the predominantly cytosolic SHP-1 becomes enriched in membranes and GEMs in association with activated Lck (58). Therefore, it is possible that the activated Lck recruited to cytolytic NKIS will also recruit SHP-1 to GEMs. Our analysis cannot determine whether SHP-1 is present in the complex as an active enzyme. In this case SHP-1 could provide a regulatory function by guiding tyrosine phosphorylation-dependent pathways toward granule exocytosis and deactivation of signals that could mediate apoptosis or cell proliferation via regulation of mitogen-activated protein kinases (25, 26, 59). Direct support for involvement of enzymatically active SHP-1 in cytolytic NK cell effector function with MHC class I negative targets has recently been provided by studies in mice transgenic for dominant-negative SHP-1. Such mice display decreased NK cytotoxicity against MHC class I-negative targets (60).

Visualization of NK cell interactions with target cells expressing self HLA class I ligands for inhibitory NK receptors clearly demonstrates that the cytolytic effector mechanisms are not being activated. MTOC, lysosomes, and talin are not polarized to the contact site with the target (Fig. 2), and the cascade of signaling events is interrupted early in such interactions (this study and Ref. 16). This deactivation of NK cell effector functions is mediated by the interactions of inhibitory NK receptors for MHC class I, as evidenced by the occurrence of cytotoxicity in the presence of anti-HLA class I mAb (Fig. 1, A and B, left panels). It has recently been shown that inhibitory KIR or CD94/NKG2 receptors interacting with cognate MHC class I ligand interrupt tyrosine phosphorylation of the activating NK receptor 2B4 (16). The inhibitory NKIS has a well-defined pSMAC containing LFA-1, and a cSMAC containing only SHP-1. Recruitment and activation of SHP-1 are tyrosine phosphorylation dependent (12–15, 52). The initial NK cell activation will induce Src kinase-mediated tyrosine phosphorylation (16), and activation of SHP-1 will then occur when the inhibitory NK receptor is recruited into the synapse as a consequence of cognate MHC-ligand interactions. The perimembrane components of Lck and SHP-1 would favor rapid initiation of the inhibitory signal transduction pathway. This could then facilitate interruption of the cytolytic signaling cascade, which also depends on Src kinase activation.

Noncytolytic NK cell conjugates were analyzed at 10 min following effector cell-target cell mixing. The majority of these conjugates display a synaptic region with uniform distribution of LFA-1 and absence of SHP-1. It is possible that these conjugates represent cell-cell interactions where the self recognition process has completed. This conclusion is supported by recent studies in T cells, where the dephosphorylation of proteins by SHP-1 and SHP-2 was completed within 5 min of incubation of the T cell membrane with ATP and SHP-1 (58). This hypothesis is further supported by three recent studies, one demonstrating down-regulation of integrin function upon inhibitory KIR interactions with cognate HLA-C molecules (61) and the others demonstrating redistribution of inhibitory Ly49 and KIR molecules on NK cells during interactions with their ligands on target cells (29, 30). Therefore, our analyses of noncytolytic NKIS support the results obtained in the other study where the inhibitory signals initiated by KIRs block aggregation and polarization of GEMs in an SHP-1-dependent manner (27).
Our studies also demonstrate that the NK cells express signaling molecules characteristic for both T cell and B cell lineages. The two Syk kinases, ZAP-70 and SYK, which have close functional similarity (62), were recruited into the cSMAC of cytolytic NKIS (Fig. 6). The two adaptor molecules, SLP-76 and BLNK, were also recruited into the cSMAC of cytolytic NKIS. It has recently been suggested that these adaptor molecules have overlapping, yet unique, functional activities (50, 51, 63). It has been previously reported that BLNK is not present in human lymphokine-activated killer cells (64). However, our studies with two different anti-BLNK Abs, including Western blot analysis of purified IL-2-activated human NK cells and NK clones did indicate the presence of this adaptor molecule in NK cells (Fig. 4). The immunofluorescence staining in NK cells of BLNK and SLP-76 demonstrate different patterns (Fig. 3), further supporting that the Abs do not cross-react between these two molecules. In another recent report SLP-76 and BLNK were both expressed in murine macrophages and were linked to signaling via Fcγ receptors (65). Our observation that a fraction of Lck, ZAP-70, BLNK, Itk, and PKC-θ colocalized with MTOC in IL-2-activated NK cells (Fig. 3, rows 1, 2, 5, 6, and 7) suggest that these signaling molecules might be translocated along with MTOC to the contact area with target cells during initiation of the cytolytic signaling cascade. The recent finding of FYK-2 association and cotranslocation with MTOC in cytolytic NK effector function supports this model for temporal and spatial regulation of cytotoxicity (28).

The ability of NK cells to distinguish self from nonself based on the presence or absence of autologous MHC class I Ags on target cells was clearly illustrated in triple-cell conjugates (Fig. 8). A single NK cell interacting simultaneously with an autologous and an MHC class I-deficient target cell displays a unidirectional cytoplasmic immune synapse with only the class I-negative target cell (Fig. 8B, compare two right side images). The ability of an NK cell to precisely direct its cytolytic machinery toward a susceptible target when surrounded by overwhelming numbers of self targets is also illustrated in the cold target inhibition assay (Fig. 8A). These findings are in agreement with previous studies in cytolytic T cells (21) and with recent studies applying video microscopy of murine NK cell interactions with target cells (66). Collectively, these results support the hypothesis that the inhibitory signals within a single NK cell are spatially and temporally restricted and are limited to interactions with nonsusceptible, resistant target cells. This localized inhibition does not lead to a general inactivation of the cytolytic effector function of the cell (Fig. 8). However, a more detailed analysis of triple-cell conjugates between a single effector cell and two susceptible target cells, two effector cells and a single susceptible target cell, and other permutations is needed to dissect the directional events required for induction of cytotoxicity and simultaneous protection of self targets. We observed polarization of talin toward two susceptible targets (2D analysis, x-y-axis) interacting simultaneously with a single effector cell, but the reorganized cytolytic NKIS with PKC-θ in the cSMAC was only observed for one of the two susceptible target cells. We have not attempted to quantitate polarization of LFA-1 and/or ICAM-1 in cytolytic or noncytolytic conjugates. Previous studies of triple-cell conjugates between a single T cell and two APCs have demonstrated accumulation of ICAM-1 at the contact sites of both APCs (67).

Our studies with IL-2-activated NK cells and NK clones have been limited to a single 10 min point. Others previously reported a temporal distribution of LFA-1 in the NKIS with central accumulation of LFA-1 (29). However, this study was performed with the CD28-positive NK-like cell line, YT, where induction of the cytolytic effector function depends on signals mediated by both CD28 and LFA-1/ICAM-1 (68, 69). Sequential analysis of the NKIS with simultaneous determination of localization of NK receptors, their ligands, and signaling molecules will provide further insight into the molecular mechanisms responsible for NK cell recognition of self vs nonself.

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