Mouse leukocyte-associated Ig-like receptor-1 (mLAIR-1) functions as an inhibitory collagen-binding receptor on immune cells

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Abstract

Leukocyte-associated Ig-like receptor-1 (LAIR-1) is a cell-surface molecule that functions as an inhibitory receptor on various immune cells. We developed mAbs to study the expression of mouse leukocyte-associated Ig-like receptor-1 (mLAIR-1) on primary immune cells and established that it is expressed on the majority of cells of the immune system, including T cells, NK cells, monocytes and dendritic cells. Furthermore, mLAIR-1 is inducibly expressed on blood granulocytes in vivo and is differentially expressed upon T cell activation in vitro. Unexpectedly, mLAIR-1 was not expressed on splenic and blood B220+ B cells. Similar to its human homolog, mLAIR-1 interacted with high affinity with a wide range of collagen molecules. Furthermore, mLAIR-1 specifically interacted in a hydroxyproline-dependent manner with synthetic collagen Gly-Pro-Hyp peptides. We show, for the first time, that mLAIR-1 cross-linking with its ligands inhibits CD3-induced T cell stimulation in vitro.

Introduction

Immune responses are tightly controlled by the opposing action of activating and inhibitory signals. Inhibitory signals are required to terminate an immune response and to prevent excessive immune reactions or autoimmune disease (1, 2). These signals can be provided by inhibitory immune receptors, often containing inhibitory signaling modules in their cytoplasmic tails [immunoreceptor tyrosine-based inhibitory motifs (ITIMs)] (2). Although all immune cells express multiple inhibitory receptors, these receptors have crucial, non-redundant functions, as underlined by receptor knockout mice that demonstrate enhanced sensitivity to autoimmune-like diseases caused by an over-activated immune system (3). Many families of inhibitory ITIM-bearing receptors have been identified in humans and their corresponding orthologs have been identified in mice and rats (1). Although mostly similar, several significant differences exist between human and mouse inhibitory immune receptors. For example, whereas humans possess ~13 killer Ig-like receptor (KIR) family genes on human chromosome 19q13.4 (4), mice completely lack these genes on the syntenic region on mouse chromosome 7. Remarkably, a completely different class of Ly49 NK receptors has evolved in mice with all the same general features as that of the human KIRs (1). Additionally, whereas the 2B4 (CD244) receptor functions as an inhibitory receptor on mouse NK cells, 2B4 activates NK cells in humans (reviewed in 1). In order to study the in vivo function of a human inhibitory immune receptor using the mouse ortholog, a comprehensive comparison between both molecules is crucial.

The human leukocyte-associated Ig-like receptor-1 (hLAIR-1) (CD305) is a member of the Ig superfamily (IgSF), which is expressed on the majority of PBMCs and thymocytes (5, 6). Cross-linking of hLAIR-1 by mAbs in vitro delivers a potent inhibitory signal that is capable of inhibiting cellular functions of NK cells (5, 6), T cells (7–9), B cells (10) and dendritic cell (DC) precursors (11). Recently, we identified orthologs of leukocyte-associated Ig-like receptor-1 (LAIR-1) in rats and mice, both sharing ~40% protein
sequence identity to hLAIR-1 (12, 13). The mouse ortholog of LAIR-1 shares potent inhibitory capacity with its human counterpart and reverse transcription (RT)-PCR analysis indicates that both proteins have a similar expression profile (12). Furthermore, the mouse leukocyte-associated Ig-like receptor-1 (mLAIR-1) cytoplasmic tail can be phosphorylated thereby recruiting SH2-containing tyrosine phosphatase (SHP)-2 (12) and C-terminal Src kinase (14) as potential signaling mediators. Interestingly, hLAIR-1 but not mLAIR-1 also recruits SHP-1 (5, 12).

Recently, we identified collagens as natural, high-affinity ligands for the LAIR-1 molecules (15). Collagens represent the most abundant type of proteins in vertebrates and play crucial roles in the development, morphogenesis and growth of many tissues (16). These trimeric molecules belong to a large family of extracellular matrix and transmembrane molecules composed of three polypeptide α chains, which contain the sequence repeat (Gly-X-Y)n, X being frequently proline (P) and, after post-translational modification, Y being hydroxyproline (O) (17). Given the moderate level of protein identity between the murine and hLAIR-1 molecules (12, 13), it is remarkable that the LAIR-1 molecules interact cross-species with various collagen molecules (13, 15). This can be explained by our finding that the interaction between hLAIR-1 and collagen is dependent on the conserved Gly-Pro-Hyp repeats present in all collagen trimers (15). Most importantly, we found that the interaction between collagen and hLAIR-1 directly inhibits immune cell activation in vitro (15) and may represent a novel mechanism of peripheral immune regulation by inhibitory immune receptors binding to extracellular matrix collagens.

Here, we characterized mLAIR-1 using mAbs and studied its interaction with its natural ligand collagen.

Methods

Cells, transfectant and cDNA

Cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured using standard techniques. Cell lines used in this study were as follows: D011.10 and B097.10 mouse T cell lines, human embryonic kidney 293T cells, mouse SP2/0 B cells and human erythro-leukemia K562 cells. The Armenian hamster fibroblast line ARHO12 was kindly provided by J. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Mouse bone marrow-derived DCs (BALB/c) were obtained as described previously (12). hLAIR1a and mLAIR-1a cloned in the pMX puro retroviral vector were described previously (15). The chimeric reporter construct was cloned in the pMX retroviral vector by fusing the extracellular domain of mLAIR-1a to the transmembrane and intracellular domain of human CD3ζ. Retroviral-based constructs were packaged by using the pCL-ecò or pCL-ampho system (18), and virus was used to infect 2B4 NFAT-GFP T cell hybridoma reporter cells (kindly provided by H. Arase and L.L. Lanier, University of California San Francisco (UCSF), USA). Three days after transduction, transfectants expressing mLAIR-1 or mLAIR-1-CD3ζ were stained with an anti-mLAIR-1 mAb (113) followed by PE-conjugated goat anti-Armenian hamster IgG (BD Biosciences) and subsequently sorted for high expression on the cell surface using a flow cytometer (FACSaria; BD Biosciences).

Antibodies

mLAIR-1-specific antibodies were produced essentially as described (19). Briefly, an Armenian hamster (Crictetus migratorius; Cytogen, West Roxbury, MA, USA) was injected intra-peritoneally (i.p.) with an ARHO12 clone stably expressing mLAIR-1 fused to a C-terminal MYC-tag (12). The mLAIR-1 transcript was derived from C57BL/6J cDNA. Five injections with 107 irradiated cells (50 Gy) in PBS were given at weekly intervals. Two weeks after the fifth injection, the hamster was boosted i.p. with 105 cells. Three days later, hamster spleen cells were fused with mouse myeloma SP2/0 cells by standard hybridoma technology. Hybridoma supernatants were tested for presence of mLAIR-1-specific antibodies by staining mLAIR-1-transfected ARHO12 cells followed by flow cytometric analysis. Selected hybridomas were sub-cloned by limiting dilution, and mAbs were purified by affinity chromatography on protein A-Sepharose columns (Amersham, Freiburg, Germany). Anti-mLAIR-1 polyclonal antibodies were described previously (12).

Flow cytometry

Flow cytometry was performed by standard procedures on a FACSCalibur (BD Biosciences). Primary cells were obtained from spleen, thymus, bone marrow and peripheral blood of wild-type BALB/c mice. To increase circulating numbers of granulocytes, when indicated, BALB/c mice were injected subcutaneously with saline or 20 μg per mouse (150 μl per mouse in PBS) polyethylene glycol-modified recombinant human granulocyte-colony stimulating factor (G-CSF) (Amgen) 3 days prior blood collection. For staining, unconjugated or biotin-conjugated Armenian hamster anti-mLAIR-1 mAb 113 was used. Additionally, the following fluorochrome-conjugated mAb were used (all from BD Biosciences): 17A2 (CD3), RM4-5 (CD4), 53-6.7 (CD8), DX5 (CD49d, pan NK cell marker), RA3-6B2 (B220) and RB6-8C5 (GR-1). A3.1 (F4/80) was obtained from Serotec. Prior to incubation with first-step mAb,Fc receptors on mouse cells were blocked with 25 μg ml⁻¹ 2.4G2 mAb (anti-CD16/CD32; PharMingen, San Diego, CA, USA). Allophycocyanine-conjugated streptavidin (BD Biosciences) and RPE-conjugated goat anti-Armenian hamster IgG (BD Biosciences) were used as secondary detecting reagents.

Surface plasmon resonance experiments

Surface plasmon resonance (BIAcore) binding studies were performed by using a BIAcore 2000 system (BIAcore AB, Uppsala, Sweden). Approximately 2000–3000 response units (RUs) of acid-soluble human collagen type I or III (Sigma) were immobilized on a CM5 biosensor chip by using the amine-coupling kit as instructed by the supplier. Immobilized triple-helical peptides composed of GCO(GPO)10GCG-NH2 [(GPO)10, also known as collagen-related peptide] and GCP(GPP)10GCG-NH2 [(GPP)10] were described previously (20) and were kindly provided by R.W. Farndale (University of Cambridge, Cambridge, UK).
Approximately 250 RU (GPP)_{10} or (GPO)_{10} peptide trimers were immobilized by using a cysteine-coupling kit according to the manufacturer’s instructions. Analysis was performed in buffer (125 mM NaCl, 2.5 mM CaCl₂, 0.005% (v/v) Tween 20 and 25 mM HEPES, pH 7.4) at 25°C at a flow rate of 20 μl min⁻¹ for collagen I and III interaction studies and 5 μl min⁻¹ for the immobilized peptides. Binding of mLAIR-1-IgG to collagen I and III was specific, because non-specific binding to an uncoated control channel was <1% compared with collagen-coated channels. In addition, an irrelevant IgG fusion protein did not bind to the collagen-coated surface. mLAIR-1-IgG dimer concentration was calculated based on a theoretical mass of 82.5 kDa (corrected for removal of leader peptide). Increasing concentrations of mLAIR-1-IgG were injected and allowed to reach an equilibrium plateau for 10 min. The delay between injections was 13 min, during which time the biosensor chip was flushed with buffer. In the peptide-binding studies, biosensor chips were regenerated by injection of 0.1 M H₃PO₄ (2 min, 5 μl min⁻¹).

Dissociation constants (Kd) and the number of binding sites expressed as the response at infinite hLAIR-1-IgG concentration (Bmax) were calculated as described previously (15). Bmax values were converted to number of mLAIR-1-IgG molecules interacting with a single collagen trimer by using the theoretical mass of mLAIR-1-IgG (82.5 kDa) versus collagen I and III (416.7 and 415.7 kDa, respectively).

The dissociation of mLAIR-1-IgG in the presence of buffer was followed for at least 13 h and Koff values were calculated by using the Bioevaluation software version 3.0.1.

**Reporter cell assay**

For ligand detection studies, 2B4 T cell hybridoma cells stably transduced with an NFAT-GFP reporter and the mLAIR-1-CD3ζ chimera were analyzed as described (15). For detection of inhibition of mouse CD3 signaling, 2B4 T cell hybridoma cells stably transduced with an NFAT-GFP reporter and the mLAIR-1 were generated. Anti-mouse CD3 (1.25 μg ml⁻¹) (PharMingen) was coated overnight at 4°C in 96-well MAXisorp flat-bottom plates (Nunc) together with 10 μg ml⁻¹ (GPO)₁₀ or (GPP)₁₀ synthetic peptides in a total volume of 100 μl per well. The next day, plates were washed and 200 μl of 2.5 × 10⁵ reporter cells per ml in medium were added to each well, and plates were incubated at 37°C for 22 h and then analyzed for GFP expression by flow cytometry.

**T cell stimulations**

For mLAIR-1 expression studies on stimulated primary T cells, single-cell preparations of BALB/c mouse spleens were depleted of erythrocytes, and 1 × 10⁶ cells were added to 96-well plates coated with anti-CD3 and anti-CD28 mAbs or anti-CD3 alone as described above. Alternatively, the cells were incubated in the presence of 5 ng ml⁻¹ phorbol 12-myristate 13-acetate (PMA) and 1.34 μM ionomycin. The cells were subsequently incubated at 37°C for 3 days. The expression levels of mLAIR-1 on T cells present in the culture were assessed after 0, 1, 2 and 3 days using the 113 antibody and anti-CD3 mAbs, as described above.

For detection of inhibition of mouse CD3 signaling in T cell lines, 2B4 T cell hybridoma cells stably transduced with an NFAT-GFP reporter and hLAIR-1a were generated. Anti-mouse CD3 (1.25 μg ml⁻¹) (PharMingen) was coated overnight at 4°C in 96-well MAXisorp flat-bottom plates (Nunc) together with 10 μg ml⁻¹ (GPO)₁₀ or (GPP)₁₀ synthetic peptides in a total volume of 100 μl per well. The next day, plates were washed and 200 μl of 2.5 × 10⁵ reporter cells per ml in medium were added to each well, and plates were incubated at 37°C for 22 h and then analyzed for GFP expression by flow cytometry on a FACS Calibur (BD Biosciences).

For detection of inhibition of mouse CD3 signaling in primary T cells, single-cell preparations of BALB/c mouse spleens were depleted of erythrocytes and labeled with CFSE (Molecular Probes, Eugene, OR, USA). CFSE-labeled splenocytes (1 × 10⁶) were incubated with anti-mouse CD3-coated 96-well plates in the presence of coated collagens I, III or BSA. Coating was performed as described above, making use of 0.5 μg ml⁻¹ anti-CD3 and 10, 3.3, 1 or 0.4 μg ml⁻¹ collagens I, III or BSA. Cells were harvested after 72 h, stained with anti-CD3, CD25 and CD69 mAbs and analyzed by flow cytometry.

**Results**

**Generation of mLAIR-1-specific mAbs**

To characterize the mLAIR-1 gene products, we generated anti-mLAIR-1-specific mAbs. An Armenian hamster fibroblast line transfected with mLAIR-1 was used to immunize an Armenian hamster. Screening of supernatants of the resulting hybridomas for specific binding to ARH012 cells expressing mLAIR-1 identified ~60 positive clones (Fig. 1A and data not shown) of which four antibodies (mAbs: 18, 53, 90 and 113) were affinity purified and tested for their capacity to recognize mLAIR-1 in flow cytometry and western blot analysis. Since all four antibodies were comparable in these assays, we choose a single mAb (113) for further characterization. The 113 mAb specifically recognized wild-type mLAIR-1 transiently over-expressed in 293T cells, whereas 293T cells transfected with irrelevant cDNA were not stained (Fig. 1B). Furthermore, the antibody specifically recognized MYC-tagged mLAIR-1 in lysates from 293T cells transiently transfected with the antigen, but not in untransfected 293T cells (Fig. 1C). Additionally, mLAIR-1 could be specifically immunoprecipitated from ARH012 cells stably expressing mLAIR-1 (Fig. 1D). Two bands were detected that specifically stained with the anti-mLAIR-1 mAbs, whether the lower additional band corresponds to proteolytic cleaved or unglycosylated protein is not known. Furthermore, endogenous mLAIR-1 precipitated from the mouse DO11.10 T cell line migrated as an ~46 kDa monomer when analyzed by SDS-PAGE under both non-reducing and reducing conditions (Fig. 1E), whereas no specific mLAIR-1 molecule was precipitated from mouse Ba/F3, B16.F10 or II A1.6 cells lines (Fig. 1E and data not shown). mLAIR-1 has a predicted theoretical mass of 29.8 kDa (12); this mass difference is in agreement with the presence of two potential sites for N-linked glycosylation at positions N34 and N90 (12). In addition to mLAIR-1 at ~46 kDa, a smaller protein migrating at ~32 kDa was evident in the DO11.10 immunoprecipitated sample. Whether this represents a distinct LAIR-1 isoform,
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A

B

C

D

E

Fig. 1. Generation of anti-mLAIR-1 mAbs. (A) Flow cytometric analysis of hybridoma supernatants. Untransfected ARHO12 cells (open histograms) and ARHO12 cells stably expressing MYC-tagged mLAIR-1 (closed histograms) were stained with the supernatant of hybridoma 113 (right panel) or Armenian hamster isotype control IgG (left panel) and visualized by PE-conjugated goat anti-Armenian hamster IgG. (B) 293T cells transiently transfected with control cDNA (open histograms) or wild-type mLAIR-1 (closed histograms) were stained with the supernatant of hybridoma 113 (right panel) or Armenian hamster isotype control IgG (left panel) and visualized by PE-conjugated goat anti-Armenian hamster IgG. (C) Western blot analysis. Cell lysates of 293T cells transiently transfected with control cDNA (−) or MYC-tagged mLAIR-1 (+) were immunoblotted using anti-mLAIR-1 mAb 113 (left panel) or anti-MYC mAb (right panel). Bands corresponding with mLAIR-1 are indicated using arrows. (D) Untransfected ARHO12 cells (−) or ARHO12 cells stably expressing MYC-tagged mLAIR-1 (+) were subjected to immunoprecipitations with anti-mLAIR-1 mAbs coupled to protein-A/G-conjugated beads. Bands corresponding with mLAIR-1 are indicated using arrows. (E) mLAIR-1 is endogenously expressed as an ~46 kDa protein on DO11.10 mouse T cells. Mouse pro-B Ba/F3 cells and DO11.10 T cells lysates were subjected to immunoprecipitation using anti-mLAIR-1 mAb 113 and immunoblotted using anti-mLAIR-1 mAbs. Bands corresponding with mLAIR-1 are indicated using arrows.

mLAIR-1 binds multiple collagens as ligands

Recently, we identified collagens as high-affinity ligands for the LAIR-1 molecules (15). We found that mLAIR-1 binds to transmembrane collagens XII, XVII and XXIII and to extracellular matrix collagen I (15). Here, we have extended these studies and show that K562 cells stably expressing mLAIR-1 are capable of binding to immobilized extracellular human collagens I, II, III, V and VI and mouse collagen II (Fig. 4A). This interaction was specifically attributable to expression of mLAIR-1, since parental K562 cells and stable KIR3DL1-expressing K562 cells did not interact with the collagen molecules (Fig. 4A). Additionally, K562 cells stably transfected with mLAIR-1 stained brightly with Oregon Green 488-labeled collagen IV, whereas the parental K562 cells did not (Fig. 4B). This shows that all major extracellular matrix molecules (collagen I–VI) can serve as ligands for mLAIR-1.

We assessed the affinity of mLAIR-1 binding to human collagens I and III by surface plasmon resonance studies using purified mLAIR-1-hIgG fusion proteins. mLAIR-1 fusion proteins bound with high affinity to collagen I (Kd = 15.3 ± 1.5 nM) and collagen III (Kd = 18.4 ± 1.5 nM) (Fig. 4C). The dissociation of mLAIR-1-hIgG from collagen I and III (Fig. 4D) was almost identical to that of hLAIR-1-hIgG.

a proteolytic cleavage product or non-glycosylated protein is unknown.

Expression of mLAIR-1

hLAIR-1 is expressed on peripheral blood CD3+CD4+ T cells, CD3+CD8+ T cells, CD3+CD56+ NK cells, CD3+CD19+ B cells, CD3+CD14+ monocytes and the majority of human fetal thymocytes (5). In addition, monocyte-derived DCs express hLAIR-1, where it plays a role in the granulocyte macrophage colony-stimulating factor (GM-CSF)-induced differentiation of peripheral blood precursors into DCs (11). Previously, we showed by RT-PCR analysis that mLAIR-1 shares a similar expression profile with its human homolog (12); here we used mAbs to examine the expression of the protein in more depth in primary cells. In splenocytes, thymocytes, bone marrow and peripheral blood cells, mLAIR-1 was expressed on CD3+CD4+ T cells, CD3+CD8+ T cells, CD3+DX5+ T cells, CD3+DX5+ NK cells and F4/80+ monocytes (Fig. 2A and data not shown). Unlike hLAIR-1 (5), mLAIR-1 was not detected on blood-derived or splenic B220+ B cells (Fig. 2A and data not shown). The highest amounts of mLAIR-1 expression were found on in vitro cultured mouse bone marrow-derived DCs (Fig. 2B). mLAIR-1 was expressed on the same immune cell types from both C57/BL6J and BALB/c mice, although C57/BL6J mice appeared to express somewhat lower amounts of the receptor (data not shown).

Since hLAIR-1 is not expressed on the surface of peripheral blood granulocytes (5), but is expressed on immature neutrophils (21), we analyzed mLAIR-1 expression on granulocytes from G-CSF-treated BALB/c mice. Immature GR-1dim granulocytes expressed no or low amounts of mLAIR-1 and mature GR-1bright granulocytes had no detectable expression (Fig. 2C). We treated animals with G-CSF causing a profound increase in granulocyte numbers in the peripheral blood, which is also associated with cells of a more immature phenotype (22). G-CSF treatment resulted in a clear up-regulation of mLAIR-1 in both immature GR-1dim and mature GR-1bright granulocytes (Fig. 2C), indicating that mLAIR-1 expression is regulated on this cell type.

To study the expression of mLAIR-1 upon activation of T cells, we stimulated CFSE-labeled splenocytes by using various stimuli and assessed LAIR-1 expression of these cells in time. Stimulation of splenic CD3-expressing T cells by PMA and ionomycin resulted in down-regulation of mLAIR-1 expression (Fig. 3A). Intriguingly, whereas αCD3 and αCD28 stimulation of splenic T cells had no apparent effect on mLAIR-1 expression, αCD3 stimulation alone induced a strong up-regulation of the antigen (Fig. 3C and B, respectively). Taken together, these data indicate that, similar to hLAIR-1, mLAIR-1 is expressed by cells of hematopoietic origin, that its expression profile partially mirrors that of its human ortholog and that the receptor is profoundly regulated on various immune cell types.
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We conclude that collagens are able to down-modulate CD3-induced T cell activation via mLAIR-1. To analyze whether collagen induces functional cross-linking of mLAIR-1, we generated 2B4 NFAT-GFP reporter cells (23) expressing a chimeric protein consisting of the extracellular domain of CD3ζ and the intracellular domain of mLAIR-1. Receptor engagement of reporter cells expressing the mLAIR-1-CD3ζ chimera, but not the parental reporter cells, via plate-bound anti-mLAIR-1 mAbs resulted in expression of GFP (Fig. 5A). This indicates that the 113 mAb induces functional triggering of this chimeric receptor. Both human collagen I and III were capable of triggering the chimeric molecule and induced GFP expression (Fig. 5B). Furthermore, (GPO)10 trimeric peptides, resulted in triggering of the receptor (Fig. 5B). Collagen I, III and (GPO)10 trimeric peptides are thus capable of cross-linking mLAIR-1.

We next investigated whether cross-linking of mLAIR-1 by extracellular matrix collagens leads to inhibition of immune cell function in vitro. As a model, we used 2B4 NFAT-GFP reporter cells (23) transfected with or without wild-type mLAIR-1. Cross-linking of the CD3 receptor on the surface of these reporter cells using plate-bound anti-mouse CD3 mAbs resulted in NFAT activation and GFP expression (Fig. 6A). Simultaneous cross-linking of mLAIR-1 via plate-bound (GPO)10 trimeric peptides inhibited CD3 activation of mLAIR-1-transfected cells, but had no effect on the parental reporter cells (Fig. 6A). Thus, mLAIR-1 transfected in these cells is capable of specifically inhibiting CD3 signaling via binding to (GPO)10 collagen peptides. As suggested by the BIAcore studies, the inhibition was hydroxyproline-dependent since (GPP)10 trimeric peptides did not inhibit the CD3 activation of these cells (Fig. 6A).

To assess whether endogenous mLAIR-1 expression on mouse immune cells is capable of inhibiting immune responses, we stimulated splenic CD3+ T cells with αCD3 mAbs in the presence or absence of collagens I or III. As expected, αCD3-induced proliferation was potently inhibited in a dose-dependent manner by both collagen I and III (Fig. 6B and C). Furthermore, collagen administration resulted in reduced expression of the activation markers CD25 and CD69 as compared with CD3 stimulation alone (Fig. 6C).

We conclude that collagens are able to down-regulate mouse CD3 signaling and thus are potential inhibitors of T cell responses in vivo. It is suggestive that the observed inhibition was caused by the mLAIR-1-collagen interaction, although we do not have blocking antibodies to formally prove this.
Discussion

In this report, we investigate the expression profile and ligand recognition of mLAIR-1. Similar to its human ortholog, this inhibitory receptor is expressed on a wide range of immune cells. Both receptors are expressed on CD3+CD4+ and CD3+CD8+ T cells, NK cells and monocytes present in spleen and peripheral blood. Unexpectedly, mLAIR-1 was not detected on splenic and blood-derived B cells.
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whereas hLAIR-1 is expressed on subsets of B cells (10). In a previous report, we did detect mLAIR-1 transcripts in mouse B cell lines (12). This discrepancy could reflect a difference between cell lines versus primary cells or could indicate that presence of mLAIR-1 transcripts in mouse B cells may not result in protein expression or membrane expression of the antigen. Alternatively, mLAIR-1 expression on various subsets of B cells in spleen and peripheral blood may be too low to detect with these mAbs.

By determining duration and magnitude of T cell responses, DCs play a central role in regulation of the immune response (24). Not surprisingly, DCs are heavily regulated by several inhibitory immune receptors (25). In line with this, hLAIR-1 is expressed on monocyte-derived DCs, where it plays a role in the GM-CSF-induced differentiation of peripheral blood precursors into DCs (11). Correspondingly, mLAIR-1 is highly expressed on mouse bone marrow-derived DCs. Since DCs are situated in many sites in the body, they will likely encounter collagens that, via LAIR-1, may establish a threshold for DC activation.

In vivo administration of G-CSF in wild-type C57BL/6J and BALB/c mice resulted in up-regulation of mLAIR-1 expression on GR1bright and GR1dim granulocytes in blood and spleen. This correlates with the greatly increased hLAIR-1 expression levels on neutrophils from G-CSF-treated humans, likely reflecting the appearance of immature granulocytes in the blood (21). This shows that immature neutrophils in peripheral blood in humans and mice express LAIR-1, whereas resting mature neutrophils do not, suggesting that the antigen may be involved in the regulation of neutrophil differentiation and function.

mLAIR-1 expression was also regulated on mouse primary CD3+ T cells. CD3 stimulation alone resulted in a profound up-regulation of the antigen, whereas simultaneous triggering of CD3 and CD28 had no apparent effect on mLAIR-1 expression. On the other hand, PMA/ionomycin stimulation of these same cells resulted in a clear down-regulation of the receptor on both mouse T cell lines and splenic T cells. This indicates that mLAIR-1 can be dynamically regulated on T cells and that different stimuli can either increase or decrease expression of the receptor.

Remarkably, although human and mLAIR-1 share a moderate level of protein homology (12), both receptors bind with high-affinity cross-species to both transmembrane and extracellular matrix collagens. The large sequence divergence indicates LAIR-1 is a rapidly evolving protein, as was previously suggested for many other genes present in the leukocyte receptor complex (LRC) on human chromosome 19 (4, 26, 27) and its syntenic region on mouse chromosome 7 where the LAIR-1 genes are encoded (12). Nevertheless, both the human and mouse proteins maintained the capacity to bind collagen molecules. Intriguingly, although the affinity of mouse and hLAIR-1 for collagens I and III is almost identical, we observed a marked difference in binding capacity to synthetic (GPO)10 trimeric peptides. This suggests that an intrinsic difference in ligand recognition between human and mLAIR-1 exists that may be caused by single amino acid differences specifically affecting (GPO)10 binding, without impacting collagen binding. In order to determine the molecular requirements for LAIR-1 binding to collagens and to address differences in collagen-binding characteristics between the various LAIR-1 molecules, co-crystallization studies of LAIR-1 in complex with trimeric (GPO)10 peptides are required.

Also glycoprotein VI (GPVI), a major player in platelet-collagen adhesive interactions leading to thrombus formation (20), binds collagen molecules as a ligand. Similar to LAIR-1, GPVI is a member of the IgSF and is encoded in the LRC on human chromosome 19 (28). These similarities are striking and may indicate that additional collagen-binding IgSF members exist.

Similar to hLAIR-1, mLAIR-1 functions as an inhibitory receptor on immune cells. We showed previously that the mLAIR-1 intracellular tail can inhibit signaling mediated by the ITAM-bearing FceRI expressed on rat basophilic leukemia (RBL) cells and that the molecule inhibits cytotoxic activity of NK cells (12). We now demonstrate that cross-linking of mLAIR-1 with plate-bound ligands directly inhibits TCR signaling in mouse T cell lines and TCR-induced activation of primary splenic T cells. This indicates that T cells may be subject to regulation via LAIR-1 in vivo as well. Of note, although all reporter cells stably expressed high amounts of mLAIR-1 on the membrane, only ~30% gave (GPO)10-induced inhibition of CD3 signaling. Potentially, the (GPO)10 trimeric peptide may not be the optimal ligand for mLAIR-1 or may not be optimally presented to the reporter cells explaining the low inhibition via the receptor. In line with this hypothesis, the full-length collagen I and III trimers were very potent in inhibiting the CD3-induced proliferation of primary T cells (Fig. 6B and C), whereas (GPO)10 trimeric peptides did not give inhibition (data not shown).

The data presented here show that mLAIR-1 is a genuine ortholog of hLAIR-1. Despite the moderate level of
sequence identity, both proteins have similar expression patterns, share a potent inhibitory capacity and bind the same collagen molecules as ligands. The functional overlap between hLAIR-1 and mLAI-1 supports the use of mouse models to assess the role of the LAIR-1 receptors in regulation of immune responses to broaden the general knowledge on the function of inhibitory receptors in immune surveillance.

Fig. 6. mLAI-1/collagen interaction inhibits CD3 signaling in mouse T cells. (A) Untransfected (top panels) or wild-type mLAI-1-transfected (bottom panels) NFAT-GFP reporter cells were incubated with immobilized anti-CD3 mAbs in the presence or absence of immobilized trimeric peptides (GPO)$_{10}$ or (GPP)$_{10}$ for 22 h and GFP expression was analyzed by flow cytometry. Percentage of GFP-negative cells as a measure of inhibited cells is indicated in each histogram. Maximal inhibition of CD3 signaling in these reporter cell assays using trimeric collagen peptides typically resulted at most in ~30% inhibition. (B and C) CFSE-labeled splenocytes were incubated with plate-bound anti-mouse CD3 mAbs in the presence/absence of indicated concentrations of coated collagens I, III or BSA. CFSE dilution was analyzed by flow cytometry after 72 h (B and C, left panel) and the cells were analyzed for expression of the activation markers CD25 (C, middle panel) and CD69 (C, right panel). Only CD3-positive splenocytes are shown. One representative experiment of three is shown for all figures.

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Abbreviations

DC     dendritic cell
GM-CSF granulocyte macrophage colony-stimulating factor
GPVi  glycoprotein VI
hLAIR-1 human leukocyte-associated Ig-like receptor-1
IgSF  Ig superfamily
i.p.  intra-peritoneally
ITIM  immunoreceptor tyrosine-based inhibitory motif
KIR   killer Ig-like receptor
LAIR-1 leukocyte-associated Ig-like receptor-1
LRC   leukocyte receptor complex
mLAIR-1 mouse leukocyte-associated Ig-like receptor-1
PMA   phorbol 12-myristate 13-acetate
RT    reverse transcription
RU    response unit
SHP   SH2-containing tyrosine phosphatase

References


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