The Soluble Leukocyte-Associated Ig-Like Receptor (LAIR)-2 Antagonizes the Collagen/LAIR-1 Inhibitory Immune Interaction[1]

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Leukocyte-associated Ig-like receptor (LAIR)-1 is a collagen-receptor that inhibits immune cell function upon collagen binding. Next to *LAIR-1*, the human genome encodes *LAIR-2*, a putative soluble homolog. In this study we show, for the first time, that the *LAIR-2* gene is broadly transcribed in human PBMC, mirroring the expression profile of LAIR-1. LAIR-2 protein is expressed as a soluble receptor exhibiting high affinity for various collagen molecules to which it binds in a hydroxyproline-dependent manner. In vitro stimulation of PBMC induces secretion of LAIR-2. We detect high amounts of LAIR-2 in urine of pregnant women, indicating that the soluble receptor is indeed produced in vivo and can be cleared from the body via urine. Furthermore, LAIR-2 levels are increased in synovial fluid of patients with rheumatoid arthritis as compared with osteoarthritis patients. We hypothesize that soluble LAIR-2 may function as a natural competitor for LAIR-1, thereby regulating its inhibitory potential. Indeed, LAIR-2 prevents binding of human LAIR-1 to collagens and LAIR-1 cross-linking in vitro, suggesting that the protein has an immunoregulatory function in vivo. Hence, we reveal a novel mechanism of immune regulation by a soluble LAIR receptor regulating the inhibitory potential of the membrane-bound LAIR-1 via competition for ligands. *The Journal of Immunology,* 2008, 180: 1662–1669.

Immune responses are 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 that often contain ITIMs essential for the inhibitory signal in their cytoplasmic tails (2). Although all immune cells express multiple inhibitory receptors and many families of inhibitory ITIM-bearing receptors have been cloned and characterized in humans and rodents (1), the means of regulation of these receptors have been less well defined. Inhibitory immune receptors can be differentially expressed on various immune cell types, and their expression is known to change upon cellular activation (3–7). Additionally, regulated and/or localized expression of their cognate ligands enables receptor and ligand to be temporally and spatially present in close proximity, allowing signaling of the inhibitory receptor. Another means of regulation is provided by the secretion of soluble receptor variants that retain the ligand binding capacity of the membrane bound inhibitory receptor. These soluble receptors are mainly obtained by proteolytic cleavage of the receptors' ectodomains or alternative splicing of mRNA transcripts (8). For example, soluble variants of the ITIM-bearing receptor PECAM-1 (CD31) have been shown to interfere with the function of the membrane-bound receptor (reviewed in Ref. 9).

The leukocyte-associated Ig-like receptor-1 (LAIR-1[3]; CD305) is an ITIM-containing inhibitory immune receptor, belonging to the Ig superfamily (10). Human (h) LAIR-1 is expressed on the majority of PBMC and thymocytes (4, 10–12). Cross-linking of hLAIR-1 by mAbs in vitro delivers a potent inhibitory signal that is capable of inhibiting immune cell function (4, 10–15). Recently, we identified collagens as natural, high-affinity ligands for the LAIR molecules (16). Interaction of hLAIR-1 with collagens directly inhibits immune cell activation in vitro and may represent a novel mechanism of peripheral immune regulation through extracellular matrix collagens (16).

Given the broad expression profile of hLAIR-1 on immune cells (10) and the high abundance of collagen molecules in the human body (17), a fine-tuned regulation of the collagen-hLAIR-1 interaction is needed to allow proper inhibitory signaling of the hLAIR-1 receptor. One level of regulation occurs by modulating hLAIR-1 expression at different stages of differentiation/activation of immune cells, as was demonstrated for B cells (12), T cells (4, 5), neutrophils (3), and dendritic cells (our unpublished observations). Furthermore, hLAIR-1 can be shed from immune cells upon cellular activation (18). Another system to regulate the interaction between collagens and hLAIR-1 may involve the putative secreted homolog LAIR-2 (CD306). LAIR-2 is encoded by a gene located in the vicinity of the *LAIR-1* gene in the leukocyte receptor complex on human chromosome 19q13.4 (10, 19). The predicted LAIR-2 protein has a single Ig-like domain sharing 84% sequence

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[3] Abbreviations used in this paper: LAIR, leukocyte-associated Ig-like receptor; hLAIR, human LAIR; SF, synovial fluid; RA, rheumatoid arthritis; OA, osteoarthritis; s, soluble; LILR, leukocyte Ig-like receptor; KIR, killer cell Ig-like receptor.

Table I. *Reactivity of LAIR-1 and LAIR-2 Abs*[a]

	Abs			
	Anti-LAIR-1 (8A8)	FMU-LAIR2.1	FMU-LAIR2.3	Polyclonal anti-LAIR-2
293T control	−	−	−	−
293T/hLAIR-1	+	−	−	+
293T/hLAIR-2 TM	−	+	+	+

[a] Reactivity of the Abs used in this study to 293T cells transfected with empty vector (control), full-length hLAIR-1, or a chimeric construct containing hLAIR-2 fused to the transmembrane region and intracellular tail of hLAIR-1 (hLAIR-2 TM). Ab reactivity was tested by FACS analysis; + indicates positive staining compared to isotype control.

homology with hLAIR-1, and the molecule lacks a transmembrane and cytoplasmic region suggesting it is a secreted protein (10). In contrast to hLAIR-1, LAIR-2 has no orthologs in mice or rats (19, 20). Whether LAIR-2 is produced in vivo is unknown. In the current study we explored whether LAIR-2 is secreted and whether it may function as a soluble competitor for the interaction between collagens and hLAIR-1. Our data reveal a novel mechanism of immune regulation by the soluble receptor LAIR-2 regulating the inhibitory potential of the membrane-bound hLAIR-1 via competition for the same ligands.

Materials and Methods

Cells

Cell lines were obtained from the American Type Culture Collection and cultured using standard techniques. Cell lines used in this study: human monocytic U937 cells; HEK293T human embryonic kidney cells; human colon carcinomas lines HT29, DLD-1, LS174, SW480, and HCT116; SKBR3 human breast cancer line; YT.2C2 human NK-like cells; 721.221 lymphoblastoid cells; THP-1 human monocyte-like cells; Jurkat human T cells; CEM human leukemia T cells; and human erythroleukemia K562 cells. K562 cells and 2B4 NFAT-GFP T cell reporter cells (provided by L.L. Lanier and H. Arase (University of California San Francisco)) were stably transfected with hLAIR-1 or hLAIR-1a-CD3ζ respectively as described previously (16).

Human PBMC were isolated from healthy donors using Ficoll-Paque density centrifugation. Cells (1.5×10^6 cells/ml) were cultured for 4 days in the presence of PMA (5 ng/ml) and ionomycin (1.34 μM), after which supernatants were harvested. Alternatively, PBMCs were stained with either anti-CD4/CD8/CD3/CD19 Abs to obtain $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells and $CD3^-CD19^+$ B cells or anti-CD16/CD14/CD3/CD56 Abs to obtain $CD3^-CD16^+CD56^+$ NK cells and $CD3^-CD14^+$ monocytes. Stained cells were sorted with a FACSAria Cell-Sorting System (BD Biosciences) and cultured as described above.

Subjects

For LAIR-2 measurements in urine: 10 healthy individuals (5 males and 5 females) and 6 healthy pregnant women (16–37 wk of gestation) volunteered in collecting their morning urine. The urine was stored at −80°C prior use. For LAIR-2 measurements in synovial fluid (SF) from 14 patients suffering from rheumatoid arthritis (RA) and 16 patients suffering from osteoarthritis (OA), SF was obtained upon joint aspiration. The average age of the RA patients was 63.7 years (range 51–79). The average age of the OA patients was 56.0 years (range 34–87). Ten of 16 RA patients were positive for rheumatoid factor. Samples were subjected to centrifugation to remove cells prior storage at −80°C.

Antibodies

The FMU-LAIR2.1 and FMU-LAIR2.3 IgG1 LAIR-2 mAbs were produced by immunization of BALB/c mice with recombinant LAIR-2 protein followed by preparation of hybridomas by using standard hybridoma techniques. Selected hybridomas were subcloned by limiting dilution, and mAbs were purified by affinity chromatography on protein A-Sepharose columns (Amersham). Biotinylated anti-human LAIR-2 Ab was obtained from R&D Systems (BAF2665). Specificity of the Abs was confirmed on transfected cells (Table I).

RT-PCR

Total RNA was isolated from several human cell lines using the TRIzol method according to the manufacturer's instructions (Invitrogen Life Technologies). Total RNA was converted to first-strand DNA with oligo$(dT)_{20}$ primers and Moloney murine leukemia virus reverse transcriptase using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). The cDNA mixtures were amplified by PCR using LAIR-2-specific forward (5′-GTTGGGGTTCAAACATTCCG-3′) and reverse (5′-TCATGGTGCATCAAATCCGG-3′) primers and the AmpliTaq Gold DNA polymerase system (Applied Biosystems). Each amplification reaction underwent 40 cycles of denaturation at 95°C for 30s, annealing for 30s at 54°C and elongation at 72°C for 50s. As a control, GAPDH transcripts were amplified from the same RNA, using GAPDH specific forward (5′-GGTACATGACAAGGTGCGGC-3′) and reverse (5′-GCATCCTGGGC TACACTGAGC-3′) primers.

The LAIR-2 isoforms were cloned using the pGEM-T easy vector system (Promega) and sequenced on an ABI 3100 sequencer (Applied Biosystems) using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The sequences were analyzed by Lasergene software (DNASTAR).

Western blot analysis

293T cells were transiently transfected with vectors encoding His-tagged LAIR-2a or wild type LAIR-2b and cultured in Optimem medium (Invitrogen Life Technologies), 48 h after transfection the supernatant was collected and separated by SDS-PAGE (12% gel) under nonreducing and reducing conditions and transferred to Immobilon-P membranes (Millipore). Western blot analysis was performed with FMU-LAIR2.1 anti-LAIR-2 mAbs, followed by peroxidase-conjugated rabbit anti-mouse mAb (DakoCytomation) as secondary Ab. Proteins were detected by ECL (GE Healthcare).

Surface plasmon resonance

The surface plasmon resonance binding studies were performed as described before (16). LAIR-2-IgG dimer concentration was calculated based on a theoretical mass of 82.5 kDa.

Flow cytometry

For intracellular LAIR-2 staining, the Zenon Mouse IgG Labeling kit (Molecular Probes) was used according to the manufacturer's instructions. Cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Interaction of Oregon Green 488-conjugated collagen IV (Molecular Probes) with hLAIR-1 or LAIR-2 was performed as follows; collagen IV was incubated with 40 μg/ml LAIR-2-IgG or control fusion protein for 30 min at room temperature. Subsequently, the mixture was added to K562 cells stably transfected with hLAIR-1 or wild-type K562 cells for 30 min, washed and cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences).

For fusion protein blocking studies, chimeric proteins of the extracellular domain of hLAIR-1 or LAIR-2 fused to the Fc region of human IgG1 were prepared as described previously (19). HT29 cells were pretreated with 10% normal mouse serum to block aspecific interactions. Subsequently, HT29 cells were incubated with unlabeled hLAIR-1-IgG or LAIR-2-IgG for 10 min at room temperature and subsequently incubated with biotinylated hLAIR-1-IgG or LAIR-2-IgG for 30 min at room temperature. Binding of biotinylated proteins was detected with allophycocyanin-conjugated streptavidin (BD Biosciences) and cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Binding of K562 transfectants to plate-bound collagen

Ninety-six-well MAXIsorp flat-bottom plates (Nunc) were coated overnight at 4°C with purified collagens I or III (2 and 5 μg/ml, respectively; Sigma-Aldrich) or BSA (5 μg/ml) in 100 μl of PBS, supplemented with 2 mM acetic acid. A total of 5×10^6 cells/ml wild-type K562 or K562 stably transfected with hLAIR-1 were assayed for their capacity to adhere to the collagens in the 96-well plates as described previously (16).

Reporter cell assay

2B4 T cell hybridoma cells were stably transduced with an NFAT-GFP reporter construct (21) and hLAIR-1-CD3ζ (16) and analyzed as described (16, 21).

Sandwich ELISA

Ninety-six-well flat-bottom MAXIsorp plates (Nunc) were coated overnight at 4°C with FMU-LAIR2.1 anti-LAIR-2 mAbs (6 μg/ml in 50 μl/

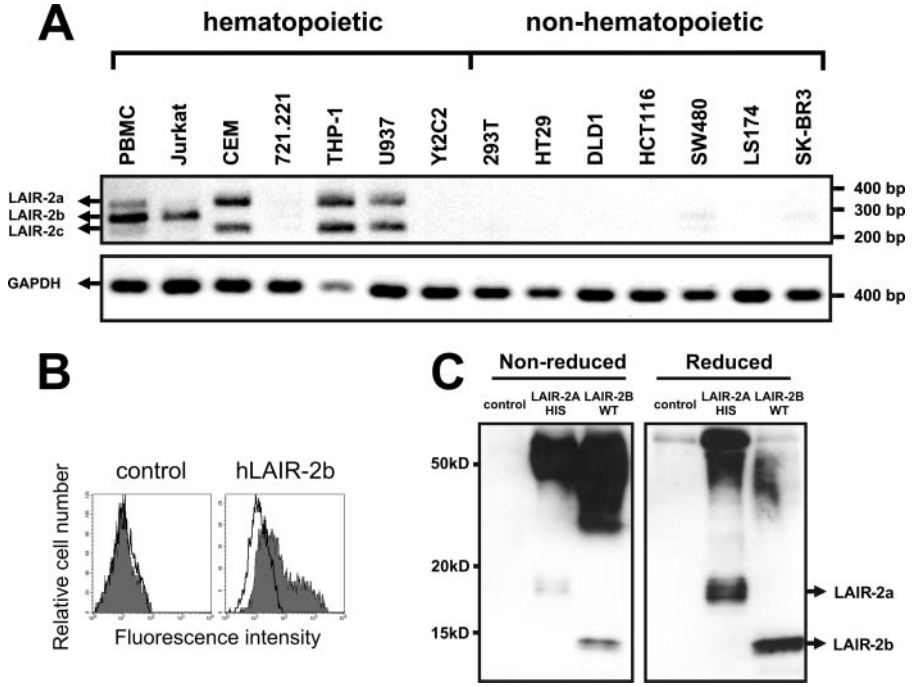

FIGURE 1. LAIR-2 is expressed as a soluble protein by various hemopoietic cell types. *A*, RNA samples obtained from human PBMCs and various human cell lines were converted to cDNA by RT-PCR. The cDNA fragments were amplified using LAIR-2- (*top panel*) or GAPDH- (*bottom panel*) specific primers. RT-PCR analysis was performed on RNA isolated from the following cells: PBMC, T cell lines Jurkat and CEM, monocytic cell lines U937 and THP-1, NK-like YT.2C2 cells, B cell line 721.221, 293T embryonic kidney cells, colon carcinoma cell lines HT29, DLD-1, LS174, SW480 and HCT116, SKBR3 breast carcinoma cells and a water control (data not shown). *B*, Permeabilized 293T cells transiently transfected with cDNA-encoding LAIR-2b (*right panel*) or control cDNA (*left panel*) were intracellularly stained using anti-LAIR-2 mAbs (filled histograms) or isotype controls (open histograms). Binding of anti-LAIR-2 mAbs was detected with PE-conjugated goat-anti-mouse IgG mAbs. *C*, Nonreducing (*left panel*) and reducing (*right panel*) Western blot analysis of supernatant from control cDNA transfected (*left lanes*), LAIR-2a-His (*middle lanes*), and LAIR-2b (*right lanes*) transfected 293T cells for presence of LAIR-2. Molecular masses are indicated.

well PBS). After washings, the plates were incubated with 3% BSA in PBS to block aspecific interactions. Meanwhile, SF were treated with hyaluronidase type IV, 20 U/ml (Sigma-Aldrich), for 20 min at 37°C to reduce viscosity. After three washes of the plate, supernatants or biological samples (urine or SF) were assayed for presence of the LAIR-2 protein. Human recombinant LAIR-2 (R&D Systems) serially diluted from 200 ng/ml was used as a reference protein. Samples were prepared in PBS containing 3% BSA and incubated for 2 h at room temperature. After three washes, the wells were incubated with a biotinylated LAIR-2 mAb (R&D Systems) for 2 h at room temperature. After washings, the wells were incubated with StreptABComplex/HRP (DakoCytomation) for 1 h and color development was performed by adding 100 μl/well ABTS reagent (Roche Diagnostics). A Mann-Whitney *U* test was performed to calculate statistical significance between the separate groups. Samples which had no detectable LAIR-2 were included in the statistical analysis; these were given the value of the detection limit (150 pg/ml). Because rheumatic factor could interfere with the LAIR-2-specific ELISA in RA SF samples, control measurements were included by replacing the anti-LAIR-2 capture Ab with an isotype-matched control Ab (BD Biosciences). The subsequent protocol was identical as described above. This isotype-matched control measurement did not result in a specific signal higher than background measurements.

Results

LAIR-2 is expressed by immune cells

To assess the cellular distribution of LAIR-2, several human cell lines were analyzed by RT-PCR specific for LAIR-2 expression. Transcripts were detected in cells of hemopoietic origin (Fig. 1*A*), corresponding with the expression pattern of hLAIR-1 (10, 4, 11, 12). LAIR-2 was detected in primary cells (PBMCs), monocytic and T cell lines, but not in nonhemopoietic cell lines. Besides the previously described LAIR-2a and LAIR-2b (14) isoforms, an additional product was evident after RT-PCR (Fig. 1*A*). Cloning, sequencing, and subsequent sequence alignment of this product indicated it was a third splice variant, which we designated LAIR-2c. LAIR-2c lacks 93 bp at the 3′end of exon 3, encoding a putative protein with a 31-aa deletion as compared with LAIR-2a. The 31-aa deletion encompasses a region in the hLAIR-1 protein where one of the conserved cysteines involved in intradomain disulfide bond formation is located (14). Thus, this splice variant does not encode an intact Ig-like domain. The DNA sequence of LAIR-2c was deposited in the GenBank database under accession number EF174570.

LAIR-2 is a secreted protein

The presence of a signal sequence and the lack of a transmembrane and cytoplasmic region suggests that LAIR-2 is a secreted protein (10). To detect expression of the LAIR-2 protein, anti-LAIR-2 mAbs were generated which specifically stained 293T cells transfected with LAIR-2, but not untransfected cells (Fig. 1*B*). The Abs did not cross-react with human LAIR-1 (Table I). As expected, LAIR-2 was present in the supernatant of 293T cells transiently transfected with LAIR-2, but was not detected in the supernatant of untransfected cells (Fig. 1*C*).

Monomeric LAIR-2a has a predicted molecular mass of ~16.3 kDa and the protein contains 10 putative *O*-linked, but no *N*-linked, sites, whereas LAIR-2b has a predicted molecular mass of ~14.7 kDa containing four putative *O*-linked glycosylation sites (data not shown). On Western blot, LAIR-2a (with His-tag) appeared as a protein of ~18 kDa, while LAIR-2b ran slightly below 15 kDa, which would fit with the predicted size (Fig. 1*C*). In addition a specific smear could be detected on nonreducing Western blot, ranging from an apparent molecular mass of ~70 to ~30

kDa. This most likely represented multimers of the protein, because the smear largely disappeared if the samples were run under reducing conditions. We conclude that the *LAIR-2* gene encodes a secreted protein.

LAIR-2 is a high-affinity collagen receptor

Recently, we identified collagens as high-affinity ligands for the LAIR-1 molecules (16). The collagen super family comprises 28 trimeric molecules each composed of three polypeptide α-chains, which contain the sequence repeat $(Gly\text{-}X\text{-}Y)_n$, X being frequently proline (P) and, after posttranslational modification, Y being hydroxyproline (O) (22). Besides hLAIR-1, LAIR-2-IgG fusion proteins interacted with transmembrane collagens XIII, XVII, and XXIII (16), suggesting that all LAIR molecules bind the same collagen molecules as ligand. To determine whether LAIR-2 has the potential to serve as a competitor for hLAIR-1 binding by blocking binding sites on human collagens, we measured binding and affinity of LAIR-2-IgG fusion proteins to collagen by surface plasmon resonance (BIAcore). As expected, LAIR-2 fusion proteins bind with high affinity of 34.6 nM ($\pm$3.5) and 41.4 nM ($\pm$3.7) to collagen I and III, respectively (Fig. 2*A*). Each collagen molecule has ~10 binding sites for LAIR-2 (Fig. 2*A*). The dissociation of LAIR-2 is biphasic and characterized by a rapid initial phase and a slower secondary phase (Fig. 2*B*).

The GPO triplet is almost exclusively present in collagenous molecules, where it comprises ~10% of the mature collagen sequence, and allows the formation of a triple helix (22). Like hLAIR-1, LAIR-2 binds common collagen motifs in a hydroxyproline-dependent manner, because it interacts with trimeric peptides containing 10 repeated GPO triplets ($(GPO)_{10}$ (23)), but not with the control $(GPP)_{10}$ trimeric peptide (Fig. 2*C*).

LAIR-2 prevents binding of hLAIR-1 to its ligand

Because LAIR-2 is a secreted protein and interacts with the same collagen molecules as hLAIR-1, LAIR-2 may function as a competitor of the hLAIR-1/collagen interaction by binding the same sites on human collagen molecules. To investigate this hypothesis, we performed LAIR-blocking experiments using human HT29 colon carcinoma cells expressing endogenous hLAIR-1 ligand(s) (16, 19, 20). Incubation of HT29 cells with biotinylated hLAIR-1-IgG fusion proteins resulted in specific staining of the cells, whereas preincubation of these cells with unlabeled LAIR-2-IgG decreased the biotinylated hLAIR-1-IgG staining (Fig. 3*A*, *top panel*). In the reverse experiment, preincubation of HT29 cells with hLAIR-1-IgG reduced the staining with biotinylated LAIR-2-IgG (Fig. 3*A*, *bottom panel*). This indicates that both human LAIR-1 and LAIR-2 fusion proteins bind the same sites on the naturally expressed collagen molecules on HT29 cells.

Additionally, Oregon Green-labeled collagen IV bound specifically to K562 cells transfected with hLAIR-1 (Fig. 3*B*, *upper panel*). This interaction was efficiently prevented by preincubation of the fluorescent collagen IV with soluble LAIR-2-IgG fusion proteins (Fig. 3*B*).

As documented before, hLAIR-1-transfected K562 cells bind firmly to plate-bound collagens I and III (16). Preincubation of plate-bound collagens I and III with LAIR-2-IgG fusion proteins blocked the binding of hLAIR-1 expressing K562 cells to collagens in a dose-dependent manner (Fig. 4*A*, *left panel*), while incubation with an irrelevant fusion protein had no effect (Fig. 4*A*, *right panel*). To assess whether wild-type LAIR-2 protein is capable of interfering with the hLAIR-1/collagen interaction, we concentrated supernatant from LAIR-2-transfected 293T cells and analyzed its blocking capacity in the above described adhesion assay. As expected, wild-type LAIR-2 present in the supernatant (Fig. 4*B*, *left panel*), but not control supernatant (Fig. 4*B*, *right panel*), blocked binding of stable human LAIR-1 expressing K562 cells to plate-bound collagen I and III. Thus, LAIR-2 binds the same sites on various collagen species as hLAIR-1, suggesting that LAIR-2 may function as a soluble competitor to hLAIR-1/collagen binding in vivo.

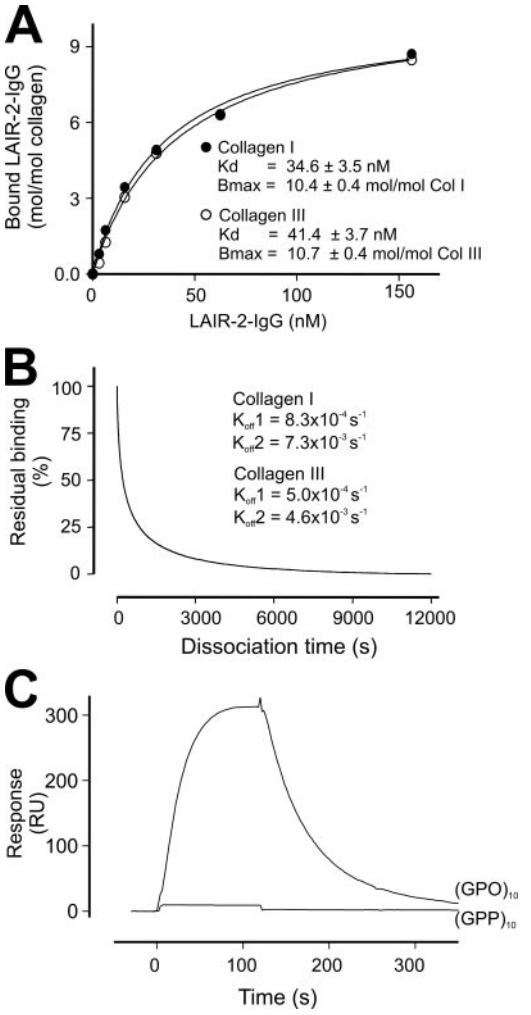

FIGURE 2. LAIR-2 is a high-affinity collagen receptor. *A*, Binding of LAIR-2 to collagen I and III as indicated by surface plasmon resonance. LAIR-2-IgG concentrations were injected at 20 μl/min sequentially through a BIAcore flow cell containing ~2000–3000 RU of immobilized collagen I (●) or III (○). Each symbol represents the resonance at equilibrium and the corresponding concentration of the LAIR-2 fusion protein. Calculated affinities (K_d) and number of maximal binding sites (Bmax) are indicated. *B*, Rate of dissociation of LAIR-2-IgG from collagen III as determined by surface plasmon resonance. The dissociation curve of LAIR-2-IgG from collagen I was comparable (data not shown). Calculated dissociation values are indicated. *C*, LAIR-2 fusion protein was injected at 5 μl/min through a BIAcore flow cell containing ~250 RU of immobilized $(GPO)_{10}$ or $(GPP)_{10}$ trimeric peptides.

LAIR-2 prevents hLAIR-1 cross-linking by collagens

By using NFAT-GFP reporter cells expressing a chimeric protein containing the extracellular domain of hLAIR-1 and the transmembrane and intracellular domain of CD3ζ, we have shown that collagen I and III are capable to functionally cross-link hLAIR-1 (16). We used this system to investigate whether LAIR-2-IgG can interfere with the activation of hLAIR-1-CD3ζ. Receptor engagement of cells expressing the hLAIR-1-CD3ζ chimera, but not the

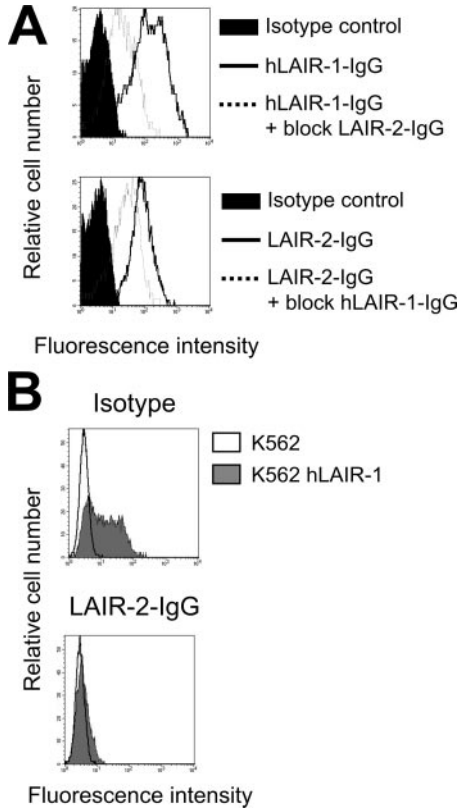

FIGURE 3. LAIR-2-IgG blocks the interaction of hLAIR-1 to endogenous collagens on HT29 cells and purified extracellular matrix collagen IV. *A*, Human colon carcinoma HT29 cells were preincubated for 30 min with LAIR-2-IgG (*top panel*) or hLAIR-1-IgG (*lower panel*) and subsequently stained with biotin-conjugated human LAIR fusion proteins. Specific binding of the biotinylated fusion proteins was detected with allophycocyanin-conjugated streptavidin. *B*, Parental (open histograms) or human LAIR-1-transfected K562 cells (closed histograms) were incubated with Oregon Green 488-labeled collagen IV which was either preincubated with 40 μg/ml LAIR-2-IgG (*lower panel*) or control Ig fusion proteins (isotype, *top panel*). Cells were analyzed by flow cytometry.

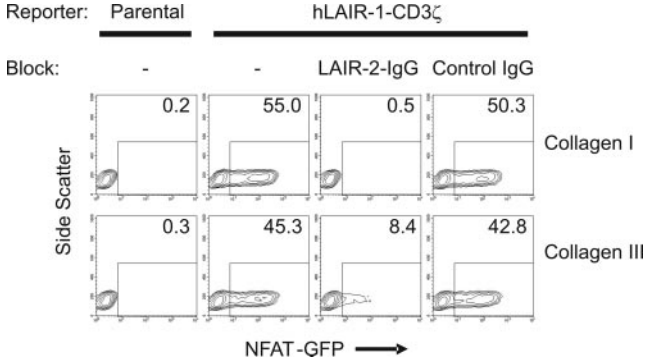

FIGURE 5. LAIR-2 blocks the functional interaction between LAIR-1 and collagen I and III. NFAT-GFP reporter cells (21) transfected with (*right three panels*) or without hLAIR-1-CD3ζ chimeric molecules (*left panels*) were incubated with immobilized collagen I (*top panels*) or III (*lower panels*) that were preincubated with or without LAIR-2-IgG or control fusion proteins. After ~20 h, GFP expression was analyzed by flow cytometry. Percentage of GFP-positive cells is indicated in each plot.

parental cells, via plate-bound collagens I and III resulted in expression of GFP (Fig. 5). Preincubation of plate-bound collagen I and III with LAIR-2-IgG fusion proteins, but not isotype matched control IgG, abrogated the NFAT-GFP activation (Fig. 5), demonstrating that LAIR-2-IgG is also capable of blocking the functional interaction between hLAIR-1 and collagens.

In conclusion, five independent assays confirmed that LAIR-2 has the capacity to interfere with human LAIR-1 binding to various classes of collagen molecules, suggesting that the protein may have a similar function in vivo.

LAIR-2 is produced by primary cells and in vivo

To allow detection of wild-type LAIR-2 in human body fluids, we developed a LAIR-2 specific sandwich ELISA that could detect LAIR-2 in solution as low as 150 pg/ml (Fig. 6*A*), but showed no cross-reactivity for hLAIR-1 (data not shown). Soluble LAIR-2 was detected in the supernatant from 293T cells transiently transfected with LAIR-2, but not from untransfected cells (data not shown).

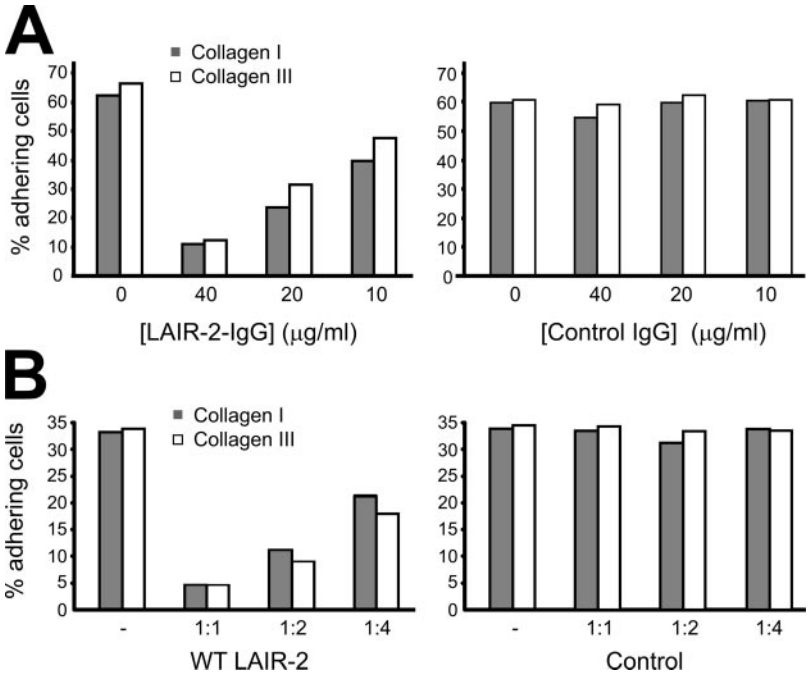

FIGURE 4. LAIR-2 blocks the binding of hLAIR-1 to collagens I and III. *A*, Ninety-six-well plates were coated with collagen I (▨) or collagen III (□). Subsequently, the wells were incubated with different indicated concentrations of LAIR-2 fusion proteins (*left panel*) or control fusion proteins (*right panel*). Fluorescently labeled K562 cells expressing hLAIR-1 were allowed to interact for ~3 h. Percentage of adhering cells relative to the input signal is shown. Cells did not adhere to wells coated with BSA. *B*, Concentrated supernatants of 293T cells transfected with LAIR-2 (WT LAIR-2, *left panel*) or control cDNA (*right panel*), were analyzed for their capacity to block adhesion of hLAIR-1 transfected K562 cells to immobilized collagens I and III, as described above. The dilution of concentrated supernatants is indicated. Concentrated supernatant of the LAIR-2 transfectant contained ~400 μg/ml LAIR-2 as determined by the ELISA described in Fig. 6.

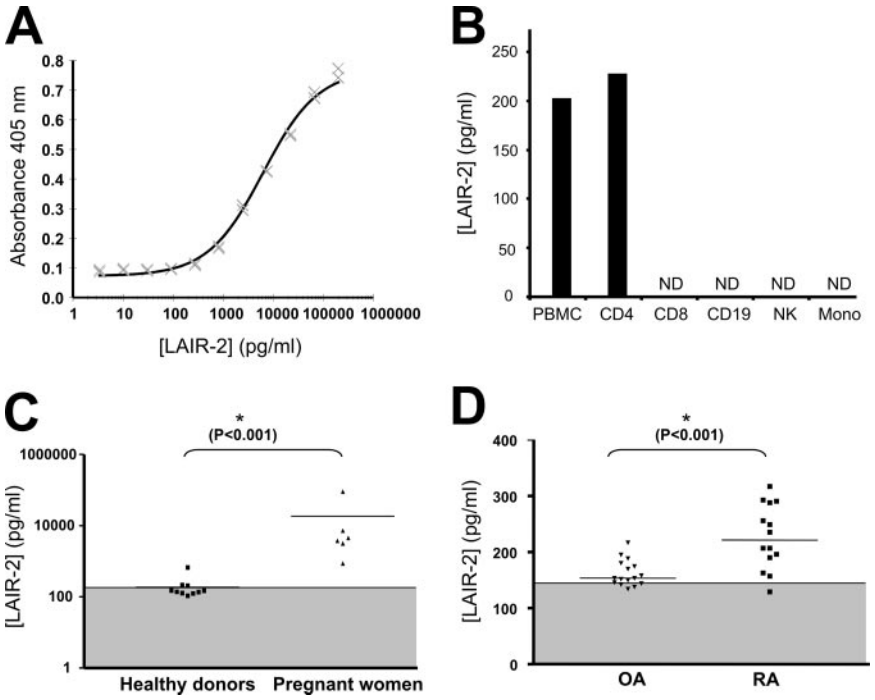

FIGURE 6. LAIR-2 is produced in vitro and in vivo. *A*, Calibration curve of LAIR-2 ELISA. The plotted values were obtained with 3, 10, 30, 91, 274, 823, 2.5×10^3, 7.4×10^3, 2.2×10^4, 6.7×10^4 and 2.0×10^5 pg/ml rLAIR-2 proteins (R&D Systems). Detection limit is 150 pg/ml. *B*, LAIR-2 production by sorted PBMC. Cells were sorted into $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD19^+$ B cells, $CD3^-CD56^+$ NK cells, and $CD3^-CD14^+$ monocytes and cultured for 4 days in the presence of PMA and ionomycin. Supernatants were harvested and LAIR-2 was detected by ELISA. Data are representative of two donors. In the absence of PMA and ionomycin, no LAIR-2 was detected in all cases. ND, not detectable. *C*, Presence of LAIR-2 in urine of nonpregnant donors (five men and five women) and six pregnant women (16–37 wk of gestation). Measurements were performed by ELISA as described in *Materials and Methods*. A Mann-Whitney *U* test indicated a statistical significant elevation of LAIR-2 levels in urine of pregnant women as compared with healthy controls ($p < 0.001$). *D*, Presence of LAIR-2 in synovial fluid of patients with rheumatoid arthritis (RA) or osteoarthritis (OA). A Mann-Whitney *U* test indicated a statistical significant elevation of LAIR-2 levels in synovial fluid of RA patients as compared with OA patients ($p < 0.001$). Shaded areas indicate the detection limit in *C* and *D*.

In vitro stimulation of PBMC with PMA and ionomycin resulted in detectable LAIR-2 expression at day 4 after stimulation in all donors tested (average 774 pg/ml, range 150-2652 pg/ml, $n = 5$). In some donors, LAIR-2 was detected after stimulation with anti-CD3 and -CD28 Abs, but this production was much lower (data not shown). To determine which cell type was producing LAIR-2, we sorted cells into $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^-CD19^+$ B cells, $CD3^-CD56^+$ NK cells, and $CD3^-CD14^+$ monocytes and subsequently assessed LAIR-2 production 4 days after stimulation with PMA and ionomycin. This indicated that $CD4^+$ T cells were the main producers of LAIR-2, while the other cell types did not produce detectable LAIR-2 levels, although in one donor the ELISA signal of the $CD8^+$ T cells was slightly above background level (Fig. 6*B*).

The protein was not detected in plasma and serum from healthy individuals and pregnant women. Unexpectedly, LAIR-2 was present in large amounts in urine from pregnant women (average: 7108 pg/ml, range: 712–20877 pg/ml), whereas the Ag was not detected in urine of men and nonpregnant women (average: 209 pg/ml, range: 150 (below detection level)-695 pg/ml) (Fig. 6*C*). Although high levels of LAIR-2 were detected in urine, LAIR-2 levels in serum of pregnant women were still below detection level of our ELISA system (data not shown). Hence we conclude that LAIR-2 is produced as a soluble protein in vivo and that the molecule can be cleared from the body via urine.

To explore whether LAIR-2 is present at sites of inflammation, we measured LAIR-2 in SF of patients with RA ($n = 14$) (Fig. 6*D*). LAIR-2 levels were compared with those found in SF from patients with OA ($n = 16$) that suffer from joint degeneration with no to mild inflammation. LAIR-2 concentrations in SF from RA patients were elevated (average: 229 pg/ml, range: 150–318 pg/ml) as compared with that in OA patients (average: 165 pg/ml, range: 150–216 pg/ml). Thus, increased LAIR-2 levels in RA SF seem to reflect the local inflammation in the joints of these patients.

Discussion

Given the broad expression profile of hLAIR-1 on immune cells (10) and the high abundance of collagen molecules in the human body (17), one can envisage that the interaction of this inhibitory receptor with its ligand is regulated. In this manuscript, we show that wild-type and chimeric LAIR-2 molecules bind the same collagen molecules as the transmembrane inhibitory receptor hLAIR-1 and that this soluble receptor can block the interaction of hLAIR-1 to both transmembrane and extracellular collagens. Hence, LAIR-2 may function as a competitor for human LAIR-1 in vivo, thereby regulating the inhibitory potential of this receptor. A similar regulatory role for soluble variants of receptors has been described for other, mainly cytokine, receptors. These soluble cytokine receptors either attenuate or promote cytokine signaling by decreasing receptor-mediated signal generation or by stabilizing ligands in the extracellular milieu (8, 24).

Soluble receptors can be generated by several means: proteolytic cleavage of receptor ectodomains, alternative splicing of mRNA transcripts, release of full-length receptors within the context of exosome-like vesicles, and cleavage of GPI-anchored receptors (reviewed in Refs. 8 and 24). Additionally, soluble receptors can be encoded by distinct genes that share homology with

transmembrane receptors. Although several genes encoding soluble cytokine receptors have been described (reviewed in (8)), this is less common for inhibitory ITIM-bearing immune receptors. A single member of the leukocyte Ig-like receptor (LILR) family of immune receptors (LILR3, also known as CD85e, Ig-like transcript 6 or leukocyte Ig-like receptor 4) lacks cytoplasmic or transmembrane domains and is thus a putative secreted molecule (25–27). Additionally, KIR3DP1 is a potential soluble member of the killer cell Ig-like receptor (KIR) family in humans. The *KIR3DP1* gene is normally silent in humans, however ~4.5% of the Caucasian population bears a recombinant allele that equips the gene with an active promoter causing it to be transcribed in these individuals (28). Nevertheless, the LILRA3 or KIR3DP1 proteins have not yet been detected in humans and it remains unknown whether the molecules can interact with the same ligands as the membrane-bound receptors to regulate their function (28, 29). Hence, our study on LAIR-2 is the first proof of a homolog of an inhibitory immune receptor expressed as a protein in humans that has the potential to bind the same ligands as its membrane-bound counterpart.

Because human LAIR-1 and LAIR-2 bind to the same collagen molecules, both receptors may compete for the same functional ligand(s) in vivo. We have shown by surface plasmon resonance that LAIR-2 is a high affinity receptor for collagens and that its affinity for human collagens I and III is comparable to that of hLAIR-1. Hence, a small variation in concentration of LAIR-2 in vivo could greatly impact the binding capacity of hLAIR-1 to collagens. By increasing the concentration of soluble LAIR-2 at a site of infection, the inhibitory capacity of membrane-bound hLAIR-1 may be subsided due to a blockage of the hLAIR-1 binding to collagens. Vice versa, a reduced concentration of LAIR-2 may result in hLAIR-1 binding to collagens, thereby allowing the inhibitory receptor to signal at sites where immune activation should be avoided.

In addition to using dimeric Ig-fusion proteins, we also demonstrate that recombinant native LAIR-2 can block the adhesion of LAIR-1 expressing cells to collagen, although higher concentrations were needed for efficient blocking. However, one could envisage that even in the presence of low levels of LAIR-2 in the circulation, locally the LAIR-2 concentrations in extracellular spaces are sufficient to antagonize LAIR-1-collagen binding.

We demonstrate that pregnant women secrete high amounts of LAIR-2 in their urine. Although the glomerular filtration rate increases during pregnancy (30), we measured comparable amounts of albumin in urine of healthy controls and pregnant women (data not shown). Hence, presence of a high concentration of LAIR-2 cannot be explained by a general increase of protein excretion during pregnancy. Presence of soluble forms of receptors in urine has also been described for other membrane receptors such as soluble (s) IL-13-binding protein (31), sTNF receptors (32), sCD58 (33), sIL-6 receptor (34), sIFN-γ receptor (34), sIL-2 receptor (35), and sCD27 (36). Like for LAIR-2, urine levels of sCD58 and sTNF receptors in healthy pregnant women are significantly elevated compared with that of non pregnant, female controls (32, 37). Because many inflammatory factors are enhanced in pregnant women, it has been postulated that pregnancy represents a state of controlled systemic inflammation (reviewed in Ref. 38). Whether a high urinary secretion of LAIR-2 during pregnancy is a reflection of increased synthesis of this protein in vivo, and/or caused by active specific secretion of the protein remains to be determined.

Interestingly, LAIR-2 levels were elevated in the joints of patients suffering from RA as compared with patients diagnosed with OA. RA is a chronic autoimmune disease that is characterized by a persistent inflammation of the joints, which results in chronic tissue destruction. In contrast, OA is a result of both mechanical and biologic events that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes, extracellular matrix, and subchondral bone and are often characterized by only moderately inflamed tissue. Increased LAIR-2 levels in the joints of RA patients therefore are associated with increased inflammation, probably accompanied with the presence of more inflammatory cells. Thus, LAIR-2 may function as a proinflammatory mediator by decreasing the inhibitory potential of the immune inhibitor hLAIR-1, resulting in more highly activated immune cells, a hallmark for autoimmune diseases such as RA. In vitro stimulation of sorted peripheral blood cells revealed that $CD4^+$ T cells are the main producers of LAIR-2. This does however not exclude other cell types as LAIR-2 producers in vivo.

To conclude, we show that LAIR-2 is a soluble, high-affinity collagen receptor expressed in humans. Our data reveal a novel mechanism of in vivo immune regulation, in which LAIR-2 may regulate the inhibitory potential of the membrane-bound hLAIR-1 via competing for the same ligands.

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Disclosures

R. J. Lebbink, N. Raynal, and L. Meyaard are named as inventors on a patent application on the LAIR-collagen interaction.

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