Leukocyte-associated immunoglobulin-like receptor 1 inhibits T-cell signaling by decreasing protein phosphorylation in the T-cell signaling pathway

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Multiple observations implicate T-cell dysregulation as a central event in the pathogenesis of rheumatoid arthritis. Here, we investigated mechanisms for suppressing T-cell activation via the inhibitory receptor leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1). To determine how LAIR-1 affects T-cell receptor (TCR) signaling, we compared 1) T cells from LAIR-1–sufficient and –deficient mice, 2) Jurkat cells expressing either LAIR-1 mutants or C-terminal Src kinase (CSK) mutants, and 3) T cells from mice that contain a CSK transgene susceptible to chemical inhibition. Our results indicated that LAIR-1 engagement by collagen or by complement C1q (C1Q, which contains a collagen-like domain) inhibits TCR signaling by decreasing the phosphorylation of key components in the canonical T-cell signaling pathway, including LCK proto-oncogene SRC family tyrosine kinase (LCK), LYN proto-oncogene SRC family tyrosine kinase (LYN), ζ chain of T-cell receptor–associated protein kinase 70 (ZAP-70), and three mitogen-activated protein kinases (extracellular signal–regulated kinase, c-Jun N-terminal kinase 1/2, and p38). The intracellular region of LAIR-1 contains two immunoreceptor tyrosine-based inhibition motifs that are both phosphorylated by LAIR-1 activation, and immunoprecipitation experiments revealed that Tyr-251 in LAIR-1 binds CSK. Using CRISPR/Cas9-mediated genome editing, we demonstrate that CSK is essential for the LAIR-1–induced inhibition of the human TCR signal transduction. T cells from mice that expressed a PP1 analog–sensitive form of CSK (CskAS) corroborated these findings, and we also found that Tyr-251 is critical for LAIR-1’s inhibitory function. We propose that LAIR-1 activation may be a strategy for controlling inflammation and may offer a potential therapeutic approach for managing autoimmune diseases.

Inhibitory receptors serve as a critical counterbalance to hold the immune system in check and protect against tissue damage induced by hyperactive immune responses or autoimmune dysfunction. Although cells may express multiple inhibitory receptors, some individual receptors are important in the regulation of particular immune functions. One such receptor is leukocyte-associated Ig-like receptor 1 (LAIR-1)² or CD305. Human LAIR-1 was first cloned in 1997; its structure was delineated, and its function as a regulator of NK cells was established (1). LAIR-1 contributes to the regulation of the immune system by binding to extracellular ligands and delivering a signal to immune cells. The ligands to which LAIR-1 binds are collagenous structures. Multiple collagens can serve as functional, high-affinity ligands for LAIR-1, including transmembrane collagens, extracellular matrix collagens, and the collagenous portion of the complement protein C1q. Structurally, LAIR-1 has an extracellular binding domain coupled to a cytoplasmic tail containing two immunoreceptor tyrosine-based inhibition motifs (ITIMs). When LAIR-1 is activated by binding to a collagenous ligand, both of the ITIMs are phosphorylated, and additional downstream events are triggered.

We have previously shown that activation of LAIR-1 can prevent collagen-induced autoimmune arthritis (CIA) (2). The precise mechanism of this activity is unknown, but immune regulation is critical. Although LAIR-1 is found on multiple immune cells, T cells were selected as the focus of this study because both Th1 and Th17 CD4⁺ T cells play a prominent role in the initiation of systemic immune responses in CIA. It has previously been shown that a subset of T cells, including naïve T cells, express high levels of LAIR-1 (3). It is also known that LAIR-1 down-regulates T-cell receptor (TCR) signal transduction, but there is no comprehensive understanding of the mechanism by which LAIR-1 exerts this activity. This study explores the mechanism by which LAIR-1 modifies T-cell function and prevents signaling through the TCR.

To accomplish these experiments, we utilized several transgenic mouse strains. The DR1 strain is a partially humanized strain developed to study experimental autoimmune arthritis

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2 The abbreviations used are: LAIR-1, leukocyte-associated immunoglobulin-like receptor 1; IL, interleukin; TCR, T-cell receptor; CSK, C-terminal Src kinase; ERK, extracellular signal–regulated kinase; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; ITIM, immunoreceptor tyrosine-based inhibition motif; CIA, collagen-induced autoimmune arthritis; 3-IB-PP1, 3-iodo-benzyl-PP1; α1(II), α chain of type I collagen; α1(II), α chain of type II collagen; Th, T-helper; RA, rheumatoid arthritis.
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(4). It has a C57Bl/6 background with a chimeric human/mouse immune response transgene and is highly susceptible to CIA. We have bred two variants of this strain, one of which is also deficient in LAIR-1 (2). We also used a mouse expressing a mutant form of C-terminal Src kinase (Csk\textsuperscript{AS}). Csk is the primary negative regulator of Src-family kinases (5). Csk\textsuperscript{AS} mice express a conditionally inhibitory CSK protein and lack endogenous CSK expression. Csk\textsuperscript{AS} catalytic activity is specifically and rapidly inhibited by 3-iodo-benzyl-PP1 (3-IB-PP1).

Collagen exists in tissue as a trimer that is extensively cross-linked. During inflammation, collagen is degraded releasing fragments of the component polypeptide chains. Both the collagen peptides and C1q are present in areas of inflammation including inflammation resulting from arthritis. These denatured fragments serve as the natural ligands for LAIR-1. In the experiments reported here, we used denatured \( \alpha \) chains of type I collagen (\( \alpha 1(II) \)), type II collagen (\( \alpha 1(II) \)), and intact C1q to engage LAIR-1. We examined both the resulting surface expression of LAIR-1 and the deactivation of critical components of the canonical T-cell signaling pathway. Individual cytoplasmic tyrosine residues in the ITIMs were modified to determine which are necessary for LAIR-1 suppression of the TCR signaling. Based on the results of these experiments, we believe that therapies directed toward stimulating LAIR-1 or augmenting the intracellular pathways used by LAIR-1 hold promise for down-regulating unwanted inflammation (6) and that this natural inhibitory molecule can be exploited to suppress the tissue injury occurring in autoimmune arthritis.

**Results**

**Inhibition of murine T cell signaling using \( \alpha 1(II) \)**

Initial experiments used spleen cells isolated from DR1 mice. Naive CD4\textsuperscript{+} T cells were isolated by negative selection from the spleens of DR1 LAIR-1–sufficient and –deficient mice. The purified T cells were pretreated with \( \alpha 1(II) \) to stimulate the LAIR-1 and were subsequently activated with antibodies to CD3 (\( \alpha \)-CD3). Proteins were collected from whole-cell lysates to evaluate for phosphorylation of the Src family tyrosine kinases Lck and Lyn. Early activation of these kinases is an early step in the canonical T-cell signaling pathway. CD3 stimulation induced phosphorylation of both Lck and Lyn in WT cells, whereas LAIR-1 engagement with \( \alpha 1(II) \) inhibited their activation. On the other hand, CD3 stimulation led to phosphorylation of both Lck and Lyn in cells lacking LAIR-1, regardless of the presence or absence of \( \alpha 1(II) \) (Fig. 1). Downstream events, specifically the activation of Zap-70, ERK1/2, JNK-2, and p38, were evaluated in a similar manner. CD4\textsuperscript{+} T cells pretreated with \( \alpha 1(II) \) and stimulated with \( \alpha \)-CD3 experienced a significant reduction in phosphorylation of Zap-70, ERK1/2, JNK-2, and p38 compared with cells not pretreated with \( \alpha 1(II) \). This inhibition depends upon the presence of LAIR-1 because the LAIR-1–deficient cells were not affected by treatment with \( \alpha 1(II) \).

**LAIR-1 in human T cells**

Having established the ability of LAIR-1 to attenuate murine T-cell receptor signaling, we next tested the effect of LAIR-1 stimulation using human T cells. T cells from Hut78, a human T-cell lymphoma cell line, were cultured overnight with a mAb recognizing human LAIR-1 or an isotype control and evaluated by flow cytometry for the surface expression of LAIR-1. The protein level of LAIR-1 on the surface of the cell was significantly up-regulated by culture with the stimulatory antibody to LAIR-1 (Fig. 2A). In a second set of experiments, Jurkat cells overexpressing human LAIR-1 were treated with either \( \alpha 1(II) \) or C1q for 5–30 min and then analyzed for the phosphorylation status of LAIR-1 as an indication of LAIR-1 activation. By 5 min after exposure of these cells to either ligand, there was significant activation of LAIR-1 (Fig. 2B). These results demonstrate that LAIR-1 can be up-regulated on the cell surface and triggered by engagement with collagenous proteins. To establish how LAIR-1 affects TCR signaling through the human canonical signaling pathway, Jurkat T cells overexpressing LAIR-1 were pretreated overnight with \( \alpha 1(II) \) and stimulated with a combination of \( \alpha \)-CD3 and \( \alpha \)-CD28. The data showed that engagement of LAIR-1 by \( \alpha 1(II) \) reduces phosphorylation of Zap-70 and the MAP kinases (ERK1/2, JNK, and p38) compared with controls stimulated by \( \alpha \)-CD3 and \( \alpha \)-CD28 without treatment with \( \alpha 1(II) \) (Fig. 3). These results are similar to those observed in murine CD4\textsuperscript{+} T cells and confirm that engagement of LAIR-1 leads to a significant suppression of T-cell signaling in both murine and human T cells.
Tyrosine phosphorylation of ITIMs in LAIR-1 is required for LAIR-1–mediated inhibition of TCR signaling in T cells

The human LAIR-1 receptor contains two ITIM modules in the cytoplasmic tail (7). The presence of these ITIM receptor subunits suggests that they are critical for the regulation of signal transduction pathways in T cells. ITIMs typically contain conserved tyrosine residues that regulate the receptor-linked signal transduction pathways in a negative fashion. To investigate whether tyrosine residues in ITIMs are critical for LAIR-1 function and to clarify which tyrosine is required for the inhibition of the T cell, we developed mutant LAIR-1 constructs in which individual tyrosine residues were replaced by phenylalanine. Jurkat cells that express LAIR-1 empty control or deletion mutants of human LAIR-1 were activated by anti-LAIR-1. Western blotting analysis confirmed the presence of LAIR-1 in the FLAG-tagged and LAIR-1 stop cell lines. The data shown are representative of three separate analyses. IB, immunoblot.

C-terminal Src kinase binds to the phosphorylated Tyr-251 in LAIR-1

LAIR-1 engages other molecules that in turn intersect with the TCR signaling pathway. Csk is a molecule implicated in the regulation of immune cells bearing ITIM-containing receptors (8). Therefore, we determined whether activation of LAIR-1 initiated recruitment of Csk to the LAIR-1 ITIMs. To that end, Jurkat cells expressing various LAIR-1 mutants were activated with collagen, and immunoprecipitation of LAIR-1 was performed.
to verify the presence of Csk in the LAIR-1 receptor signaling complex. Csk co-precipitated with activated LAIR-1, whereas replacement of the Tyr-251 residue of LAIR-1 completely abrogated Csk binding. Replacement of the Tyr-281 residue had no effect (Fig. 5). These data establish that Csk binds to the phosphorylated tyrosine residue 251 in LAIR-1 and suggest that Csk is involved in the regulation of the immune system by the ITIM bearing LAIR-1.

**LAIR-1–mediated inhibition of TCR signal transduction requires Csk**

It is known that Csk can be a negative regulator of basal and inducible receptor signaling (9), and from our previous experiments it was clear that Csk binds to phosphorylated LAIR-1. To address the necessity of Csk for LAIR-1 signaling, we used the CRISPR–Cas9 genome editing system to delete the Csk gene in Jurkat T cells. Several Csk haploid mutant cell lines were developed, and Western blotting and PCR analyses were used to detect protein or RNA for Csk in the resulting clones. Two Jurkat mutants, Csk PO-61 and Csk PO-101, had decreased amounts of Csk, compared with the control cell (Control CO-2) (Fig. 6A). These cells were selected for functional testing to determine whether TCR activation with anti-CD3 and anti-CD28 in the presence or absence of the LAIR-stimulator α1(II) would alter the production of IL-2. As shown in Table 1, when
LAIR-1 was stimulated by α1(II), there was a significant decrease in cytokine production (IL-2) in the control cells, whereas both cells with the knockdown of Csk had no change in the secretion of IL-2. Similarly, overnight culture with α1(II) followed by TCR activation with anti-CD3 and anti-CD28 significantly decreased the phosphorylation of ZAP-70 in control cells, whereas the two cell lines with the knockdown of Csk showed no change in activation of ZAP-70 following TCR activation in the presence of α1(II) (Fig. 6B). These data demonstrate that Csk not only binds to the phosphorylated Tyr-251 residue of LAIR-1 but also is a critical molecule for the attenuation of the TCR signaling induced by LAIR-1 engagement.

**Discussion**

LAIR-1, also known as CD305, is an immune inhibitory receptor that regulates immune system balance and protects against tissue damage and autoimmune dysfunction (11). In this study, LAIR-1 engagement by α chains of collagen or C1q led to inhibition of TCR signaling and decreased activation levels of key components of the canonical T cell signaling pathway, including Lck, Lyn, Zap-70, and the three MAP kinase (ERK1/2, JNK1/2, and p38). Although both ITIMS of LAIR-1 can be activated, point mutants of LAIR-1 showed that only the first ITIM with Tyr-251 is essential for the LAIR-1 inhibitory function. Moreover, CRISPR–Cas9 genome editing demonstrated that the nonreceptor tyrosine protein kinase, Csk, bound Tyr-251 of LAIR-1 and was required for the LAIR-1–induced inhibition of the human TCR signal transduction. This finding was corroborated using CD4+ cells from Csk<sup>As</sup> transgenic mice in which inhibition of the Csk transgene abrogated the LAIR-1–mediated suppression.

Although LAIR-1 is found on almost all cells of the immune system and its inhibitory functions have been described in a variety of cellular systems, this study demonstrates that LAIR-1 is a major player in down-regulating TCR signaling in both human and murine T cells (6, 7, 11–14). These results are especially interesting because CD4<sup>+</sup> T cells, both Th1 and Th17, play a prominent role in the pathogenesis of autoimmune responses in autoimmune arthritis, because levels of LAIR-1 can be up-regulated on the surface of T cells. These data suggest that this inhibitory receptor can be used as a critical counterbalance to hold the immune system in check, when T cells become dysregulated (15).

The discovery that collagens are functional ligands for LAIR-1 (15, 16) disclosed a novel role for collagen in the regulation of immune function. α chains of type I or type II collagen and C1q were used to engage LAIR-1, and each could activate LAIR-1 in the absence of the other chains. Collagen is the most abundant protein in animals and is a key component of extracellular matrices, providing essential structural support for connective tissues. All collagens possess triple-helical regions, consisting of three polypeptide chains, that are characterized by repeating Gly-Xaa-Yaa sequences. Amino acids in positions Xaa and Yaa are often proline and 4-hydroxyproline, respectively (17). It has been found that GPHyp, the relatively common sequence in the triple-helical collagen domain, is recognized by LAIR-1, whereas GPP is not (18–20). Because each of the 18 polypeptide chains of C1q contains an N-terminal colla-
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Figure 6. Csk is essential for the suppression of TCR-induced ZAP-70 activation by LAIR-1. A, Jurkat cells, a control CRISPR (clone CO-2), or Csk CRISPR (clones PO-61 and PO-101) cells were examined for Csk protein expression by Western blotting analysis (left panel) or for Csk mRNA by RT-PCR (right panel). Actin was used as control. The data presented are representative of three separate analyses. B, Jurkat cells, a control CRISPR (clone CO-2), or Csk CRISPR (clones PO-61 and PO-101) cells were pretreated with medium (no) or type II collagen (α1(II)) overnight and then stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time period. Whole-cell lysates were collected and analyzed for activation of ZAP-70 by Western blotting analysis using anti–phospho-ZAP70 Tyr-493 (α-pZAP70) antibody. The membrane was stripped and reblotted with non–phospho-specific antibody for ZAP-70 (α-ZAP70). These data are representative of three separate analyses. C, CD4+ T cells from Csk KO mice were pretreated with either vehicle (no), PP1* (3-IB-PP1, 10 μM), α1(I) (200 μg/ml), or PP1* + α1(I) overnight and stimulated with anti-CD3 antibody (10 μg/ml) for 5 min. Whole-cell lysates were prepared and analyzed for activation of ZAP-70 by Western blotting analysis using an anti–pZAP70 Tyr-493 antibody. The membrane was stripped and reblotted with a non–phospho-specific antibody for ZAP-70 (α-ZAP70). In the absence of α-CD3, none of these agents were able to elicit T-cell activation (data not shown). The data shown are representative of three separate analyses.

Table 1
IL-2 concentrations

| Cell line          | +α-CD3 | +α-CD3 + BI | Media |
|--------------------|--------|-------------|-------|
| POCO2 control      | 296 ± 52 | 133 ± 43*  | <10   |
| PO61 Csk KO        | 375 ± 22 | 412 ± 35   | <10   |
| PO101 Csk KO       | 280 ± 15 | 285 ± 25   | <10   |

*p ≤ 0.05 when stimulation with CD3 + BI is compared with stimulation with α-CD3 alone.

Our data showing that LAIR-1 binds and utilizes Csk to down-regulate TCR signaling are similar to observations made with the B-cell receptor (27). A yeast trihybrid system and chick B cells were used to show that phosphorylated LAIR-1 bound Csk through its SH2 domain. Following activation of the N-terminal tyrosine in LAIR-1, B-cell receptor signaling was attenuated. Because chick DT40 B cells lack both SHP-1 and SHP-2, these data show that LAIR-1 can utilize Csk to suppress receptors without requiring any other SH2 domain–containing phosphatases (27), although in some circumstances LAIR-1 does bind SHP-1 (13). In the case of T cells, LAIR-1 receptor engagement activates its ITIMs, leading to the recruitment of Csk to the receptor site. Csk then delivers a negative signal to the ITAM-bearing TCR.

The general assumption is that lack of inhibition predisposes for autoimmune diseases. When testing was done in synovial fluids of RA patients, LAIR-1 expression was found to be decreased in circulating CD4+ T cells in RA patients compared with both OA patients and healthy individuals. Upon stimulation with TNFα, LAIR-1 expression decreased in Th1 and Th2 CD4+ T cells from healthy donors. Although some studies indicate that LAIR-1 can be elevated in CD14+ monocytes and local CD68+ macrophages in synovial tissues from RA patients, more work must be done to determine whether LAIR-1 may exert different functions on T cells and monocytes/macrophages (11). When antibodies that stimulate LAIR-1 were used therapeutically to treat autoimmunity in a murine model of arthritis (collagen-induced arthritis), the antibodies significantly suppressed arthritis if administered in vivo (2). Mice genetically deficient in the LAIR-1 receptor developed more severe arthritis than WT controls (2). These data suggest...
that LAIR-1 may be a potential therapeutic target for suppressing RA.

Manipulating LAIR-1 may be a strategy for regulating inflammation and disease activity in systemic lupus erythematosus as well. Son and Diamond (13) have demonstrated that C1q can engage LAIR-1 and suppresses the secretion of interferons in human monocytes. Moreover, an absence of C1q has been shown to lead to enhanced IFN production by both human and mouse pDCs. These findings suggest that C1q/LAIR-1 signaling mediates a major inhibitory pathway for the innate immune response and that the C1q collagen tail, which engages LAIR-1, inhibits TLR signaling. A recent report (28) showed that collagenous domains of C-type lectin surfactant protein D bind to LAIR-1 and regulate FcαR-mediated reactive oxygen production in neutrophils. These results suggest that LAIR-1 engagement by collagen-like domains may be a therapeutic strategy for controlling inflammation in autoimmune diseases such as RA, systemic lupus erythematosus, and many other inflammatory conditions (28).

Conclusion

Our study has elucidated the regulation and function of LAIR-1 on T-cell receptor activation. The data show that once LAIR-1 is triggered by its ligand, either collagen or C1q, both ITIMS are phosphorylated, leading to the binding of CSK to Tyr-251 of LAIR-1. This CSK recruitment then delivers a negative signal to the ITAMs of the T-cell receptor, causing a down-regulation of the canonical T-cell receptor signaling pathway. These data suggest that LAIR-1 could be a potential therapeutic target in autoimmune diseases, as well as other diseases with an immune component.

Experimental procedures

Preparation of cartilage-derived type II collagen (CII) and skin-derived type I collagen

Native type II collagen was solubilized from fetal calf articular cartilage and native type I collagen from bovine hides by limited pepsin digestion and purification as described earlier (29). The purified collagen was dissolved in cold 10 mM acetic acid at 4 mg/ml and stored frozen at −70 °C until used. In some experiments, type I collagen from Advanced Biomatrix (Carlsbad, CA) was used. α1(II) and α1(I) represent the constituent protein chains of bovine CII and CI, respectively, isolated by carboxymethyl-cellulose chromatography. C1q, which contains Gly-Xaa-Yaa sequences resembling collagen was purchased from Sigma–Aldrich.

Animals

C57BL/6 mice expressing the chimeric (human/mouse) DRB1*0101 construct were obtained from Taconic Biosciences, (Hudson, NY) and bred in the animal core facility of the Rheumatic Diseases Research Core Center, University of Tennessee. The chimeric DRB1*0101 construct has been previously described, as has the production of transgenic mice expressing this construct (4). Mice transgenic for a CII-specific TCR in the context of DR1 were developed and bred in our animal core facility as described previously (30). LAIR-1 KO (knockout) mice (16) were cross-bred to B6.DR1 transgenic mice with a B6 background for 12 generations. Genomic DNA was obtained from blood samples, and PCR was used to identify mice homozygous for either the LAIR-1−/− or LAIR−/− and expressing the DR1 transgene. CskAS mice were purchased from the Jackson Laboratory (Bar Harbor, ME). A description of their generation has been previously reported (9). Briefly mice that contained a modified Csk transgene were crossed to Csk+/− mice and intercrossed. The mice were genotyped with PCR, and pups were selected that contained both a Csk−/− deletion and the Csk transgene.

All mice were fed standard rodent chow (Ralston Purina Co., St. Louis, MO) and water ad libitum. Sentinel mice were routinely tested for a panel of mouse pathogens. All animals were kept until the age of 7–10 weeks before being used for experiments. All animal care and housing requirements set forth by the National Institutes of Health Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources were followed, and animal protocols were reviewed and approved by the University of Tennessee and Veterinary Affairs Animal Care and Use Committees.

Flow cytometry

Human Hut78 cells or modified Jurkat cells were collected, and the phenotype was determined by multiparameter flow cytometry using an LSRII flow cytometer (Becton Dickinson). The Cells were labeled with fluorochrome antibodies including FITC-conjugated anti-CD4 and phycoerythrin-conjugated LAIR-1. All were used according to the manufacturer’s recommendations. A minimum of 10,000 cells was analyzed from each sample, and the final analysis was performed using FlowJo software (Tree Star, Ashland, OR).

CD4+ T-cell isolation and activation

Spleens were collected from either LAIR-1–sufficient or –insufficient DR1 mice, and single-cell suspensions were prepared by mechanical disruption in complete Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 IU/ml of penicillin, 100 µg/ml streptomycin, 2.5 µM β-mercaptoethanol, and 2 mM L-glutamine. CD4+ naive T cells were isolated using a CD4+ T-cell isolation kit (Miltenyi Biotec, Auburn, CA) based on a negative selection protocol. The purity of the recovered CD4+ T cells was determined by flow cytometry after staining with anti-CD4 mAb, and they were >95% pure. In some experiments the cells were cultured for varying lengths of time (30 s to 60 min) with α1(II) or α1(I) (100 µg/ml). After an overnight culture at 37 ºC, the cells were collected and lysed, and insoluble materials were removed by centrifugation at 10,000 × g at 4 °C for 15 min.

Analysis of protein phosphorylation

To study the proximal signaling events following TCR stimulation, CD4+ T cells were isolated from mice. The lysates of whole cells following stimulation with CD3/CD28 or collagen were separated using SDS-PAGE gels and electrotransferred onto nitrocellulose membranes. After transfer, the membrane was blocked in TBS with Tween 20 buffer (TBST) containing 5% BSA for 1 h and incubated for 2 h with phospho-specific

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antibodies. The membrane was then incubated with a secondary antibody (Bio-Rad) for 1 h and subjected to ECL detection (ECL2 Western blotting kit; Thermo Scientific/Pierce) according to the manufacturer’s protocol. To detect protein levels, the membranes were stripped and reprobed with antibodies pan-specific for the proteins of interest.

Generation of LAIR-1-overexpressing Jurkat cells

Retroviral vectors expressing WT or various mutant forms of human LAIR-1 were developed as previously described (31). Briefly, the coding region of each was amplified by PCR using human cDNA as a template and then inserted into a MSCV-IRES-GFP retroviral vector. 1 × 10⁷/10 ml of HEK293T cells in D10 (Dulbecco’s modified Eagle’s medium + L-Glu + sodium pyruvate + 10% fetal bovine serum) medium were seeded in the morning. After 9 h, the cells were transfected with the MSCV construct encoding empty control, WT, or various mutant forms of human LAIR-1 and helper DNAs using the calcium method (10 μg of MSCV + 3 μg of 3240 helper DNA + 3 μg of pSR αG (VSV-G envelop) + 450 μl of endo-free H2O + 50 μl of 2.5 M CaCl₂ (in 10 mM HEPES buffer) + 500 μl of 2× HEPES buffer). The next morning, the medium was changed with fresh D10 medium. Virus particles in D10 medium were harvested after 1 and 2 days. The virus particles were 20× concentrated by ultracentrifugation (25,000 rpm for 90 min at 4 °C). 5 × 10⁸/ml of Jurkat cells were infected with the MSCV-IRES-GFP retrovirus that expressed empty control, WT, or mutant form of human LAIR-1 in the presence of 8 mg/ml of Polybrene. After 1 day, the medium was changed with fresh D10 medium. Further selection was done by GFP-positive sorting. Six days later, the levels of LAIR-1 in these cells were analyzed by SDS-PAGE followed by Western blotting analysis using anti-human LAIR-1 antibodies (1:1000 dilution factor).

Generation of Csk haploid deficient Jurkat T cells using the CRISPR/Cas9 genome editing method

Control CRISPR/Cas9 plasmids (sc-418922) and Csk/Cas9 CRISPR plasmid, (sc-400992) were purchased from Santa Cruz Biotechnology and used to develop Csk haploid-deficient Jurkat cells. Briefly, plasmids consisting of Csk-specific 20-nt guide RNA sequences derived from the GeCKO (v2) library were purchased from the manufacturer (Nucleofector II, Lonza). gRNA sequences directed poration (Nucleofector II, Lonza). gRNA sequences directed for murine Lck, Lyn, ZAP-70 (pZAP-70–Tyr-493), JNK (pSAPK/JNK-Thr-183/185), p38 (pp38 MAP kinase–Thr-180/Tyr-182), or ERK (pp44/42 MAP kinase–Thr-202/204), as well as their anti-non–phospho-specific counterparts were acquired from Cell Signaling Technology, Inc. (Beverly, MA). The anti-human LAIR-1 and their anti-phosphotyrosine-specific antibodies were purchased from R&D Systems, Inc. (Minneapolis, MN). An antibody against the non–phospho-specific Lyn was purchased from Santa Cruz Biotechnology. Antibodies against murine CD3 and CD4 and human CD3 and CD28 were purchased from BD Biosciences, and an antibody against Csk (Csk Antibody (E-3), sc-166560) was purchased from Santa Cruz Biotechnology.

Chemicals—The 3-IB-PP1 inhibitor has been described (10) and was used at a concentration of 10 μM (Sigma–Aldrich).

Cell lines—Jurkat cells (clone E6–1) and Hut78 cells, both immortalized human T-cell lines, were purchased from ATCC.

Measurement of cytokines

To measure cytokines, Jurkat T cells were stimulated with anti-CD3 in the presence or absence of collagen. Supernatants were collected at 72 h and analyzed for the presence of IL-2 using a Bio-Plex cytokine assay (Bio-Rad) according to the manufacturer’s protocol.

Statistical analysis

Cytokine levels and mean fluorescence were compared using the Mann–Whitney test.

Reagents

Antibodies—The following antibodies were used for these studies: anti-phospho- and non–phospho-specific antibodies for murine Lck, Lyn, ZAP-70 (pZAP-70–Tyr-493), JNK (pSAPK/JNK-Thr-183/185), p38 (pp38 MAP kinase–Thr-180/Tyr-182), or ERK (pp44/42 MAP kinase–Thr-202/204), as well as their anti-non–phospho-specific counterparts were acquired from Cell Signaling Technology, Inc. (Beverly, MA). The anti-human LAIR-1 and their anti-phosphotyrosine-specific antibodies were purchased from R&D Systems, Inc. (Minneapolis, MN). An antibody against the non–phospho-specific Lyn was purchased from Santa Cruz Biotechnology. Antibodies against murine CD3 and CD4 and human CD3 and CD28 were purchased from BD Biosciences, and an antibody against Csk (Csk Antibody (E-3), sc-166560) was purchased from Santa Cruz Biotechnology.

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