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Cutting Edge: A Role for the Adaptor Protein LAT in Human NK Cell-Mediated Cytotoxicity

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Stimulation of NK cell-mediated cytotoxicity involves the coupling of proximal Src and Syk family protein tyrosine kinases to downstream effectors. However, the mechanisms linking these second messenger pathways are incompletely understood. Here, we describe a key role for the LAT (p36) adaptor protein in human NK cell activation. LAT is tyrosine phosphorylated upon stimulation of NK cells through FcγRIII receptors and following direct contact with NK-sensitive target cells. This NK stimulation induces the association of LAT with several phosphotyrosine-containing proteins. In addition to the biochemical evidence showing LAT involvement in NK cell activation, a genetic model shows that LAT is required for Fcε-dependent phosphorylation of phospholipase C-γ. Furthermore, overexpression of LAT in NK cells leads to increased Ab-dependent cell-mediated cytotoxicity and "natural cytotoxicity," thus demonstrating a functional role for LAT in NK cells. These data suggest that LAT is an important adaptor protein for the regulation of human NK cell-mediated cytotoxicity. The Journal of Immunology, 1999, 162: 2453–2456.  

Natural killer cells are a subpopulation of CD16+CD56+ large granular lymphocytes (1). They have multiple immune functions, including mediation of resistance to intracellular microbial infections, control of tumor growth, and regulation of inflammatory responses. Unlike B and T lymphocytes, NK cells do not express Ag-specific receptors. Rather, their activation is initiated by the FcγRIII receptor (Ab-dependent cell-mediated cytotoxicity (ADCC)) or, in a less defined manner, through a direct interaction with certain malignant or virus-infected target cells ("natural cytotoxicity") (2).

The biologic functions of NK cells differ from the functions of other lymphocyte subpopulations, e.g., B and T cells. However, the mechanism of NK activation bears homology to that employed by other lymphocytes. Although B cell receptor, TCR, and FcR differ in their extracellular portions, these multichain immune recognition receptors (MIRR) all have immunoreceptor tyrosine-based activation motifs in their signal-transducing subunits and all share certain common features in their intracellular signaling pathways (3). FcγRIII cross-linking on NK cells leads to the activation of Src and Syk family kinases (4–6). Downstream of Syk family kinases are, among other molecules, phospholipase C-γ (PLC-γ) (7–9), the guanine nucleotide exchange factor Vav (10), and the adaptor protein SLP76 (11–13), all of which are important regulators of NK cell-mediated cytotoxicity (7, 8, 14, 15).

Early studies of lymphocyte signaling showed that an ~36-kDa protein undergoes tyrosine phosphorylation upon stimulation of T and NK cells (16–19). Recently, this protein was cloned from T cells and named linker for activation of T cells (LAT) (20). This protein lacks intrinsic catalytic activity, but it appears to function as an adaptor integrating signals initiated by MIRR stimulation. LAT is a transmembrane protein and has multiple tyrosine residues that, upon phosphorylation, promote specific protein-protein interactions with Src homology (SH)-2-containing proteins. Palmitoylation of LAT seems to be important for its targeting to glycolipid-enriched membrane subdomains in which specific interactions with other proteins may occur (21). Upon TCR cross-linking, phosphorylated LAT associates with several signaling proteins such as PLC-γ1, Vav, SLP76, Grb2, and the p85 subunit of the phosphatidylinositol 3-kinase (PI3K) (20). A LAT mutant bearing substitutions at the two C-terminal tyrosine residues fails to associate with specific interacting proteins, suggesting that the full adaptor function of LAT is dependent on the inducible tyrosine phosphorylation of this protein. Moreover, the same LAT mutant inhibits TCR-induced transcriptional activity of AP-1 and NFAT when transiently expressed in Jurkat T cells. Therefore, phosphorylated

Abbreviations used in this paper: ADCC, Ab-dependent cell-mediated cytotoxicity; PLC, phospholipase C; MIRR, multichain immune recognition receptors.

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LAT seems to be playing a role in the nucleation of several signaling complexes important for T cell activation.

The function of LAT in NK cells is unknown. Phosphorylated p36 has been implicated as a direct target of the killer cell inhibitory receptor-associated phosphatase SHP-1 during inhibitory signaling in NK cells (19). Phosphorylated p36 has also been found to bind to the adaptor protein Grb2 during the FcR stimulation of NK cells (18, 22). This association may be involved in the activation of downstream effectors such as Ras and phosphatidylinositol 3-kinase. However, the precise role for LAT during NK cell activation and particularly its potential role in NK cell-mediated cytotoxicity have not been described. In this paper, we provide genetic, biochemical, and functional evidence that supports a key role for LAT in the regulation of human NK cell-mediated cytotoxicity.

Materials and Methods

Cell lines and reagents

Unless otherwise indicated, all chemicals were obtained from Sigma (St. Louis, MO). Human NK clones were cloned and passaged as previously described (23). Briefly, CD16+ PBL from healthy donors were cloned by limiting dilution in RPMI 1640 supplemented with 10% human serum and 20 U/ml of IL-2. Freshly isolated PBMC were obtained from healthy human donors and were monocyte-depleted by adherence to tissue-culture flasks. The human Jurkat T cell line, murine mastocytoma line P815, and anti-CD3-producing hybridoma OKT3 (murine IgG2a) were obtained from American Type Culture Collection (Manassas, VA). The HLA class I-deficient C1R and 721.221 cell lines were kindly provided by Peter Cresswell (Yale University, New Haven, CT) and Peter Parham (Stanford University, Palo Alto, CA), respectively. The LAT-deficient ANJ3 clone was derived by selection of randomly mutagenized Jurkat E6 cells for stable somatic mutants bearing defects in TCR-induced Ca2+ mobilization (24). Anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antisera to LAT was obtained by immunization of rabbits with keyhole limpet hemocyanin-conjugated LAT peptide (amino acids 106–131). Polyclonal antisera to Vav, SLP76, and PLC-γ1 have been previously described (Refs. 14, 15, and 7, respectively).

Cell stimulation

Cells were stimulated as previously described (25) and lysed in buffer containing 20 mM Tris-HCl, 40 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM Na3P04, 0.1% BSA, 1 mM NaVO4, 1 mM PMSF, 5 μg/ml apro- tin, 5 μg/ml Leupeptin, and 1% Triton X-100 (pH 7.4). Insoluble material was removed by centrifugation for 5 min at 14,000 rpm. Cell lysates were subjected to immunoprecipitation for 1–2 h with the indicated rabbit anti-serum bound to protein A-Sepharose beads. Eluted proteins were resolved by SDS-PAGE and transferred electrophoretically to Immobilon-P membranes (Millipore, Bedford, MA). Tyrosine phosphorylated proteins were detected with 4G10 mAb, followed by sheep anti-mouse IgG coupled to horseradish peroxidase (Amersham, Buckinghamshire, England) and with the enhanced chemiluminescence detection system (Amersham). LAT, PLC-γ1, SLP76, and Vav were analyzed with specific rabbit antisera and detected with protein A-horseradish peroxidase.

Vaccinia viruses

Recombinant vaccinia viruses encoding myc-tagged, wild-type LAT (myc-LAT wt), a double tyrosine mutant (myc-LAT Y171/191F), CD16, and γ constructs were made as previously described (14). Cells (2 × 10⁶/ml) were infected at a multiplicity of infection of 20:1 for 1 h in serum-free RPMI 1640 at 37°C and then for the remainder of the indicated infection time at 10⁶ cells/ml in RPMI 1640 supplemented with 10% bovine calf serum.

Cytotoxicity assays

The Cr51-release assays measuring direct NK cell-mediated cytotoxicity or reverse ADCC were performed as previously described (23).

Results and Discussion

The role of LAT as an adaptor protein in TCR-initiated signaling depends on its ability to become tyrosine phosphorylated upon T cell stimulation (20). Since tyrosine phosphorylation of a 36-kDa protein has been detected after NK cell stimulation (18, 19), we were interested in testing the potential involvement of LAT in signaling by NK cells. For that purpose, human NK cell clones were stimulated with anti-FcγRIII Ab (A and B) or with NK-sensitive C1R target cells (C) for the indicated period of time. Target cells do not express the LAT protein (“alone”). LAT immunoprecipitates from lysates (1% Triton X-100) were subjected to SDS-PAGE and, upon transfer to the membrane, probed with anti-phosphotyrosine or anti-LAT Ab.

![FIGURE 1](http://www.jimmunol.org/)
However, the nature of specific molecules involved in this interaction is, for the most part, not known. To test a possible involvement of LAT in signaling from these yet unidentified receptors, NK cells were stimulated directly with the tumor target cells. As with the FcγRIII cross-linking, stimulation of NK cells with HLA class I-deficient, NK-sensitive C1R cells led to an increase in the tyrosine phosphorylation of LAT (Fig. 1C), although the kinetics of LAT phosphorylation were somewhat slower. Similar results were obtained when NK cells were stimulated with 721.221 NK-sensitive target cells (data not shown). The above experiments suggest that LAT is involved in signaling pathways leading to ADCC and certain forms of "natural cytotoxicity." However, it needs to be emphasized that since the triggering receptors on NK cells utilized during natural cytotoxicity have been incompletely characterized, different NK-sensitive targets may potentially initiate different sets of second messengers.

Following TCR stimulation of T cells, LAT associates with a number of signaling molecules, including PLC-γ and Grb2 (20). We also detected coimmunoprecipitation of LAT with PLC-γ following NK cell stimulation with anti-FcR Ab (data not shown). Association of LAT with PLC-γ implied its potential role in coupling receptor stimulation to PLC-γ tyrosine phosphorylation. To test if LAT is necessary for the activation of PLC-γ, we used a LAT-deficient Jurkat subline, ANJ3, as a genetic model (Fig. 2A). Wild-type Jurkat, ANJ3, or ANJ3 cells reconstituted with the myctagged wild-type LAT (ANJ3-wt65, ANJ3-wt67) were infected with CD16- and γ-encoding recombinant vaccinia viruses. FcγRIII was equally expressed on the surface of each of the T cell lines (data not shown). Stimulation of ANJ3 cells through FcγRIII or TCR leads to the tyrosine phosphorylation of multiple cellular proteins, similarly to the stimulation of parental Jurkat cells (Fig. 2B). However, although LAT does not seem to be required for the phosphorylation of many of the signaling proteins (SLP-76 and ZAP-70, for example), a selective loss of phosphorylation is detected for a band that migrates in the same position as PLC-γ. To confirm the identity of this band as PLC-γ, we repeated the experiment using PLC-γ1 immunoprecipitates. As shown in Fig. 2C, PLC-γ1 is tyrosine phosphorylated upon stimulation of Jurkat cells (lanes 2 and 3). This inducible phosphorylation is completely blocked in the LAT-deficient ANJ3 cells (lanes 5 and 6). Reconstitution of the wild-type LAT into ANJ3 cells rescues the phosphorylation of PLC-γ1 (lanes 8 and 9). These results directly demonstrate the regulatory role of LAT in the activation of PLC-γ induced by the TCR and FcγRIII receptors.

We next wanted to functionally test the potential role of LAT in NK cell cytotoxicity. NK cells were infected with vaccinia viruses encoding the wild-type (LAT wt) or the double tyrosine mutant of LAT previously shown to block AP-1- and NFAT cell-dependent transcription in T cells (LAT.Y171/191F; LAT.2YF) (20). Equal expression of the two LAT constructs was demonstrated by Western blot analysis with the anti-LAT Ab (data not shown). The effect of LAT overexpression on NK cell cytotoxicity was tested against the murine mastocytoma P815 cells bearing anti-FcγR mAb (reverse ADCC; Fig. 3) or against NK-sensitive cell lines C1R (Fig. 3) and 721.221 (data not shown). Overexpression of the wild-type LAT, but not the Y171/191F mutant increases cytotoxicity of NK cells in the reverse ADCC and "natural cytotoxicity" assays (Fig. 3A). However, some variations between different NK clones were observed (Fig. 3B). Among potential explanations for the observed variations are differences in receptor densities or levels of LAT expression between different NK clones.

The results of cytotoxicity assays support the regulatory role of LAT in NK cell signaling. However, the mechanism by which overexpression of an adaptor protein increases NK cytotoxicity is not clear. It is likely that clustering of signaling molecules upon receptor cross-linking results in the cellular activation only when a threshold level of mobilized downstream molecules is reached. Since LAT interacts with multiple signaling proteins (Ref. 20 and data not shown), the positive effect of LAT overexpression on NK cytotoxicity may be due to a more rapid kinetics of mobilization of key downstream effectors to the receptor complex. In contrast to the transcriptional activation of Jurkat T cells, in most of our clones LAT.2YF did not have a "dominant-negative" effect on cytotoxicity, which implies that different cellular functions may depend on different activation kinetics or different sets of second messengers.

Another potential function of LAT is the regulation of inhibitory signaling in NK cells. Interaction with MHC class I-expressing target cells led to dephosphorylation of p36 and prevented its association with PLC-γ and adaptor protein Grb2 (19). The loss of association between PLC-γ and p36 corresponded with diminished IP3 production but did not influence phosphorylation of PLC-γ
FIGURE 3. Overexpression of the wild-type but not the tyrosine mutant of LAT enhances NK cytotoxicity. NK clones were infected for 6 h with LAT wt or LAT.2YF-expressing recombinant vaccinia viruses or with the wild-type vaccinia virus as a control. CI851-labeled target cells (10^3/well, with 150 ng/ml of anti-FcγRIII Ab for P815 target cells) were incubated for 4 h with infected NK cells at the indicated E:T ratio. The release of CI851 into the supernatant was determined, and the results are expressed as percent specific cytotoxicity (A) or as lytic units per 10^6 target cells (B).

upon stimulation, thus implicating spatial restraints as a mechanism of regulating PLC-γ activity. In other studies, PLC-γ was found to be dephosphorylated upon killer cell inhibitory receptor engagement (26, 27). Thus, the regulation of signals initiated by the inhibitory receptors on NK cells and the potential role of LAT in inhibitory signaling are not resolved.

In this paper, we have shown through biochemical and functional assays the importance of LAT for the activation of human NK cells. The role of the association of LAT with other signaling proteins, as well as the exact place of LAT in the signaling cascade from MIRRs, awaits future investigations.

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