Differential contribution of the immunoreceptor tyrosine-based inhibitory motifs of human leukocyte-associated Ig-like receptor-1 to inhibitory function and phosphatase recruitment

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Abstract

Leukocyte-associated Ig-like receptor (LAIR)-1 is an inhibitory receptor expressed on most human leukocytes. It contains two immunoreceptor tyrosine-based inhibitory motifs (ITIM) in its cytoplasmic tail and recruits phosphatases upon phosphorylation. Here we show that both ITIM are required for full inhibition of cellular responses and optimal phosphatase recruitment. Mutation of the C-terminal ITIM still allows partial inhibition of the cytotoxic activity of the NK-like YT.2C2 cells, while mutation of the N-terminal ITIM completely abolishes this inhibitory activity. In contrast, in rat basophilic leukemia (RBL) cells, both mutants of LAIR-1 are partially effective. This is reflected in phosphorylation of these mutants in the different cell types upon pervanadate treatment. However, in both YT.2C2 cells and RBL cells, only the mutant containing the N-terminal ITIM recruits Src homology 2 domain-containing tyrosine phosphatase-2 (SHP-2), while the mutant containing the C-terminal ITIM does not. In RBL cells the mutant containing only the N-terminal ITIM also binds SHP-1, although to a lesser extent than wild-type LAIR-1. We find that in Jurkat T cells Lck is required for the association of SHP-1 with LAIR-1. Co-expression with Lck in 293T cells leads to phosphorylation of both wild-type LAIR-1 and the mutant containing only the N-terminal ITIM, while the mutant lacking this ITIM is not phosphorylated. These results indicate that Lck, or another Src family kinase, is essential for the consecutive phosphorylation of the N- and C-terminal ITIM. Our data imply that the N-terminal ITIM is dominant in LAIR-1 signaling, but that both ITIM contribute to an optimal inhibitory function.

Introduction

A balance between cell activation and inhibition is crucial for the regulation of an immune response. Over the past few years, a large number of inhibitory receptors have been identified (1–3). Leukocyte-associated Ig-like receptor (LAIR)-1 is a human inhibitory receptor that is broadly expressed in the immune system (4). It inhibits BCR signaling (5) and the cytotoxic activity of T cells (6,7) as well as of NK cells (4,7,8). LAIR-1-mediated signaling also inhibits the differentiation of peripheral blood precursors towards dendritic cells (9).

A common feature of most inhibitory immune receptors is the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIM) in the intracellular domain (1–3). These ITIM are involved in the recruitment and activation of the Src homology 2 (SH2) domain-containing tyrosine phosphatases (SHP)-1 and SHP-2 or the SH2 domain-containing inositol phosphate 5'-phosphatase (SHIP), which dephosphorylate key components involved in cell activation (2,3). As inhibitory immune receptors can contain one to four ITIM, the question arises whether ITIM function as independent units or whether they have a cooperative function in the recruitment of phosphatases. In addition, although most studies have focused on the role of ITIM, inhibitory receptors have been
Both ITIM of LAIR-1 contribute to full inhibition

LAIR-1 contains two ITIM in its intracellular domain and becomes phosphorylated upon treatment with pervanadate (4,12,13), but the kinase responsible for LAIR-1 phosphorylation is not known. For several ITIM-bearing receptors it has been shown that phosphorylation is mediated by a Src family kinase (14–19). Previously Xu et al. reported that phosphorylation of LAIR-1 in 293T cells is indeed inhibited by the Src family kinase inhibitor PP1, although PP1 may inhibit other tyrosine kinases as well (12,20–22). We previously demonstrated that phosphorylated LAIR-1 recruits both SHP-1 and SHP-2 (4), but there is controversy since others have reported that LAIR-1 only recruits SHP-1 and not SHP-2 (12). LAIR-1 has been found as a major binding partner of SHP-1 in Jurkat T cells in which LAIR-1 is constitutively phosphorylated (23). Previously it has been shown that recruitment of SHP-1 requires both ITIM, but this has not been linked to biological function (12).

Here we report that LAIR-1 is phosphorylated by Src family kinases, and recruit both SHP-1 and SHP-2 upon antibody-mediated cross-linking. Both ITIM are required for optimal phosphatase recruitment and full inhibitory function, although mutants with only one functional ITIM are still capable to inhibit cellular responses of the NK-like YT.2C2 cells and basophilic cells.

Methods

Cell lines and culture

YT.2C2 is a human NK-like tumor cell line that was kindly provided by Dr K. Smith (24). YT.2C2 stably transfected with human LAIR-1 and 721.221 cells expressing human FcγRII were generated at the DNAX Research Institute (Palo Alto, CA), and have been described before (4,6). RBL-2H3 is a rat basophilic leukemia (RBL) cell line (25). The Lck-deficient Jurkat clone JCaM1.6 and the Lck-reconstituted JCaM1.6 cells (26) were generously provided by Dr A. Weiss (Howard Hughes Medical Institute, San Francisco, CA). All cells were grown in RPMI 1640 media (Gibco, Paisley, UK) supplemented with 10% FCS (Integro, Dieren, The Netherlands) and antibiotics.

Antibodies

Monoclonal mouse IgG1 antibodies directed against human LAIR-1, 8A8 and DX26, have been described before (4). Monoclonal IgE anti-TNP was generously provided by Professor Dr L. Aarden (Sanquin Research, Amsterdam, The Netherlands). Goat anti-mouse F(ab)_2 fragments were purchased from Southern Biotechnology Associates (Birmingham, AL). Monoclonal anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal rabbit anti-SHP-1 (C19), anti-SHP-2 (C18) and anti-Lyn antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For western blot analysis of whole-cell lysates, monoclonal anti-SHP-2 antibody was used (BD Transduction Laboratories, Franklin Lakes, NJ). Polyclonal anti-Lck antibody was a generous gift of Dr J. Borst (Dutch Cancer Institute, Amsterdam, The Netherlands). For western blot analysis horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Denmark) and HRP-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL) were used.

cDNA constructs and transfectants

cDNA encoding for human LAIR-1a was cloned into the pCDNA3.1/zeo vector (Invitrogen, Breda, The Netherlands). Tyr→Phe mutations were introduced at Y253 and Y283 (located in the first and second ITIM respectively) by PCR-based mutagenesis. The sequence of the constructs was confirmed by automated DNA sequencing. To generate stable transfectants expressing wild-type or mutant LAIR-1, cells were transfected by electroporation. Stable transfectants were selected in 50 μg/ml zeocin (Invitrogen, Carlsbad, CA). RBL transfectants were subsequently cloned by the limiting dilution method. To assess the expression levels of the LAIR-1 mutants on RBL clones and YT.2C2 cell lines the cells were stained with DX26 anti-LAIR-1 antibody and phycocerythrin (PE)-conjugated goat anti-mouse IgG, and measured by flow cytometry.

Human Lck cDNA (27), cloned into the pmT2 expression vector, was generously provided by Dr J. Borst (Dutch Cancer Institute, Amsterdam, The Netherlands).

Cytotoxicity assay

721.221 cells stably expressing FcγRII were labeled with 51Cr and used in a 4-h cytotoxicity assay as described (28). To engage LAIR-1 mutants, 10 μg/ml 8A8 anti-LAIR-1 antibody was added. The maximum release was determined by lysing target cells with 10% Triton X-100. The percentage of specific lysis was calculated as: [(c.p.m. specific 51Cr release ± c.p.m. spontaneous 51Cr release)/(c.p.m. maximum 51Cr release ± c.p.m. spontaneous 51Cr release)] × 100. Data are expressed as the mean of triplicate cultures.

Degranulation assay

The assay to measure the extent of degranulation of RBL clones was adapted from Yamashita et al. (29). Briefly, RBL cells transfected with LAIR-1 or mutant molecules were coated with anti-TNP-IgE and 10 μg/ml 8A8 anti-LAIR-1 IgG or anti-TNP-IgE alone at 37°C for 20 min. Cells were washed and 1.5 × 10^6 cells were incubated with 10 ng/ml BSA-TNP in 150 μl RPMI with 1% FCS in the presence or absence of 10 μg/ml goat anti-mouse F(ab)_2 fragments at 37°C for 1 h. The culture supernatant was incubated with an equal volume of 5 mg/ml p-nitrophenyl-β-D-glucuronide (Sigma, St Louis, MO) in 1 M acetate buffer, pH 4, for 4 h. Then 60 μl of the reaction was added to 140 μl 250 mM glycine buffer, pH 11.5, containing 1% SDS and the absorbance at 405 nm was measured. Measurements were performed using triplicate cultures. Spontaneous release was determined by adding BSA instead of BSA-TNP to the primed cells. The percentage of inhibition of degranulation by LAIR-1 mutants was calculated as: percentage of inhibition = [(OD_{405} without LAIR-1 cross-linking – OD_{405} with LAIR-1 cross-linking)/(OD_{405} without LAIR-1 cross-linking – OD_{405} spontaneous release)] × 100.

LAIR-1 cross-linking and pervanadate treatment

For phosphorylation studies, cells were washed twice in PBS and 15 × 10^6 cells were incubated with 50 μM pervanadate in 3°C for 1 h. The culture supernatant was incubated with an equal volume of 5 mg/ml p-nitrophenyl-β-D-glucuronide (Sigma, St Louis, MO) in 1 M acetate buffer, pH 4, for 4 h. Then 60 μl of the reaction was added to 140 μl 250 mM glycine buffer, pH 11.5, containing 1% SDS and the absorbance at 405 nm was measured. Measurements were performed using triplicate cultures. Spontaneous release was determined by adding BSA instead of BSA-TNP to the primed cells. The percentage of inhibition of degranulation by LAIR-1 mutants was calculated as: percentage of inhibition = [(OD_{405} without LAIR-1 cross-linking – OD_{405} with LAIR-1 cross-linking)/(OD_{405} without LAIR-1 cross-linking – OD_{405} spontaneous release)] × 100.
PBS at 37°C for 15 min. For LAIR-1 cross-linking on Jurkat cell lines, cells were washed in PBS twice and incubated with 50 µg/ml 8A8 in PBS at 10^7 cells/100 µl on ice for 30 min. The cells were washed to remove excess antibody and incubated with 10 µg/ml goat anti-mouse F(ab)_2 fragments at 37°C for 2 min. Alternatively, the cells were left on ice in PBS without antibodies. The cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40 and 0.02% sodium azide) supplemented with protease inhibitors (Complete Mini EDTA-free protease inhibitor cocktail tablets; Roche, Mannheim, Germany), 1 mM phenylmethylsulfonyl fluoride and 50 µM pervanadate.

For SHP-1 and SHP-2 recruitment studies, YT.2C2 cells and transfectants were washed in serum-free medium, and incubated with 50 µg/ml 8A8 in PBS at 10^7 cells/100 µl on ice for 30 min. The cells were transferred to a 37°C water bath for 1 min and immediately washed with ice-cold PBS containing 50 µM pervanadate. RBL cells (30 x 10^6) cells and transfectants were treated with pervanadate as described above. The cells were lysed in Triton lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 and 0.02% sodium azide) supplemented with protease and phosphatase inhibitors. Cell lysates were cleared by centrifugation and used for immunoprecipitation as described below.

**Co-expression studies**

293T epithelial cells were seeded in six-well plates, and transfected with both 0.8 µg/well wild-type LAIR-1 or mutant LAIR-1 and 0.8 µg/well pMT2-Lck or empty expression construct, using FuGENE 6 (Roche) as transfection agent. After 24 h of incubation, the cells were washed with PBS once and lysed in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors as described above. Where indicated, the cells were treated with 50 µM pervanadate at 37°C for 15 min. Cell lysates were subjected to immunoprecipitation as described below. Aliquots of cell lysates were taken for the analysis of Lck expression by western blotting.

**Immunoprecipitation and western blot analysis**

For immunoprecipitation Protein A/G PLUS-agarose beads (Santa Cruz Biotechnology) were coated with DX26 anti-LAIR-1 antibody. Immunoprecipitation was performed overnight or for 90 min (SHP-1 and SHP-2 recruitment studies) in the presence of 0.5% BSA. Immune complexes were washed with NP-40 lysis buffer or Triton wash buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 0.02% sodium azide) supplemented with 1 mM phenylmethylsulfonyl fluoride and 250 µM sodium orthovanadate 5 times, and boiled in non-reducing Laemmli sample buffer. For the analysis of the expression of Src family kinases, SHP-1 and SHP-2 in 293T, Jurkat, YT.2C2 and RBL cells, the cells were washed with PBS, and lysed by boiling in Laemmli sample buffer. Proteins were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blot analysis was performed with anti-phosphotyrosine, 8A8 anti-LAIR-1, anti SHP-1, anti-SHP-2, anti-Lck or anti-Lyn antibodies followed by HRP-linked secondary antibodies. Proteins were detected by enhanced chemiluminescence (Amersham, Little Chalfont, UK).

**Results**

The N-terminal ITIM of LAIR-1 is required for the inhibition of cytotoxic activity of NK cells

Many studies have indicated an important role for ITIM in the inhibitory function of ITIM-bearing receptors (2,3). Both ITIM of LAIR-1 are necessary for the recruitment of SHP-1 in 293T cells (12), but the contribution of each ITIM to the inhibitory function in immune cells is not known. Therefore, we investigated the requirement of LAIR-1 ITIM to the inhibition of the cytotoxic activity of YT.2C2 cells and the degranulation of basophilic cells.

Triggering of LAIR-1 by addition of mAb to LAIR-1 transfectants of the NK cell line YT.2C2 inhibits the cytotoxic activity towards FcγRII-bearing target cells (6) (Fig. 1B, upper panels). We created mutants of LAIR-1, which contained Tyr→Phe mutations in the N-terminal ITIM (LAIR-1-FY), the C-terminal ITIM (LAIR-1-YF) or both ITIM (LAIR-1-FF). When stably transfected into YT.2C2 cells, these mutants were expressed at the cell surface at similar levels as wild-type LAIR-1 (Fig. 1A), which is comparable to the LAIR-1 expression levels on primary NK cells (4). All transfectants showed cytotoxic activity, although the efficacy of target cell lysis varied, probably due to the oligoclonal nature of the cell lines (Fig. 1B). Cross-linking of LAIR-1 molecules that were mutated in the N-terminal ITIM (i.e. LAIR-1-FY and LAIR-1-FF) did not lead to an inhibition of the cytotoxic activity of YT.2C2 transfectants. In contrast, the LAIR-1-YF mutant, that still contained an intact N-terminal ITIM, did inhibit cytotoxic activity upon cross-linking, although less effectively than wild-type LAIR-1 (Fig. 1B). These results show that the N-terminal ITIM of LAIR-1 is required for the inhibition of cytotoxic activity in YT.2C2 cells. The C-terminal ITIM does not function individually, but is necessary for full inhibitory action of LAIR-1 in YT.2C2 cells.

Mutants of LAIR-1 containing only one functional ITIM retain partial inhibitory capacity in RBL-2H3 cells

We next investigated whether LAIR-1 could inhibit signaling mediated by the high-affinity IgE receptor FcεRI, a well-defined ITAM-bearing receptor. We transfected RBL-2H3 cells with either wild-type human LAIR-1 or LAIR-1 containing mutated ITIM, as described above. Each mutant was stably expressed at the cell surface at a similar level as wild-type LAIR-1 (Fig. 2A). Incubation of the transfectants with anti-TNP-IgE, and subsequent triggering with TNP-conjugated BSA, led to the degranulation of the cells and the release of β-glucuronidase. Cross-linking of stably transfected LAIR-1 with mAb and anti-mouse Ig on an RBL clone resulted in an inhibition of degranulation of ~75% (Fig. 2B). In contrast, anti-LAIR-1 antibodies had no effect on the degranulation of non-transfected RBL-2H3 cells (data not shown). Thus, LAIR-1 was able to inhibit activation of RBL-2H3 cells via FcεRI. Mutation of both ITIM of LAIR-1 abolished the inhibitory effect on the degranulation (Fig. 2B, LAIR-1-FF), indicating that the ITIMs are required for inhibition. In contrast to YT.2C2 cells, mutation of either the N-terminal ITIM (LAIR-1-FY) or the C-terminal ITIM (LAIR-1-YF) alone resulted in a decrease, but not in a complete loss, of inhibitory function (Fig. 2B). Thus, LAIR-1 molecules containing only one functional ITIM are still partially...
Both ITIM of LAIR-1 contribute to full inhibition

Fig. 1. The N-terminal ITIM of LAIR-1 is required for inhibition of cytotoxic activity of NK cells. (A) Expression of LAIR-1 on YT.2C2 cells stably transfected with either wild-type LAIR-1 or LAIR-1 containing Tyr → Phe mutations in the N-terminal ITIM (FY), the C-terminal ITIM (YF) or both ITIM (FF). Non-transfected YT.2C2 cells were taken as a control. The cells were stained with DX26 anti-LAIR-1 antibody and PE-conjugated goat anti-mouse IgG (solid histogram) or PE-conjugated goat anti-mouse IgG alone (open histogram) and measured by flow cytometry. (B) YT.2C2 cells and YT.2C2 cells expressing wild-type or mutant LAIR-1 were assayed for lysis of 721.221 cells stably transfected with the human FcγRII at different E:T ratios in the absence (circles) or presence (squares) of 10 μg/ml 8A8 anti-LAIR-1 antibody. Data are representative of four independent experiments.
active, while both ITIM are required for full inhibition of degranulation. This indicates that the relative contribution of each ITIM to the inhibitory function of LAIR-1 differs between experimental systems.

Both ITIM of LAIR-1 are required for optimal phosphorylation

The different abilities of LAIR-1 mutants to inhibit cell activation raises the question whether this is the result of a phosphorylation difference between the two ITIM. Therefore we investigated whether the LAIR-1 mutants could be phosphorylated in YT.2C2 cells. We treated the cells with pervanadate as wild-type LAIR-1 becomes extensively phosphorylated upon pervanadate treatment (12,13) (Fig. 3A). Interestingly, the mutant that still contained the N-terminal tyrosine residue was phosphorylated while phosphorylation of the single mutant that only contained the C-terminal tyrosine residue was barely detectable (Fig. 3A). This corresponds to the relative ability of these mutants to inhibit the cytotoxic activity of YT.2C2 cells. Although the LAIR-1 mutant that only contained the C-terminal tyrosine was poorly phosphorylated, this tyrosine was most likely phosphorylated in wild-type LAIR-1. Wild-type LAIR-1 ran at a higher mol. wt in SDS-PAGE than the LAIR-1-YF mutant after pervanadate treatment (Fig. 3A, lower panel), suggesting that both tyrosine residues are phosphorylated in wild-type LAIR-1. These results suggest that phosphorylation of the N-terminal ITIM may be required for the phosphorylation of the C-terminal ITIM.

In RBL cells, in which both LAIR-1 mutants with one functional ITIM can still inhibit cell function, both mutants were phosphorylated, but to a much lesser extent than wild-type LAIR-1 (Fig. 3B). This is in contrast to the observation in YT.2C2 cells, although again the N-terminal ITIM was phosphorylated more efficiently than the C-terminal ITIM.

Lck is required for SHP-1 recruitment by LAIR-1 in Jurkat cells

The kinase responsible for the initial tyrosine phosphorylation of LAIR-1 is not known. In a yeast two-hybrid system, LAIR-1 can be phosphorylated by Lck (23). We therefore investigated whether Lck is involved in the phosphorylation of LAIR-1 in Jurkat cells. As SHP-1 recruitment is associated with LAIR-1 phosphorylation (4,12,13), we compared the binding of SHP-1 to LAIR-1 in Jurkat cells and the Lck-deficient Jurkat cell line JCaM1.6 (26). Both cell lines showed equal LAIR-1 cell-surface expression (data not shown). SHP-1 was constitutively associated with LAIR-1 in wild-type Jurkat cells (Fig. 4). The association did not increase upon LAIR-1 cross-linking. These
data are consistent with observations by Sathish et al., who also reported constitutive LAIR-1 phosphorylation and SHP-1 association in Jurkat cells (23). In JCaM1.6 cells, association of SHP-1 with LAIR-1 was not detectable and SHP-1 binding was restored in JCaM1.6 in which Lck is reintroduced (Fig. 4). These results suggest that in Jurkat cells Lck is required for the phosphorylation of LAIR-1 and the subsequent recruitment of SHP-1.

**Lck requires the N-terminal ITIM for the phosphorylation of LAIR-1**

In both YT.2C2 transfectants and RBL clones, the mutant of LAIR-1 that contains only a functional N-terminal ITIM is more extensively phosphorylated than the mutant that contains only a functional C-terminal ITIM (Fig. 3). This raises the question whether Src family kinases may preferentially phosphorylate the N-terminal ITIM. To address this question we co-expressed wild-type or mutant LAIR-1 with Lck in 293T cells, which do not express endogenous Lck (Fig. 5B, upper panel). As shown in Fig. 5A, both wild-type LAIR-1 and single mutants can be phosphorylated upon pervanadate treatment when transfected in 293T cells, probably by an endogenous Src family kinase that can be inhibited by PP1 (12). As in YT.2C2 cells and RBL clones, the phosphorylation of the mutants is less efficient. Co-expression of Lck resulted in constitutive phosphorylation of wild-type LAIR-1 (Fig. 5A). Interestingly, the mutant that contains only a functional N-terminal ITIM was phosphorylated upon co-expression of Lck, while the mutant lacking this ITIM was not detectably phosphorylated. However, both ITIM are required for maximal phosphorylation of LAIR-1. Thus, the N-terminal ITIM of LAIR-1 may be phosphorylated first and subsequently the C-terminal ITIM.

As LAIR-1 is phosphorylated in 293T cells upon pervanadate treatment in the absence of Lck, another kinase must be able to phosphorylate LAIR-1 as well. Several groups have indicated Lyn as a kinase that is involved in the phosphorylation of ITIM-bearing receptors (17–19). Jurkat cells express Lck, whereas 293T, YT.2C2 and RBL cells express Lyn (Fig. 5B).

**Both ITIM of LAIR-1 are required for the recruitment of SHP-1, but not for SHP-2, recruitment in YT.2C2 cells**

Wild-type LAIR-1 becomes phosphorylated after stimulation with mAb (12) and data not shown). We therefore investigated whether LAIR-1 and the LAIR-1 mutants associate with SHP-1 and SHP-2 in YT.2C2 transfectants after antibody stimulation. Although binding of SHP-2 to LAIR-1 has been a matter of debate (12), we found that wild-type LAIR-1 recruited both SHP-1 and SHP-2 (Fig. 6A and B). Mutation of both ITIM, which results in abrogation of the inhibitory function of LAIR-1, abolished recruitment of both SHP-1 and SHP-2.

Mutants containing only one functional ITIM did not recruit SHP-1. However, SHP-2 was recruited to the mutant that contained a functional N-terminal ITIM, which was still capable of inhibition of cytotoxic activity of YT.2C2 cells. Thus, whereas both ITIM of LAIR-1 are required for the recruitment of SHP-1, SHP-2 can be recruited to the single N-terminal ITIM in YT.2C2 cells.

**The N-terminal ITIM is sufficient for phosphatase recruitment in RBL cells**

In contrast to YT.2C2 cells, both single mutants of LAIR-1 were partially effective in RBL cells. We therefore investigated whether this is reflected by the recruitment of phosphatases to the mutant molecules in these cells. As the phosphorylation of LAIR-1 mutants in RBL cells is limited (Fig. 3B), we decided to
accumulated phosphorylated molecules by pervanadate treatment. Upon pervanadate treatment both wild-type and the mutant containing a functional N-terminal ITIM recruited SHP-2, whereas the mutant lacking the N-terminal ITIM failed to bind SHP-2 (Fig. 6B). Surprisingly, the mutant containing only a functional N-terminal ITIM also recruited SHP-1 in these cells, although much less than wild-type LAIR-1. This is in contrast to the lack of SHP-1 recruitment by this mutant in YT.2C2 cells (Fig. 6A) and 293T cells (12). Western blot analysis showed that RBL cells have a higher expression of SHP-1 compared to SHP-2 than YT.2C2 cells (Fig. 6C). Thus, the recruitment of SHP-1 by the mutant that contains only a functional N-terminal ITIM in RBL cells may be a consequence of a relative high abundance of this phosphatase. We did not detect binding of SHP-1 to the mutant lacking the N-terminal ITIM (Fig. 6B).

Discussion

Here we show that LAIR-1-mediated inhibition of immune responses depends on the presence of functional ITIM and that both ITIM contribute differently to the inhibitory function. We investigated the functional requirement of LAIR-1 ITIM using two different cell lines. Wild-type LAIR-1 inhibited the cytotoxic activity of human YT.2C2 NK cells as well as the degranulation of RBL cells. LAIR-1-mediated inhibition was abolished when the tyrosine residues of both ITIM were mutated to phenylalanine residues. Thus, unlike CD5 and FcγRIIB, which contain domains outside an ITIM that are involved in the inhibition of cell activation pathways (10,11), LAIR-1-mediated inhibition requires at least one functional ITIM.

Interestingly, the mutants containing only one functional ITIM behave differently in the two cell types studied. In the NK cell-like YT.2C2 cells, a mutant lacking the N-terminal ITIM is not effective in inhibition of cell function at all, while in the RBL cells both mutants are still partially able to inhibit degranulation. This could be due to differences in the activation signal that needs to be inhibited. YT.2C2 cells lyse susceptible target cells in a manner that requires CD28 and lymphocyte function-associated antigen-1, but for which the primary activation signal is not known (30). RBL-2H3 is a RBL cell line that degranulates upon cross-linking of the FcεRI, an ITAM-bearing receptor for which the signaling pathway has been well defined (31). Alternatively, there might be differences in the kinases that phosphorylate the ITIM in RBL versus YT.2C2 cells. In the YT.2C2 cells, the LAIR-1 mutant that contains only a functional C-terminal ITIM is not detectably phosphorylated, while in RBL cells both mutants still can be phosphorylated, although to a much lesser extent than wild-type LAIR-1. This might result in different recruitment of downstream signaling molecules. However, the different inhibitory capacity of the mutants in the different cell lines cannot be explained by SHP-1 or SHP-2 recruitment, as we did not find recruitment of these phosphatases by the mutant lacking the N-terminal ITIM in either cell line.

In 293T cells, phosphorylation of LAIR-1 and the subsequent recruitment of SHP-1 is inhibited by the Src family kinase inhibitor PP1 (12), suggesting that LAIR-1 is phosphorylated by a Src family kinase. One Src family kinase that may phosphorylate LAIR-1 is Lck. In a yeast two-hybrid system, Lck has been shown to phosphorylate LAIR-1 and induce association with SHP-1 (23). In addition, Lck is required for the phosphorylation of ITIM of killer cell Ig-like receptor (KIR) and Ig-like transcript-2 in Jurkat cells (14,15). Our finding that SHP-1 recruitment to LAIR-1 is not detectable in Lck-deficient Jurkat cells supports the hypothesis that LAIR-1 is indeed phosphorylated by Lck. Furthermore, co-expression of Lck and LAIR-1 mutants in 293T cells resulted in the phosphorylation of both wild-type LAIR-1 and a mutant containing only the N-terminal ITIM. This corresponds to the phosphorylation of these molecules in pervanadate-treated YT.2C2 transfectants, suggesting that also in these cells a Src-family kinase is responsible for LAIR-1 phosphorylation. Other Src family kinases may be involved in LAIR-1 phosphorylation in different cell types, as co-expression of Src and Fyn also resulted in the phosphorylation of wild-type LAIR-1 in 293T cells (data not shown).

In YT.2C2 cells, wild-type LAIR-1 is phosphorylated on both ITIM, as it runs at a higher mol. wt than the mutant containing only the N-terminal ITIM and it recruits SHP-1, which requires both ITIM. This suggests that the N-terminal ITIM is phosphorylated first, and that phosphorylation of the C-terminal ITIM depends on the N-terminal ITIM and perhaps is mediated by another kinase. Thus, when only a functional C-terminal ITIM is present, LAIR-1 may not be sufficiently phosphorylated.
to inhibit the cytotoxic activity of YT.2C2 cells. In contrast, the
N-terminal ITIM of LAIR-1 was sufficient for SHP-2 recruitment.
Thus, SHP-2 might mediate the inhibitory effect of this mutant
in YT.2C2 cells. We cannot exclude, however, that another, yet
unidentified, protein is involved.

In RBL cells, the mutant containing only the N-terminal ITIM
recruited both SHP-1 and SHP-2, while no SHP-1 recruitment
was found in YT.2C2 cells. However, compared to the
recruitment of SHP-2, the recruitment of SHP-1 by this mutant
was very inefficient. By western blot analysis of the expression
of both phosphatases in the cell lines, we found a much
stronger signal for SHP-1 in RBL cells than in YT.2C2 cells.
This result suggests that RBL cells have a relatively high
expression of SHP-1. This could explain why the mutant
containing only the N-terminal ITIM was capable of recruiting
SHP-1 in RBL cells, while it failed to do so in YT.2C2 cells.
Thus, whereas SHP-2 can bind to a single N-terminal ITIM,
SHP-1 requires both ITIM and binds only very inefficiently to a
single ITIM.

Interestingly, the sequence of the N-terminal ITIM of LAIR-1,
VTYAQL, is conserved in several ITIM-bearing receptors (32-
36). Of these receptors, the KIR most closely resemble LAIR-1.
KIR contain two ITIM, of which the N-terminal ITIM has the
VTYAQL sequence, and recruit SHP-1 and SHP-2. Both ITIM
are required for the recruitment of SHP-1 (37,38). However,
whereas a KIR mutant containing only the C-terminal ITIM is no
longer effective, a KIR mutant containing only the N-terminal
ITIM still recruits SHP-2 (39) and has inhibitory capacity
(37,38). Recently, Yusa et al. described that SHP-2 is recruited
to single VTYAQL ITIM of KIR2DL4 and KIR2DL3, and that this
is sufficient for inhibitory signaling (39,40). Similarly, the
VTYAQL ITIM of gp49B and PIR-B seem to contribute most
to the inhibitory effect, but are neither sufficient nor absolutely
required (29,41,42). We therefore postulate that this con-
served sequence serves as a basal ITIM that recruits SHP-2
and has inhibitory capacity, but whose function may be
extended by the presence of other ITIM.

The mutant lacking the N-terminal ITIM is partially effective
in RBL cells and is phosphorylated in 293T cells, indicating
that the C-terminal ITIM of LAIR-1 may be phosphorylated
when sufficient kinase activity is present. However, in RBL
cells the phosphorylation of the mutants containing only one
functional ITIM is very inefficient compared to wild-type LAIR-
1, which may indicate that little phosphorylation is required for
inhibitory function in these cells. Limited phosphatase recruit-
ment may be sufficient to inhibit a cellular response, when the
phosphatases are recruited closely to the receptor mediating
the activation signal. Recently, SHP-1 was shown to localize in
the center of the contact area between NK cells and resistant
target cells (43). In a similar way, LAIR-1/phosphatase
complexes may localize to those regions of the plasma
membrane where cell activation would normally occur. The
limited phosphorylation makes an investigation into the
phosphatases recruited by the mutant containing only the C-
terminal ITIM difficult. We did not detect recruitment of SHP-1
or SHP-2 by this mutant. Alternatively, LAIR-1 could mediate
inhibition of cell activation by recruiting another, yet uniden-
tified protein that binds to the mutant containing only the C-
terminal ITIM in RBL cells. Uehara et al. reported that PIR-B
contains an ITIM that does not recruit SHP-1, SHP-2 or SHIP,
yet exerts inhibitory effect in RBL cells (44). Thus, other
molecules may exist that mediate the inhibitory function of
ITIM-bearing receptors.

We conclude that although the N-terminal ITIM is the major
determinant in LAIR-1 signaling, both ITIM of LAIR-1 co-
operate in the recruitment of SHP-1 and SHP-2, resulting in
optimal inhibitory function.

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Abbreviations

HRP horseradish peroxidase
ITIM immunoreceptor tyrosine-based inhibitory motif
KIR killer cell Ig-like receptor
LAIR leukocyte-associated Ig-like receptor
PE phycoerythrin
RBL rat basophilic leukemia
SH2 Src homology 2 domain
SHIP SH2-containing phosphatase
SHIP2 -containing inositol phosphate 5'-phosphatase

References

Both ITIM of LAIR-1 contribute to full inhibition

receptor for cellular and viral MHC class I molecules. *Immunity* 7:273.


