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*J Immunol* 2011; 186:2998-3005; Prepublished online 26 January 2011;
doi: 10.4049/jimmunol.1002438

http://www.jimmunol.org/content/186/5/2998

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/01/26/jimmunol.1002438.DC1

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Cytotoxic lymphocytes kill target cells through polarized release of the content of lytic granules at the immunological synapse. In human NK cells, signals for granule polarization and for degranulation can be uncoupled: Binding of β2 integrin LFA-1 to ICAM is sufficient to induce polarization but not degranulation, whereas CD16 binding to IgG triggers unpolarized degranulation. In this study, we investigated the basis for this difference. IL-2–expanded human NK cells were stimulated by incubation with plate-bound ligands of LFA-1 (ICAM-1) and CD16 (human IgG). Surprisingly, LFA-1 elicited signals similar to those induced by CD16, including tyrosine phosphorylation of the TCR ζ-chain, tyrosine kinase Syk, and phospholipase C-γ. Whereas CD16 activated Ca2+ mobilization and LAT phosphorylation, LFA-1 did not, but induced strong Pyk2 and paxillin phosphorylation. LFA-1–dependent granule polarization was blocked by inhibition of Syk, phospholipase C-γ, and protein kinase C, as well as by paxillin knockdown. Therefore, common signals triggered by CD16 and LFA-1 bifurcate to provide independent control of Ca2+-dependent degranulation and paxillin-dependent granule polarization. The Journal of Immunology, 2011, 186: 2998–3005.

Integrins play important roles in immunity, providing the adhesion required for cell conjugate formation as well as extravasation from blood vessels to sites of inflammation (1–3). The β2 integrins are expressed on leukocytes and are important for antibacterial responses as seen in leukocyte adhesion deficiency patients, who lack a functional β2-chain (CD18) (4). The αβ2 (CD11a:CD18) integrin LFA-1 is important for the adhesion of T cells and NK cells to target cells. Ab blockade of LFA-1 on CTLs (5) and NK cells (6) impairs cytotoxicity, as does inhibition of LFA-1 on NK cells with small molecule inhibitors (7). We have previously shown that binding of LFA-1 on NK cells to ICAM-1 on target cells is sufficient to induce the accumulation of perforin-containing granules and the microtubule organizing center (MTOC) at the site of NK cell contact with the target cell (8–10). Cytotoxicity occurs when this polarization signal is combined with a second signal that triggers degranulation. This behavior contrasts with that of LFA-1 in T cells, which requires "inside-out" signaling for its own activation (11). In T cells, perforin polarization requires engagement of the TCR, and costimulation with LFA-1 greatly enhances polarization of perforin-containing granules (12).

In NK cells, stimulation of LFA-1 results in phosphorylation of Vav1 (13), a guanine nucleotide exchange factor for the GTPase Rac1. Vav1 regulates cytoskeletal dynamics (14–16) and is required for NK cell cytotoxicity (17–21). Small interfering RNA (siRNA)-mediated knockdown of Vav1 in human NK cells reduces calcium mobilization and degranulation (22), which are responses not induced by LFA-1 in NK cells (9), suggesting that Vav1 signaling controls multiple pathways. In NK cells, β1 integrin engagement results in phosphorylation of both Pyk2 and paxillin (23), molecules that regulate the cytoskeleton. Phosphorylation of paxillin is observed after β2 integrin engagement in neutrophils (24, 25), and LFA-1 stimulation in human NK cells induces tyrosine phosphorylation of Pyk2 and possibly paxillin (26).

Signaling by β1 and β2 integrins in macrophages and neutrophils results in degranulation and is dependent on the ITAM-containing adapter proteins DAP12 and FcR y-chain (27). Cells from mice lacking these two adapters failed to respond to integrin ligands, as did cells lacking the tyrosine kinase Syk (28). The dependence of the response on the Src homology 2 (SH2) domains of Syk suggests binding to the phosphorylated ITAMs, as opposed to the SH2 domain-independent recruitment of Syk to the β2 integrin cytoplasmic tail, which has been described (29, 30). ITAM-dependent integrin signaling has been seen in other cell systems (31–33).

We investigated whether LFA-1 in NK cells induces ITAM-dependent signals, and whether molecules such as paxillin are involved in LFA-1–induced perforin polarization. In this report, we demonstrate that β2 integrin engagement by ICAM-1 alone leads to tyrosine phosphorylation of the TCR ζ-chain, Syk, and phospholipase C (PLC)-γ1 and PLC-γ2 in human NK cells. Inhibition of Syk and PLC-γ enzymatic activity prevents perforin polarization in response to ICAM-1 expressed on target cells. Comparison to a known ITAM-dependent signaling pathway in NK cells, the CD16 response, reveals surprising similarity in the proximal biochemical responses, despite very different downstream outcomes with regard to perforin polarization and degranulation. Further investigation reveals differential requirement of the adapter proteins paxillin and LAT for responses to LFA-1 and CD16, suggesting the importance of different signaling complex assemblies or compartmentalization of signaling molecules to determine functional outcomes.
Materials and Methods

Cells

Human NK cell populations were isolated from peripheral blood by negative selection using an NK cell isolation kit (Stemcell Technologies). NK populations were resuspended with irradiated autologous PBL feeder cells in IMDM (Invitrogen) supplemented with 10% human serum (Valley Biomedical), 10% purified IL-2 (Hemagen Diagnostics), 100 U/ml recombinant human IL-2, 1 mg/ml PHA (Sigma-Aldrich) and 100 mg/ml pyrophosphate, 150 mM NaCl, 0.5% Triton X-100, 1 mM sodium ortho-pyrophosphate, 100 mg/ml monensin and 5 mg/ml antiphosphotyrosine Ab (clone 4G9) and protein A-agarose and re-suspended in the same medium without PHA or feeder cells. Alternatively, in experiments including stimulation through CD16, NK cells were cultured with irradiated autologous PBLs as feeder cells in OpTmizer T cell expansion medium (Invitrogen) supplemented with 10% purified IL-2, 100 U/ml recombinant human IL-2, and 5 mg/ml PHA. Subsequent passages of the cells were performed in the same medium, without PHA or feeder cells. All IL-2-expanded NK populations were used between week 3 and 4 of expansion. Culture, maintenance, and transfection of Drosophila Schneider line 2 (S2) cells were performed as described (34, 35). 293T cells were cultured in IMDM supplemented with 10% FBS.

Abs and reagents

Abs against CD56 (clone B159), CD16 (clone 3G8), CD11a (clone HI111), Pyk2 (clone 11), and ICAM-1 (clone HA58, PE-conjugated) were purchased from BD Biosciences (San Jose, CA). Anti-phospho-tyrosine Ab 417) and anti-LAT (no. 06-807) were from Millipore. TCR (clone G9), DAP12 (C-20), Syk (4D10), PLC-γ1 (1249), and PLC-γ2 (Q-20) Abs were purchased from Santa Cruz Biotechnology. Anti-perforin Ab clone 8G9 was acquired from Endogen. Anti-phosphoserine protein kinase C (PKC) substrate Ab (no. 2261) was purchased from Cell Signaling Technology. Purified human IgG (15029) and sodium phenyl phosphate (P7751) were from Sigma-Aldrich. Goat anti-mouse IgG F(ab)2 was from Jackson Immunoresearch Laboratories. Syk inhibitor II [2-(2-aminoethylamino)-4-(3-trifluoromethoxyanilino)-pyrimidin-5-carboxamide], bisindolylmaleimide, U73122, and U73433 were purchased from Millipore. CellTracker Green, Fluo-4, Fura Red, and 4-60 MOPS SDS-PAGE gels were purchased from Invitrogen.

Production and purification of His-tagged ICAM-1

A cDNA encoding the extracellular domain of the mouse ICAM-1 with a C-terminal 6× His tag was generated by PCR with the following primers: forward, 5'-TGGCAACGCCCCACCATGCTTCAAAACCGTGTCAGCCGAC-3' and reverse, 5'-CTCTAGACTAATGATGGTGGATGATGGTTATTTTG-AGAGTGGCAGATCAGTCTGCAGTAGFCC-3'. The PCR product was ligated in the pCR2.1-TOPO vector (Invitrogen) and confirmed by sequencing. The insert was digested with SalI and BamHI and cloned into the SalI and BamHI sites of pBabe+CMV-Puro. This plasmid was transfected into 293T cells via FuGENE (Roche), and a stable transfectant was selected with 100 μg/ml G418. The insert was digested with SalI and BamHI and subcloned into the SalI and BamHI sites of pCD-38R. This plasmid was transfected into 293T cells via FuGENE (Roche), and a stable transfectant was selected with 100 μg/ml G418. The insert was digested with SalI and BamHI and subcloned into the SalI and BamHI sites of pBabe+CMV-Puro. This plasmid was transfected into 293T cells via FuGENE (Roche), and a stable transfectant was selected with 100 μg/ml G418. The insert was digested with SalI and BamHI and subcloned into the SalI and BamHI sites of pBabe+CMV-Puro. This plasmid was transfected into 293T cells via FuGENE (Roche), and a stable transfectant was selected with 100 μg/ml G418. The insert was digested with SalI and BamHI and subcloned into the SalI and BamHI sites of pBabe+CMV-Puro. This plasmid was transfected into 293T cells via FuGENE (Roche), and a stable transfectant was selected with 100 μg/ml G418.

Stimulation of NK cells for immunoprecipitation

Ten-centimeter Petri dishes were incubated overnight, at 37°C, with 5 ml 50 mM sodium carbonate solution (pH 9.6) containing 10 μg/ml either purified ICAM-1 or purified human IgG, or 15 mg/ml sodium carbonate solution containing no protein. Plates were washed twice with PBS and blocked at 4°C for 30 min with 5% BSA in PBS. Cells were then washed twice with PBS. NK cells were harvested, washed in PBS, and resuspended in cold, serum-free IMDM at ∼4×106 cells/ml. Five milliliters (∼20×106 cells) was added to each coated and blocked plate. The plates were placed at 4°C for 15 min and then moved to 37°C for 20 min. The medium and unbound cells were removed from each plate, placed into a 15 ml conical tube, and the unbound cells were pelleted. The pelleted cells were lysed in lysis buffer (50 mM Tris-HCl [pH 6.1], 10 mM NaCl, 0.5% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride), and this 1 ml lysate was added to the plate from which the cells were harvested. Plates were placed on a rocker at 4°C for 10 min, the lysates were moved to 1.5 ml Eppendorf tubes, and nuclei were pelleted at 16,100×g. In experiments including pharmacological inhibitors, NK cells were pretreated with the inhibitor, in their standard culture medium, for 45 min at 37°C, and the inhibitor was included in the stimulation. Anti-phosphotyrosine immunoprecipitations (IPs) were performed with 30 μl agaroase-conjugated 4G10l for 1 h at 4°C. IPs were washed in lysis buffer, and bound proteins were eluted with 40 μl 100 mM sodium phenyl phosphate in PBS. Eluates were diluted with 2X Laemmli buffer containing 10% 2-ME. Other IPs were performed with 1 μg Ab and protein A-agarose, and resolved by standard SDS-PAGE.

Perforin polarization assays

Perforin polarization induced by target cells was performed as described (8, 9), with modifications. Target cells were prelabelled with 1 μg/ml CellTracker Green for 30 min at room temperature in their standard culture medium. In experiments including pharmacological inhibitors, NK cells were pretreated with the inhibitor, in their standard culture medium, for 45 min at 37°C. Inhibitors were included in the assay. After fixation and permeabilization, intracellular perforin was stained with 3 μg/ml anti-perforin Ab and a 1:2000 dilution of Alexa 568-labelled goat anti-mouse secondary Ab. Complete z-stacks of ∼40 fields were acquired by confocal microscopy. Three-dimensional reconstruction of the interfaces and visual analysis of the z-stacks were used to determine whether intracellular perforin was concentrated at the point on NK cell contact with the target cell in each conjugate pair.

CD107a surface expression assay

The CD107a assay was performed as described (9), with some modifications. IL-2–expanded NK populations were maintained in serum-free OpTmizer T cell expansion medium (Invitrogen) supplemented with 10% FBS in the presence of 6 μg/ml monensin and 5 μFIC-labeled anti-CD107a Ab (BD Biosciences). Cells were mixed with pipetting and incubated for 2 h at 37°C. The cells were pelleted at 300 × g and resuspended in 200 μl FACS buffer (PBS plus 2% FBS) containing 2 μl PE-Cy7–labeled anti-CD56 Ab (clone NCA16.2; BD Biosciences) per 1×106 cells. Flow cytometry was performed at 4°C for 30 min and cells were washed twice with FACS buffer. The CD107a expression of CD56+ NK cells was analyzed by flow cytometry.

Determination of intracellular calcium mobilization

NK cells were labeled for 30 min at 37°C in their standard culture medium with 1 μg/ml Fluo-4 and 1 μg/ml Fura Red. For experiments involving Ab stimulation, cells were washed twice with PBS and resuspended at 10×106 cells/ml in cold, serum-free IMDM. Cells (2×106, 200 μl) were stained with 1 μg indicated stimulating Ab for 30 min on ice. Cells were washed twice with IMDM, resuspended in 1 ml IMDM, and transferred to FACS tubes. Cells were warmed at 37°C for 5 min and then placed on the flow cytometer. Data were acquired for 30 s, the cells were removed from the flow cytometer, and 4 μg cross-linking goat anti-mouse F(ab)2 was added. Cells were vortexed and replaced on the flow cytometer, and data were acquired for a total of 5 min. For experiments involving stimulation with target cells, a protocol used for monitoring calcium flux after APC stimulation of T cells was adapted (36). Labeled NK cells (1×105) were placed into a FACS tube along with an equal number of target cells in 800 μl warm, serum-free IMDM. The tubes were placed on the flow cytometer and data were acquired for 30 s to establish a baseline. Tubes were removed from the flow cytometer, and cells were pelleted at 100 × g for 30 s. After centrifugation, the cells were gently resuspended with a micro-pipette and the tubes were placed back on the flow cytometer. Data were acquired for a total of 5 min. Analysis was performed in FlowJo (Tree Star). Fluorescently labeled NK cells were gated, and the ratio of Fluo-4 to Fura Red was calculated. Due to variability in the baseline value from sample to sample, the results are presented as normalized to the starting value, such that the ratio at time 0 is set to 1.

siRNA Transfections

NK cells expanded in the serum-free OpTmizer T cell expansion medium were nucleofected with 300 pmol of siRNA duplexes in the solution from the human NK cell transfection kit, using Nucleofector program U-001 (Lonza). The oligos used to achieve knockdown of paxillin were 5'-MUUGACGACCCACACUGGUUUGU-3' and 5'-ACAAAGAAGGCTC-3'. For the sense and antisense strands, respectively, as described (37). The siRNA for LAT was part of TriFECTa Dicer substrate kit from Integrated DNA Technologies. The oligos used were 5'-GCACAUCUC-3'.
Results

LFA-1 binding to ICAM-1 results in TCRζ and Syk phosphorylation

NK cell activation by LFA-1 was determined by stimulating IL-2–expanded human NK cells with purified, plate-bound soluble ICAM-1 stimulation induced six histidines at the end of the intracellular domain. Tyrosine phosphorylated proteins were immunoprecipitated with agarose-conjugated mAb 4G10, eluted with sodium phenylphosphate, resolved by SDS-PAGE along with whole-cell lysates, and immunoblotted for phosphotyrosine (Fig. 1A). Strong tyrosine phosphorylation of five proteins, with approximate molecular masses of 115, 105, 65, 55, and 48 kDa, was induced by ICAM-1 stimulation, as these proteins were easily visualized. Longer exposure of the blot revealed several less heavily phosphorylated proteins with approximate molecular masses of 160, 140, and 18 kDa (Fig. 1A).

Recent reports have demonstrated that integrins in leukocytes, including LFA-1, signal through ITAM-containing adapter molecules (27). We therefore investigated whether the small phosphoprotein observed around 18 kDa was one of three ITAM-bearing adapter proteins expressed in NK cells: the TCR-ζ chain, the FcR-γ chain, and DAP12 (38–40). IPs were performed from NK cell lysates using Abs to the TCR-ζ chain, the FcR-γ chain, and DAP12. Their tyrosine phosphorylation status was determined by immunoblotting with 4G10 mAb (Fig. 1B). TCR-ζ, but not FcR-γ or DAP12, was detectably phosphorylated. Each IP was further immunoblotted for all three chains, as controls for the IP and to test for heterodimer formation (Fig. 1B). Interestingly, the TCR-ζ IPs contained substantial amounts of FcR-γ, consistent with the existence of a ζ-γ heterodimer known to be expressed in NK cells. Curiously, it appears that only the TCR-ζ chain in those IPs is phosphorylated, as tyrosine phosphorylation of the FcR-γ chain was not observed in TCR-ζ IPs and TCR-ζ phosphorylation was not observed in FcR-γ IPs. This suggests a preferential phosphorylation of TCR-ζ homodimers. Although DAP12 was difficult to detect by immunoblot, phosphorylation could be detected in DAP12 IPs following pervanadate stimulation of NK cells (data not shown). Given that ITAM-mediated signaling is known to proceed through the recruitment of the Syk/ZAP70 family of kinases (41), we next examined Syk and ZAP70.

We have previously demonstrated that engagement of LFA-1 by ICAM-1 expressed on Drosophila S2 cells causes polarization of perforin-containing granules in the NK cell toward the site of cell–cell contact (8–10). To test for the importance of Syk in this response, the same assay was employed in the presence of a selective inhibitor of Syk, Syk inhibitor II (IC50 = 41 nM), which is reportedly effective against ZAP70 (IC50 = 11.2 μM) and other kinases only at much higher concentrations (IC50 = 5.1–22.6 μM). Preincubation of NK cells with 1 μM Syk inhibitor II reduced binding of NK cells to ICAM-1 (Table I), as determined in a bead binding assay using an ICAM-1-Fc fusion protein that carries a mutation in the CD16 binding site of the Fc (9), although considerable binding to ICAM-1 remained. Because the polarization assay scores only NK cells that are in definite contact with target cells, and therefore favors the NK cells that retain binding to ICAM-1 (Fig. 2A), it is likely that inhibition of polarization would reflect genuine inhibition of the polarization response, and not the consequence of inhibition of adhesion. Upon mixing of NK cells with S2 cells expressing ICAM-1, the Syk inhibitor completely blocked polarization of perforin to the site of contact with ICAM-1–expressing S2 cells (Fig. 2B). Western blotting also revealed the importance of Syk in the LFA-1 signaling response, as much of the tyrosine phosphorylation seen after ICAM-1 stimulation was blocked or reduced by pretreatment with the Syk inhibitor (Fig. 2C). Two proteins, Pyk2 (115 kDa) and paxillin (65 kDa), were identified as tyrosine phosphorylated following stimulation, and they correspond to bands observed in Fig. 1A. Inhibition of Syk significantly reduced phosphorylation of Pyk2 and nearly abolished phosphorylation of paxillin. With the complete block of perforin polarization observed with the Syk inhibitor, the differential effects of the inhibitor on these proteins may reflect a greater importance of paxillin in LFA-1–induced polarization.

LFA-1 and CD16 induce ITAM-based signals but different responses

Integrin engagement in neutrophils induces degranulation and release of reactive oxygen species in an ITAM- and Syk-dependent manner (27). We have previously shown that whereas engagement of LFA-1 on freshly isolated NK cells induces perforin polarization, it does not induce degranulation (9). In contrast, in freshly isolated NK cells, the ITAM-utilizing low-affinity receptor for IgG, CD16, induces sustained calcium flux (45) and degranulation.

**Figure 1.** Tyrosine phosphorylation of TCRζ and Syk induced by LFA-1 engagement in IL-2–expanded human NK cells. A, NK cells were stimulated at 37°C for 20 min on plates coated with either purified ICAM-1 or BSA. Cells were lysed, and tyrosine phosphorylated proteins were immunoprecipitated with 4G10-agarose. IPs were resolved by 4–20% SDS-PAGE and immunoblotted with biotinylated anti-phosphotyrosine Ab (4G10). The left and right panels represent short and long exposures of the resulting chemiluminescent reaction. B, NK cells were stimulated and lysed. TCRζ, DAP12, and the FcR-γ chain were immunoprecipitated, resolved by SDS-PAGE, and immunoblotted for phosphotyrosine. The blot was stripped and probed in succession with anti-TCRζ, anti-DAP12, and anti-FcRγ Abs. C, Anti-phosphotyrosine IPs prepared as in Fig. 1A were immunoblotted with an anti-Syk Ab.

Syk inhibition blocks LFA-1–induced perforin polarization

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with adhered to poly-D-lysine–coated slides, fixed, permeablized, and stained to represent a representative image of treated or untreated NK cells in contact with S2-ducted with NK cells from different donors. *Target cell interface. Error bars represent SD of three experiments conducted with NK cells from different donors. + indicates adherent cells.

**Table I.** Binding of NK cells to ICAM-1–coated beads following treatment with pharmacological inhibitors or siRNA transfection

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<th>Treatment</th>
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<td>DMSO</td>
<td>62.1 ± 0.3</td>
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<td>37.2 ± 12.9</td>
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<tr>
<td>U73 343</td>
<td>62.3 ± 2.6</td>
</tr>
<tr>
<td>U73122</td>
<td>19.9 ± 11.6</td>
</tr>
<tr>
<td>Bisindolylmaleimide</td>
<td>33.7 ± 15.4</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>75.9 ± 8.0</td>
</tr>
<tr>
<td>LAT siRNA</td>
<td>79.1 ± 9.6</td>
</tr>
<tr>
<td>Paxillin siRNA</td>
<td>75.5 ± 12.3</td>
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NK cells were either pretreated with the indicated inhibitors or transfected with the indicated siRNA oligos. Cells were incubated with ICAM-1–coated beads for 20 min at 37°C and fixed with 4% paraformaldehyde. Binding of NK cells to beads was monitored by flow cytometry.

(9). If both LFA-1 and CD16 use ITAM-bearing adapters and the Syk kinase to transduce signals, why are the downstream responses so different?

To investigate the signaling pathways induced by these receptors, the tyrosine phosphorylation response of NK cells was monitored following stimulation with plate-bound ICAM-1 or purified human IgG (Fig. 3A). The most prominent tyrosine phosphorylation induced by both receptors, as detected by 4G10-agarose IP and 4G10 immunoblotting, and observed in a short exposure of the blot, is surprisingly similar, with major bands around 105, 65, and 55 kDa. A prominent band seen around 18 kDa following CD16 stimulation is likely the TCR-ζ-chain. Other bands uniquely induced in the CD16 response were observed around 34 and 26 kDa. Weaker bands, observed upon longer exposure, were seen around 45 and 25 kDa only after CD16 stimulation. LFA-1 stimulation produced weaker phosphorylation of the 18-kDa band (possibly TCR-ζ), as well as weak phosphorylation of higher molecular mass proteins (150 kDa and above) that were not observed following CD16 stimulation. Phosphorylation of TCR-ζ and Syk was confirmed following both stimuli (Fig. 3B), with stronger phosphorylation after CD16 stimulation. The multiple bands in the Syk blot following IgG stimulation are likely due to Syk ubiquitination. Changes in TCR-ζ mobility occur following tyrosine phosphorylation, with distinct molecular masses corresponding to phosphorylation at specific sites (46). The largest band observed in the IgG stimulation after 4G10-agarose IP and TCR-ζ immunoblot is likely the fully phosphorylated 23 kDa form of TCR-ζ.

Although TCR-ζ and Syk phosphorylation were stronger following CD16 stimulation in comparison with LFA-1 stimulation, the two responses displayed similar kinetics, with greater phosphorylation after 20 min in both cases, phosphorylation was reduced by an inhibitor of Syk (Supplemental Fig. 1). In both cases, phosphorylation was monitored through the surface expression of CD107a by NK cells following target cell conjugation (Fig. 3C). Stimulation with *Drosophila* S2 cells coated with a polycyonal rabbit anti-serum, which engages CD16, induced significant CD107a surface expression. In contrast, ICAM-1–expressing S2 cells induced no degranulation, as seen previously in freshly isolated cells. To further highlight the differences in CD16- and LFA-1–mediated signaling, calcium flux induced by both receptors was determined. Calcium mobilization was observed following Ab cross-linking of CD16, but not LFA-1 (Fig. 3D). Ab-coated S2 cells induced calcium flux, whereas ICAM-1–expressing S2 cells did not (Fig. 3D). Calcium flux via flow cytometry may not reveal transient calcium fluxes, such as those observed in platelets following integrin engagement (48), leaving the possibility that LFA-1 engagement in NK cells induces transient calcium oscillations. However, our results contrast with published data in isolated primary T cells, in which LFA-1 stimulation causes a TCR-dependent, slow, and sustained calcium mobilization (49).

**FIGURE 2.** LFA-1–dependent responses are ablated by inhibition of the Syk kinase. A, NK cells pretreated for 45 min at 37°C in culture medium with 1 μM Syk inhibitor II or DMSO carrier were mixed with CellTracker Green-labeled S2 cells or S2 cells expressing ICAM-1 for 20 min at 37°C, adhered to poly-α-lysine–coated slides, fixed, permeabilized, and stained with an anti-perforin Ab and Alexa 568 goat anti-mouse secondary Ab. A representative image of treated or untreated NK cells in contact with S2-ICAM-1 cells is shown. B, Treated or untreated NK cells in conjugate with S2 or S2-ICAM-1 cells were scored for polarization of perforin toward the target cell interface. Error bars represent SD of three experiments conducted with NK cells from different donors. *p < 0.05 relative to DMSO-treated NK cells plus S2-ICAM-1 cells. C, NK cells were pretreated for 45 min at 37°C in culture medium with 1 μM Syk inhibitor II (Calbiochem) or DMSO carrier. Cells were then stimulated with purified ICAM-1 on plates for 20 min at 37°C and lysed. Pyk2, CasL, and paxillin IPs and whole-cell lysates (WCL) were resolved by SDS-PAGE and immunoblotted for phosphotyrosine.

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**FIGURE 2.** LFA-1–dependent responses are ablated by inhibition of the Syk kinase. A, NK cells pretreated for 45 min at 37°C in culture medium with 1 μM Syk inhibitor II or DMSO carrier were mixed with CellTracker Green-labeled S2 cells or S2 cells expressing ICAM-1 for 20 min at 37°C, adhered to poly-α-lysine–coated slides, fixed, permeabilized, and stained with an anti-perforin Ab and Alexa 568 goat anti-mouse secondary Ab. A representative image of treated or untreated NK cells in contact with S2-ICAM-1 cells is shown. B, Treated or untreated NK cells in conjugate with S2 or S2-ICAM-1 cells were scored for polarization of perforin toward the target cell interface. Error bars represent SD of three experiments conducted with NK cells from different donors. *p < 0.05 relative to DMSO-treated NK cells plus S2-ICAM-1 cells. C, NK cells were pretreated for 45 min at 37°C in culture medium with 1 μM Syk inhibitor II (Calbiochem) or DMSO carrier. Cells were then stimulated with purified ICAM-1 on plates for 20 min at 37°C and lysed. Pyk2, CasL, and paxillin IPs and whole-cell lysates (WCL) were resolved by SDS-PAGE and immunoblotted for phosphotyrosine.

**TABLE I.** Binding of NK cells to ICAM-1–coated beads following treatment with pharmacological inhibitors or siRNA transfection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% NK Cells Bound to ICAM-1-Fc Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>62.1 ± 0.3</td>
</tr>
<tr>
<td>Syk inhibitor II</td>
<td>37.2 ± 12.9</td>
</tr>
<tr>
<td>U73 343</td>
<td>62.3 ± 2.6</td>
</tr>
<tr>
<td>U73122</td>
<td>19.9 ± 11.6</td>
</tr>
<tr>
<td>Bisindolylmaleimide</td>
<td>33.7 ± 15.4</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>75.9 ± 8.0</td>
</tr>
<tr>
<td>LAT siRNA</td>
<td>79.1 ± 9.6</td>
</tr>
<tr>
<td>Paxillin siRNA</td>
<td>75.5 ± 12.3</td>
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PKC substrate (anti–phospho-serine motif) Ab.

FIGURE 4. PLC-γ and PKC are required for perforin polarization in response to LFA-1 engagement. A, NK cells were stimulated at 37˚C for 20 min on plates coated with BSA, purified ICAM-1, or purified human IgG. Cells were lysed, and tyrosine phosphorylated proteins were immunoprecipitated with 4G10-agarose, and immunoprecipitates were resolved by SDS-PAGE and immunoblotted for phosphoryrosine. B, 4G10-agarose immunoprecipitates from NK cells stimulated as in Fig. 3C for 20 min were immunoblotted for Syk and TCRγ. C, NK cells were mixed with S2 cells, S2 cells plus antiserum, or S2-ICAM-1 cells (right panel). Fluo-4 and Fura Red intensities were monitored for 5 min total, the ratio of Fluo-4 to Fura Red was calculated, and the ratios for each time course were normalized to the starting ratio at time = 0. Data are expressed as fold changes in the ratio from the starting time point.

PKC family, we tested the involvement of PKC in the response to LFA-1. Stimulation of NK cells with ligands for either LFA-1 or CD16 induced serine phosphorylation that is detected by an Ab directed against a motif that corresponds to preferred substrates of PKC (Fig. 4C). Several bands were induced by LFA-1 or CD16 engagement, and at least three bands were induced only by LFA-1. These proteins have not been identified. Inhibition of PKC activity reduced perforin polarization in response to LFA-1 engagement (Fig. 4D), suggesting that PKC activity induced by DAG production was involved in the LFA-1 response. Treatment with the PKC inhibitor reduced binding to ICAM-1 (Table I), but again substantial binding remained.

Role of paxillin and LAT in the responses to LFA-1 and CD16

We next chose to investigate potential differences in signaling that might explain the differing outcomes of LFA-1 and CD16 engagement. We assayed for the phosphorylation status of proteins known to be involved in integrin signaling and ITAM-mediated signaling in different systems. As shown in Fig. 2A, Syk-dependent paxillin phosphorylation is induced by LFA-1 engagement. Stimulation through CD16 resulted in a lesser amount of paxillin phosphorylation than LFA-1 stimulation (Fig. 5A). In comparison, LAT, a protein involved in TCR signaling (53), is phosphorylated following CD16 stimulation and very

PLC-γ2 were tyrosine phosphorylated following engagement of LFA-1 or CD16 (Fig. 4A). PLC-γ activation following LFA-1 stimulation is surprising, given the lack of calcium flux following LFA-1 stimulation. Inhibition of PLC activity resulted in a block in perforin polarization in response to ICAM-1 (Fig. 4B), indicating that PLC-γ1/2 are involved in this response. Treatment of the NK cells with the PLC inhibitor also reduced binding to ICAM-1 (Table I), although some binding remained.

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weakly phosphorylated by LFA-1 stimulation (Fig. 5A). To determine whether the differential patterns of phosphorylation correlated with differential involvement in functional outcomes, CD16-mediated degranulation and LFA-1-mediated perforin polarization were determined after siRNA-mediated knockdown of paxillin and LAT (Fig. 5B). Knockdown of LAT reduced CD16 induced degranulation by a statistically significant degree (by about a third; Fig. 5C). Knockdown of paxillin reduced degranulation to a lesser extent, which did not achieve statistical significance. Degranulation of NK cells after control siRNA transfection was generally less than degranulation seen in transfected NK cells (compare Fig. 3A and Fig. 5C). This reduction was consistent across multiple siRNA oligos, and it appears to be a result of nucleofection (data not shown). Perforin polarization was reduced by a statistically significant amount following paxillin knockdown (Fig. 5D), and it was unaffected by LAT knockdown. Therefore, we conclude that paxillin is involved in LFA-1–mediated polarization, whereas LAT-dependent signaling is required for CD16-mediated degranulation but is dispensable for LFA-1–mediated polarization.

**Discussion**

In this report, we have described a signaling network used by the integrin LFA-1 to induce perforin polarization in NK cells. We have used protein biochemistry, pharmacological inhibitors, and siRNA-mediated protein knockdown to demonstrate the phosphorylation of the TCRζ-chain, Syk, and PLC-γ1/2 and a requirement for paxillin in this signaling pathway. By stimulating NK cells with natural ligands for LFA-1 or CD16, we have shown that LFA-1 and CD16 share signaling properties (TCRζ, Syk and PLC-γ phosphorylation), yet initiate different functional outcomes. The basis for this difference was explored. Knockdown of protein expression by siRNA transfection showed that paxillin contributed to LFA-1–dependent perforin polarization, whereas LAT contributed to CD16-mediated degranulation.

A requirement for ITAM-containing adapter molecules in integrin signaling was reported in macrophages and neutrophils (27). These cells normally express DAP12 and the FeRγ-chain, and in the absence of these two adapters the response to both fibrinogen, a ligand for integrin αMβ2, and ICAM-1 was defective. Deletion of Syk also abrogated these integrin-dependent responses. In comparison, NK cells express three ITAM-containing adapters: TCRζ, FeRγ, and DAP12. We show that engagement of LFA-1 resulted in detectable tyrosine phosphorylation of only the TCRζ-chain. NK cells are known to express a heterodimer of TCRζ and FeRγ (39). Even in conditions where this heterodimer was detected in our IPs, we detected phosphorylation of only the TCRζ chain (Fig. 1B), suggesting that homodimers of TCRζ are used preferentially in LFA-1 signaling. Complete ablation of integrin responses in myeloid cells required the deletion of both DAP12 and FeRγ, and it is possible that other ITAM-containing adapters could replace TCRζ in integrin signaling in NK cells despite our inability to detect phosphorylation of these molecules in normal human NK cells.

Previous reports have established a link between LFA-1 and TCR signaling chains. In Jurkat T cells, clustering of the integrin β2 cytoplasmic tail results in calcium flux, and this response is defective in cells lacking the TCRζ-chain (54). This study was performed using overexpressed fusion proteins, rather than intact integrin chains, but it highlights a possible functional tie between β2 integrins and the TCRζ-chain in T cells. Additionally, cross-linking and capping LFA-1 on primary CTL induces a capping of CD3 signaling chains (55), which was interpreted as LFA-1 favoring the formation of the immunological synapse in T cells. Despite the evidence linking integrins and ITAMs, no protein–protein interaction between an integrin chain and an ITAM-containing adapter has been demonstrated, and ITAM signaling by integrins remains somewhat mysterious (33).

In human NK cells, we have previously shown that LFA-1 engagement results in polarization of perforin containing granules, but does not induce degranulation (8, 9). Conversely, CD16 engagement induces degranulation, but does not induce perforin polarization (9). Interestingly, in mouse NK cells, stimulation through LFA-1 alone is not sufficient to induce perforin polarization, but polarization occurs following simultaneous engagement of LFA-1 and NKG2D (56). This difference between mouse and human NK cell responses to LFA-1 may reflect genuine differences between species, or the fact that NK cells in humans may have been primed in vivo.

We have shown previously (and confirmed in this study) that CD16 stimulation induces calcium flux in human NK cells. In contrast, cross-linking of LFA-1 by Abs or engagement with ICAM-1 on target cells induces no degranulation or calcium mobilization (Fig. 3). The lack of degranulation and calcium flux in response to LFA-1 engagement on NK cells stands in contrast to previous reports in other cell types. LFA-1 engagement in neutrophils induces degranulation and release of antimicrobial products such as reactive oxygen species (27). β2 integrin Mac-1–mediated phagocytosis of opsonized particles initiates calcium mobilization in human neutrophils (57), and Ab cross-linking of the β2-chain in primary human CD4+ T cells induces robust calcium flux (49). Perhaps this represents true cell type-specific differences, although these details remain to be elucidated. Certainly, calcium mobilization is considered a typical response in...
ITAM-dependent signaling (51), and the involvement of TCRζ and Syk in a signaling pathway that does not induce calcium, as reported in this study, is novel.

CD16 engagement induces TCRζ and Syk phosphorylation, along with calcium flux and degranulation, but without perforin polarization. The similarities in proximal intracellular signaling downstream of LFA-1 and CD16 are striking given the obvious difference in the ultimate downstream outcomes. Interestingly, PLC-γ1 and PLC-γ2 are tyrosine phosphorylated in response to both stimuli. The phosphorylation of PLC-γ1 and PLC-γ2 following LFA-1 engagement is surprising, given the role of the PLC-γ product inositol 1,4,5-triphosphate in calcium flux (58). However, LFA-1 induced no calcium flux in NK cells. These observations suggest an unknown bifurcation in the signaling pathways used by LFA-1 and CD16. This could be a threshold effect, as the observed biochemical responses to CD16 stimulation were stronger (particularly with TCRζ, Syk, and PLC-γ1/2) than the responses to LFA-1. Alternatively, there may be qualitative differences, including different kinetics, in the responses. However, the point at which signals by LFA-1 and CD16 diverge is unknown, and the reason for the failure of LFA-1 to initiate calcium flux remains to be elucidated.

PLC-γ phosphorylation following integrin engagement has been observed in other cell types: PLC-γ1 following LFA-1 engagement in T cells (49) and PLC-γ2 in neutrophils following β2 cross-linking (59). A recent report revealed that the MTOC in T cells moves to sites of localized DAG production (60). The MTOC movement in response to TCR stimulation is PLC-γ-dependent, as DAG is also a product of PLC-γ activity. Our results with PLC-γ1 and PLC-γ2 in LFA-1 signaling are consistent with these observations.

Signaling pathways that influence integrin affinity are referred to as inside-out signals (61, 62). Binding of integrins to their ligands is controlled by the activation status of the integrin, with distinct conformations of the integrin ranging from low- to high-affinity states (63, 64). In turn, engagement of integrins by ligands initiates outside-in signaling, which leads to biological responses. We have previously shown, in freshly isolated human NK cells, that LFA-1 binding to ICAM-1 leads to further activation of LFA-1, such that outside-in signaling by LFA-1 results in its own inside-out activation, and that stimulation by IL-2 leads to activation of LFA-1 (34). However, the pharmacological inhibitors used in this study in several biochemical experiments with IL-2-expanded NK cells caused a reduction in binding to ICAM-1 (Table I). The residual binding was sufficient to score for polarization in an assay that considers only cells that are in contact with target cells. Our results with siRNA transfections, in which paxillin knockdown inhibited perforin polarization without reduction of binding to ICAM-1, indicate that it is possible to separate integrin activation and outside-in signaling by using tools of sufficient specificity. In this respect, NK cells offer a unique tool to study outside-in signaling by LFA-1, given that binding to ICAM-1 is not completely dependent on outside-in signaling.

To identify molecules that could explain the disparate functional outcomes of LFA-1 and CD16 engagement, we investigated the activation of other downstream signaling molecules. Pyk2 and paxillin were preferentially phosphorylated downstream of LFA-1, as compared with CD16. LAT was almost exclusively phosphorylated after CD16 engagement. We therefore tested their potential roles in LFA-1 signaling for perforin polarization and in CD16 signaling for degranulation in IL-2-expanded human NK cells. Knockdown of LAT by siRNA transfection reduced CD16-induced degranulation, but had no effect on LFA-1-induced polarization. Knockdown of paxillin significantly reduced polarization through LFA-1, and it had a lesser effect on CD16-mediated degranulation. The role of LAT downstream of the ITAM-dependent CD16 signaling was expected. LAT is best known for its role in TCR signaling, in which it serves downstream of ITAMs as a tyrosine phosphorylated docking platform for the assembly of a large, multimolecular complex, including PLC-γ1 and Vav1 (65). Paxillin is an adapter protein that interacts with actin binding proteins (66, 67) and proteins with actin regulating functions (68, 69). It is phosphorylated downstream of integrins in human NK cells (23). Paxillin associates with Pyk2 (70–74), which was tyrosine phosphorylated downstream of LFA-1 in NK cells (Fig. 24). Paxillin localized to the MTOC in T lymphoblasts (75), and a complex of Pyk2, paxillin, and the MTOC localizes to cytotoxic synapses in NK cells (76), creating a direct link between this adapter protein and a crucial component of the cytotoxic granule polarization machinery. We have shown a selective role of paxillin downstream of LFA-1 in perforin polarization. While further understanding will require a more detailed analysis of paxillin-containing protein complexes formed following LFA-1 stimulation, our results are a first step toward understanding the signaling pathways used by LFA-1 in perforin polarization in NK cells.

Disclosures

The authors have no financial conflicts of interest.

References


