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Ig-like transcript 2 (ILT2)/leukocyte Ig-like receptor 1 (LIR1) Inhibits TCR Signaling and Actin Cytoskeleton Reorganization

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Ig-like transcript 2 (ILT2)/leukocyte Ig-like receptor 1 (LIR1) is a receptor, specific for MHC class I molecules, that inhibits lymphoid and myeloid cells. Here, we analyzed the molecular and cellular mechanisms by which ILT2 modulates T cell activation in primary CTLs and transfected T cell lines. We found that cross-linking with the TCR and the activity of Src tyrosine kinase p56Lck are required for phosphorylation of ILT2 and subsequent recruitment of Src homology protein 1. In contrast, ILT2 triggering resulted in reduced phosphorylation of TCR and linker for activation of T cells, which led to reduced TCR-ZAP70 complex formation, as well as extracellular signal-related kinase 1 and 2 activation. Furthermore, ILT2 inhibited both superantigen and anti-TCR Ab-induced rearrangement of the actin cytoskeleton. The inhibitory effect mediated by ILT2 is probably concentrated at the APC-T cell interface because both TCR and ILT2 were strongly polarized toward the APC upon engagement by their specific ligands. Thus, ILT2 inhibits both signaling and cellular events involved in the activation of T cells. The Journal of Immunology, 2001, 166: 2514–2521.

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3 Abbreviations used in this paper: LAT, linker for activation of T cells; PV, pervanadate; GAM, goat anti-mouse; ERK, extracellular signal-related kinase; TSST-1, toxic shock syndrome toxin-1; MFI, mean fluorescence intensity; KIR, killer cell Ig-like receptor; ITIM, immunoreceptor tyrosine-based inhibition motif; ITAM, immunoreceptor tyrosine-based activation motif; ILT2, Ig-like transcript 2; LIR1, leukocyte Ig-like receptor 1; SHP, Src homology protein.
Materials and Methods

Cells and transfectants
Jurtak and J. CaM 1.6 (deficient of p56^lck^) (32) cells were grown in RPMI-1640/10% FCS. ILT2 and CD4-^z^ CDNA was transfected in Jurtak or J. CaM 1.6 cells by electroporation as previously described (33), and stable transfectants were selected in G418-containing medium. ILT2 and CD4-^z^ expression on transfected cells was assessed by FACS analysis and immunoblotting using mAb GHI/75 or GK1.5. 721.221 (or 721.221 transfected with HLA-B^2705^) cells are MHC class I-deficient EBV-transformed human B cell lines (22). LOQ22.7 and OKT8-24 CD8^+^ T cell clones were isolated and maintained as previously described (22).

Antibodies
PY20, anti-SHP-1, and anti-ZAP70 mAbs were obtained from BD Transduction Laboratories (Lexington, KY). Anti-CD3 mAb was kindly donated by A. Lanavecevich (Institute for Research in Biomedicine, Bellinzona, Switzerland). F(ab')^2^ of goat anti-mouse (GAM) IgG H+L or mouse anti-human IgG Fc-specific were from Jackson ImmunoResearch (West Grove, PA). HRP-conjugated goat anti-mouse and PE-conjugated anti-CD69, FITC-conjugated CD25, or anti-TCR^z^ mAb were obtained from Immunotech (Marseille, France). Anti-phospho-extracellular signal-related kinase (ERK) or ERK Abs were obtained from New England Biolabs (Beverly, MA). Anti-LAT Ab was obtained from Upstate Biotechnology (Lake Placid, NY). Src kinase inhibitors PP1 and PP2 were obtained from Calbiochem (La Jolla, CA). Alexa-conjugated phallolidin was obtained from Molecular Probes (Eugene, OR).

Immunoprecipitation and immunoblotting
Cells treated with mAbs or pervanadate (PV) (200 mU sodium orthovanadate and 200 mU H_2O_2 at 37°C for 10 min) as indicated were lysed in 1% Triton X-100 or 1% Brij97 lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl with added inhibitors, 0.75 mU aprotinin, 10 mU leupeptin, 3 mU pepstatin A, 1 mM PMFSF, 0.4 mM EDTA). PV-treated cells were pre-cleared with protein G beads (Amersham Pharmacia, Uppsala, Sweden). Thereafter, lysates were subjected to immunoprecipitation with the indicated mAbs as previously described (34). For whole-cell lysate analysis, cells were lysed in Laemmli sample buffer. Immunoprecipitates and whole-cell lysates were separated by standard SDS-PAGE, transferred to polyvinylidene difluoride membranes (Amersham Pharmacia) and immunoblotted with the indicated mAbs. Bound Abs were visualized using ECL (Amersham Pharmacia).

Cell stimulations
Two million cells/ml were incubated at 37°C with the indicated mAbs and F(ab')^2^; GAM Abs (Jackson ImmunoResearch) as cross-linker. After stimulation, cell aliquots were lysed and subjected to anti-phosphotyrosine and anti-ERK blotting using PY20 (BD Transduction Laboratories) and either anti-phospho-ERK or ERK Abs (New England Biolabs). Alternatively, lysates were subjected to immunoprecipitation with the indicated mAbs as previously described (34). For whole-cell lysate analysis, cells were lysed in Laemmli sample buffer. Immunoprecipitates and whole-cell lysates were separated by standard SDS-PAGE, transferred to polyvinylidene difluoride membranes (Amersham Pharmacia) and immunoblotted with the indicated mAbs. Bound Abs were visualized using ECL (Amersham Pharmacia).

Confluent microscopy
Cells were washed in PBS and fixed for 10 min with 1% parafomaldehyde/PBS. The cells were permeabilized for 10 min at room temperature with washing buffer (HEPES-buffed PBS, containing 0.1% saponin) and stained with primary Abs at room temperature for 10 min. Cells were washed three times in washing buffer and stained with FITC-conjugated secondary Abs at room temperature for 10 min. In experiments involving stimulation of cells with Ab-coated beads or TSST-1-pulsed APCs, cell-bead/APC conjugates were attached to polylysine (1 mg/ml) coated coverslips, fixed, permeabilized, and stained with Alexa-conjugated phallolidin or the indicated mAbs. Confocal microscopy was performed on an MRC-1000 (Bio-Rad, Richmond, CA) connected to an Axiovert 100 M microscope (Zeiss, Oberkochen, Germany).

Results
In T cells, Src kinase p56^lck^ is required for phosphorylation of ILT2, which recruits SHP-1 to VxYxxLV cytoplasmic motifs
ILT2 was previously shown to inhibit superantigen-induced T cell-mediated cytotoxicity by a subset of CD8^+^ T cells (22). To investigate the molecular mechanisms responsible for this inhibitory function, we first examined the phosphorylation status of ILT2 in T cells after cross-linking ILT2 with the TCR (with specific mAbs and a cross-linker) or following treatment of cells with the phosphatase inhibitor PV. Jurtak T cells transfected with ILT2 CDNA (Jurtak-ILT2) were either treated with PV or subjected to ILT2-TCR cross-linking. Thereafter, ILT2 was immunoprecipitated from cell lysates, and its phosphorylation status was analyzed by Western blot. Both PV treatment and ILT2-TCR cross-linking induced substantial tyrosine phosphorylation of ILT2. Some ILT2 phosphorylation was also observed following ligation of the TCR alone (data not shown). In contrast, ILT2 was not phosphorylated following cross-linking of ILT2 alone (Fig. 1A). To analyze which tyrosine kinase was involved in the phosphorylation of ILT2, Jurkat-ILT2 cells were pre-treated with an inhibitor of Src-tyrosine kinase p56^lck^ (PP2) before stimulation with PV. This treatment significantly reduced the phosphorylation of ILT2 (Fig. 1B). The same result was obtained with Src-tyrosine kinase p56^lck^ inhibitor PP1 (data not shown). In another approach, ILT2 was transfected into J. CaM 1.6, a mutant of Jurkat that lacks expression of the tyrosine kinase p56^lck^ (p56^lck^). No phosphorylation of ILT2 was observed upon PV treatment or after TCR-ILT2 cross-linking in the J. CaM 1.6-ILT2 transfectants (Fig. 1, C, and D). Thus, p56^lck^ is required for phosphorylation of ILT2. Because it was previously shown that phosphorylated ILT2 recruits SHP-1 in B cells and NK cells (22), we verified whether this also occurs in T cells. SHP-1 clearly associated with ILT2 following PV treatment (Fig. 1E). This association was dependent upon p56^lck^ activity, because no association was observed in J. CaM 1.6-ILT2 transfectants (Fig. 1E). We finally examined which of the four cytoplasmic tyrosine-based motifs of ILT2 bind to SHP-1. Phosphorylated and unphosphorylated peptides spanning the four tyrosine motifs were conjugated with Sepharose beads and incubated at 37°C with lysate from Jurkat-ILT2 cells for 2 h. The association of SHP-1 to the peptides was then analyzed by Western blot. SHP-1 bound to the peptides in the following order: pY2 >> pY3 > pY1, whereas little or no binding was observed with pY4, control peptide, or nonphosphorylated peptides.
forms of the peptides (Fig. 1F, and data not shown). Taken together, our results show that in T cells ILT2 is tyrosine phosphorylated following cross-linking to the TCR. Tyrosine-phosphorylated ILT2 recruited SHP-1, which preferentially bound to the VxYxxV motif in ILT2 in vitro. Tyrosine phosphorylation of ILT2 and the association with SHP-1 required the presence and activity of p56Lck.

**ILT2 ligation reduces phosphorylation of TCRζ, LAT, and ERK1/2**

Because ILT2 recruited the phosphatase SHP-1 upon phosphorylation, we next examined whether this would affect the phosphorylation of proteins involved in TCR-induced signaling pathways. TCR was cross-linked either alone, with ILT2, or with another cell surface protein, MHC class I, in Jurkat-ILT2 cells. Cell lysates from stimulated cells were analyzed by Western blot using an anti-phosphotyrosine mAb. The results showed that the phosphorylation of two proteins of molecular mass 20 and 36–44 kDa was clearly decreased following TCR-ILT2 coligation as compared with TCR cross-linking alone or with TCR-MHC-I coligation (Fig. 2, A and B). To assess whether the proteins of molecular mass 20 and 36 kDa corresponded to TCRζ and LAT, tyrosine-phosphorylated proteins were precipitated from stimulated Jurkat-ILT2 cells and analyzed by Western blot analysis using an anti-TCRζ mAb or a LAT Ab. Alternatively, TCRζ and LAT were precipitated, and the phosphorylation state of these proteins was analyzed by Western blot. In control experiments, TCR was cross-linked either alone, with ILT2, or with another cell surface protein, MHC class I, in Jurkat-ILT2 cells. Cell lysates from stimulated cells were analyzed by Western blot using an anti-phosphotyrosine mAb. The results showed that the phosphorylation of both TCRζ (Fig. 2, C–E) and LAT (Fig. 2, F–I) was reduced upon TCR-ILT2 ligation. In addition, the reduction in TCRζ phosphorylation was associated with reduced recruitment of ZAP70 (Fig. 2E). In control experiments, TCR-MHC-I cross-linking did not reduce phosphorylation of TCRζ or LAT (Fig. 2, D, G, and I). ILT2-induced reduction of LAT and TCRζ phosphorylation was also observed in a CD8±ILT2± T cell clone (LOQ22.7) (Fig. 2I, and data not shown). Because phosphorylated LAT links to activation of the mitogen-activated protein kinases ERK1 and 2, we also examined whether TCR-ILT2 coligation affected the activation of ERK1 and 2. Activation of both ERK1 and ERK2 in LOQ22.7 cells decreased following ILT2-TCR cross-linking as compared with cross-linking of TCR alone or TCR-MHC-I cross-linking (Fig. 3). This was also observed in Jurkat-ILT2 cells (data not shown). Taken together, these experiments show that TCR-ILT2 coligation reduces TCR-mediated phosphorylation of TCRζ, LAT, and ERK1 and 2, as well as the TCRζ-ZAP70 complex formation.

**TCR-ILT2 coligation inhibits TCR-dependent rearrangement and polymerization of the actin cytoskeleton**

One of the consequences of TCR engagement is modification of the actin cytoskeleton, which is absolutely required for T cell activation and cytotoxicity (7, 35–37). Because TCR-ILT2 coligation decreased T cell activation, we speculated that it might also affect polymerization of actin following engagement of TCR. To examine this possibility, cells were incubated with beads coated with either anti-CD3 mAb, anti-ILT2 mAb, anti-CD3 and -ILT2 mAbs, or anti-CD3 and -MHC-I mAbs and analyzed by confocal microscopy using phalloidin, which binds to polymerized actin (F-actin). Upon triggering with anti-CD3 or anti-CD3/MHC-I-coated beads, actin strongly accumulated at the bead-cell contact...
region. In contrast, when cells were incubated with anti-CD3/ILT2 beads, we observed a significant reduction in the accumulation and polarization of actin (Fig. 4A). Anti-ILT2 beads did not induce polarization of actin. This was observed with Jurkat-ILT2 and OKT8-24 cells (Fig. 4, A and B). Thus, TCR-ILT2 coligation specifically inhibited TCR-mediated actin polymerization. To quantify the changes in actin polymerization, Jurkat-ILT2 or OKT8-24 cells were stimulated with soluble anti-TCR and/or anti-ILT2 mAbs and cross-linker for different time periods, stained with phalloidin, and analyzed by FACS. Engagement of TCR alone or together with MHC-I led to a ~50% increase in the amount of F-actin after 5 min, which declined with time (Fig. 4C). Upon TCR-ILT2 coligation, only small increases in F-actin were observed, in agreement with the results obtained with Ab-coated beads (Fig. 4, C and D). Cross-linking of ILT2 alone did not increase F-actin (data not shown). To determine whether ILT2 could also inhibit actin polymerization induced by engagement of the TCR with superantigen, OKT8-24 cells were incubated with 721.221 or 721.221 cells transfected with the ligand for ILT2, HLA-B27. Both types of cell had been pulsed with the superantigen TSST-1. Binding of TCR to TSST-1-pulsed 721.221 induced strong actin accumulation at the contact site as compared with OKT8-24 cells incubated with nonpulsed 721.221 cells (Fig. 5A). FACS analysis showed a 30–40% increase in F-actin in superantigen-stimulated OKT8-24 cells. However, upon TSST-1 presentation by HLA-B27-positive cells, actin polymerization was strongly reduced (Fig. 5B). Taken together, these results demonstrate that ILT2 inhibits TCR-induced actin polymerization.

**Targets of ILT2, TCRζ and LAT, are involved in actin polymerization**

ILT2-mediated reduction of TCR-induced actin polymerization could be due to reduced tyrosine phosphorylation of TCRζ, LAT, or other substrates. In support of a role for tyrosine phosphorylation, TCR-induced actin polymerization was strongly reduced in cells with impaired tyrosine phosphorylation of TCRζ (and other proteins). These included cells treated with an inhibitor (PP2) of p56lck, which is responsible for TCRζ phosphorylation (38), and cells deficient in p56lck (Fig. 6A). In direct support of a role for TCRζ in actin polymerization, Ab-mediated cross-linking of a fusion protein containing the extracellular and transmembrane region of murine CD4 and the cytoplasmic tail of TCRζ was sufficient to induce actin polymerization in Jurkat (Fig. 6B). Another target of ILT2-mediated dephosphorylation that could be involved in actin polymerization is LAT. Although the role of LAT in TCR-induced actin polymerization is not known, LAT has been reported to interact with proteins involved in cytoskeletal changes (5, 39). To examine whether LAT is required for actin polymerization, we used the LAT-deficient Jurkat cell line (ANJ3) (5). Stimulation of this cell line with an anti-TCR mAb, either in soluble form or coated on beads, did not induce actin polymerization. In contrast, transfection of ANJ3 cells with LAT cDNA completely restored

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**FIGURE 2.** ILT2 triggering reduces phosphorylation of TCRζ and LAT. A and B, Jurkat-ILT2 cells were stimulated with anti-TCR mAb and cross-linker (GAM) in the presence or absence of ILT2 (A) or MHC-I (B) ligation. Thereafter, cell lysates were analyzed in Western blot for tyrosine-phosphorylated proteins. C and D, Jurkat-ILT2 cells were stimulated with anti-TCR mAb and cross-linker (GAM) in the presence or absence of ILT2 or MHC-I ligation. Thereafter, precipitated tyrosine-phosphorylated proteins were analyzed in Western blot with TCRζ Ab. Reprobing with ZAP70 showed the amount of ZAP70 that coprecipitated with TCRζ (lower right panel). E, Alternatively, TCRζ precipitate was analyzed in Western blot using an anti-phosphotyrosine mAb. Reprobing with LAT confirmed an equal amount of these proteins in each lane (upper right panel). F and G, Cells were stimulated with anti-TCR mAb and cross-linker (GAM) in the presence or absence of ILT2 or MHC-I ligation. Thereafter, tyrosine-phosphorylated proteins were precipitated and analyzed in Western blot with a LAT Ab. H and I, Alternatively, LAT precipitate from Jurkat-ILT2 or OKT8-24 cells was analyzed in Western blot using an anti-phosphotyrosine mAb. Reprobing with LAT confirmed an equal amount of these proteins in each lane (right). J, Jurkat-ILT2 cells were stained with MHC-I mAbs (white profile) or irrelevant mAb (black profile) and analyzed by FACS. Cells and mAbs used in precipitation and Western blots, as well as the molecular mass, are indicated in the figure.
TCR and ILT2 are both polarized toward the target cell upon engagement with specific ligands

We finally analyzed the localization of both ILT2 and TCR on T cells upon recognition of their respective ligands, HLA-B27 and TSST-1, on APCs. OKT8-24 cells were incubated with 721.221 cells or an HLA-B27 transfectant of 721.221 pulsed with TSST-1. Thereafter, cells were analyzed by confocal microscopy. As expected, TCR was clearly recruited to the contact region between the T cell and the TSST-1-pulsed APC. Cells not pulsed with TSST-1 did not induce TCR polarization (Fig. 7A). ILT2 was also polarized toward the APC expressing an MHC-I ligand, whereas no polarization was observed using class I-negative APCs (Fig. 7B). ILT2 polarization did not require TSST-1 stimulation (data not shown). This demonstrated that upon recognition of their ligands both TCR and ILT2 are polarized toward the APC, indicating that the inhibitory effect mediated by ILT2 is probably concentrated at the contact region between T cell and APC.

**Discussion**

The cross-talk between TCR and ILT2

In this study, we analyzed the interaction between the TCR and ILT2, an inhibitory receptor that has a broad specificity for MHC class I molecules. We found that, upon TCR-ILT2 cross-linking, ILT2 is phosphorylated on tyrosines. In addition, using PV, we showed that tyrosine phosphorylation and p56\(\zeta\)k were required for SHP-1 recruitment to ILT2. In vitro binding experiments showed that SHP-1 binds preferentially to phosphopeptides spanning the cytoplasmic ILT2 VxpYxxL/V motif. This indicates that the Src homology 2 domains of SHP-1 choose ITIMs, which are preceded by a V in the Y-2 position, in agreement with previous studies on KIRs and paired Ig-like receptor B ITIMs (40–43). TCR-ILT2 cross-linking resulted in reduced phosphorylation of the ITAMs of TCR\(\zeta\), reduced recruitment of ZAP70, and also decreased phosphorylation of LAT and ERK1/2. Reduced ZAP70 recruitment to TCR\(\zeta\) could explain the reduction of LAT phosphorylation because LAT can be a substrate for ZAP70 (44). Reduced LAT phosphorylation as well as reduced TCR\(\zeta\)-ZAP70 complex formation may in turn explain the deactivation of ERK1 and 2 because both ZAP70 and LAT have proved important for activation of ERKs (5, 45). However, both ERK1/2 and a protein of 36 kDa (most probably LAT) have been shown to constitute direct substrates for tyrosine phosphatases (46–49). In addition, ERK1- and ERK2-mediated phosphorylation of p56\(\zeta\)k influence the activity of this kinase (50), which is known to phosphorylate TCR\(\zeta\). Therefore, our results do not exclude the possibility that ERK1 and 2 (and LAT) are directly dephosphorylated by SHP-1, which in turn negatively affects activation of p56\(\zeta\)k and phosphorylation of TCR\(\zeta\) ITAMs. Thus, although we favor the first model, future experiments are required to identify precisely the mechanism responsible for the observed reduction in tyrosine phosphorylation of TCR\(\zeta\), LAT, and ERK1/2.

In contrast to TCR-ILT2 coligation, engagement of ILT2 alone induced neither ILT2 phosphorylation nor association with SHP-1. Upon stimulation, TCR associates with Src kinase p56\(\zeta\)k (9, 38,
Thus, activation (phosphorylation) of ILT2 required interaction with the TCR. In addition, previous studies showed that in NK cells, phosphorylation of KIR3DL1 and KIR2DL1 required p56lck and the CD16-ζ complex (29), whereas in B cells, phosphorylation of paired Ig-like receptor B required the Src kinase Lyn and the FcεRI-Fcγ complex (52). Thus, the activity of inhibitory receptors apparently requires ITAM-containing receptors and their associated tyrosine kinases. One model that could explain this is that the ITAM of the activating receptor (TCR/FcR/B cell receptor) recruits the tyrosine kinase, which phosphorylates the ITIM. However, following ITIM phosphorylation and recruitment of SHP-1, the ITAM and/or associated proteins are dephosphorylated, thus preventing that TCR/FcR/B cell receptor activation proceeds further. This model further predicts: 1) the initial activation of TCR, involving recruitment of kinases, cannot be inhibited by inhibitory receptors because it is in fact required for their activation; 2) therefore, the phosphorylation and activity of ITIMs are regulated by their own substrates, the ITAMs; and 3) dephosphorylation of ITAM (by ITIM), resulting in loss of associated proteins (including the kinases that activate the ITIM), may down-regulate not only the ITAM but also the ITIM itself.

**Modulation of TCR induced reorganization of the actin cytoskeleton by ILT2**

This is the first study to demonstrate that an inhibitory receptor can negatively affect cytoskeletal changes triggered by the TCR. TCR-ILT2 cross-linking markedly reduced actin polymerization as compared with T cells stimulated by cross-linking of TCR alone. Furthermore, coengagement of ILT2 and TCR by HLA-B27 and TSST-1, respectively, reduced TCR-mediated actin polymerization as compared with T cells stimulated with TSST-pulsed class I-negative APCs. The inhibition of actin polymerization may reduce the polarization of TCR (and adhesion/costimulatory molecules) toward the target cell and/or the generation of supramolecular activation clusters (7–9). This in turn would affect the coordinated recruitment of signaling proteins and thus the activation of the T cell. The mechanism by which ILT2 inhibits TCR-triggered actin polymerization is most likely based on dephosphorylation and subsequent lack of recruitment/activation of proteins.
patterns were observed for other inhibitory receptors (53). Another function for ILT2 and other inhibitory receptors may be to terminate an immune response. Following TCR stimulation, the TCR is internalized and targeted for lysosomal degradation (54). The decrease in surface-expressed TCRs during the course of T cell activation may change the balance in favor of inhibitory receptors and thus lead to down-regulation of the response. Finally, ILT2 may protect effector/memory T cells from activation-induced cell death, thereby preserving a pool of Ag-specific T cells.

Concluding remarks

In conclusion, we have described the molecular mechanisms behind activation of ILT2 and showed that a cross-talk exists between ILT2 and TCR, which may involve the regulation of ILT2-ITIM activation/phosphorylation by TCR-ITAMs, as well as the TCRζ-ITAM being a direct substrate for the ILT2-ITIM-SHP-1 complex. ILT2 ligation inhibited both signaling and cellular events important for T cell activation. Proximal signaling events including TCRζ phosphorylation and recruitment of ZAP70 were inhibited, as were downstream events such as LAT phosphorylation and mitogen-activated protein kinase activation. Furthermore, TCR-induced reorganization of the actin cytoskeleton was strongly reduced. Because actin cytoskeletal changes are involved in both T cell and NK cell-mediated killing, this will very well represent a common target for ILTs and KIRs in these cells.

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