LAIR and collagens in immune regulation

Robert Jan Lebbink

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(met een samenvatting in het Nederlands)

Proefschrift

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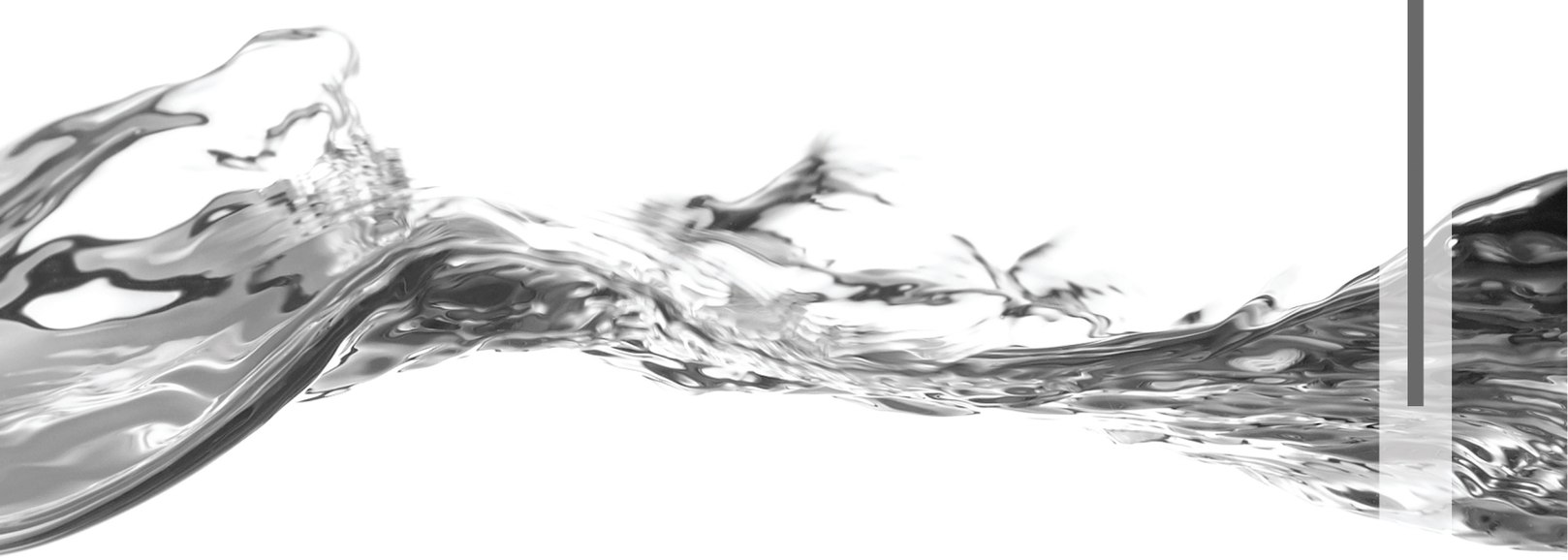
1 CHAPTER

Introduction to LAIR-1 and scope of this thesis



The immune system constantly receives opposing signals that on the one side activate immune cells allowing them to eradicate diseased cells and pathogens, and on the other side inhibit these same cells to limit and ultimately terminate an immune response[1;2]. A correct balance is crucial for effective defence against pathogens without affecting healthy tissues. One of the mechanisms of negative regulation in the immune system is provided by specialized inhibitory receptors that upon interaction with their ligands attenuate activation signals initiated by activating receptors. Loss of inhibitory signaling is often associated with autoreactivity and unchecked inflammatory responses, illustrating the essential role these systems play in immune regulation[1;2]. This thesis is focused on the function of such an inhibitory receptor called leukocyte-associated immunoglobulin-like receptor (LAIR)-1.

Structure of LAIR-1

Human LAIR-1 (Leukocyte associated Ig-like receptor-1, CD305) is a type I transmembrane glycoprotein of 287 amino acids containing a single extracellular Ig-like domain and two Immuno receptor Tyrosine-based Inhibitory Motifs (ITIMs) in its cytoplasmic tail[3]. Almost all inhibitory immune receptors contain one or several ITIMs in their cytoplasmic tail, which serve as docking sites for downstream effectors that mediate the inhibition of cellular action, such as proliferation, differentiation and cytotoxicity. These effectors include the phosphatases SHP-1,2 and SHIP as well as the tyrosine kinase Csk[4;5]. LAIR-1 is structurally related to several other inhibitory immunoglobulin superfamily (IgSF) members, including human killer cell Ig-like receptors (KIRs), human FcαR, human leukocyte Ig-like receptors (LILRs, also known as Ig-like transcripts (ILTs), leukocyte Ig-like receptors (LIRs), monocyte-macrophage inhibitory receptors (MIRs) and CD85), mouse gp91 or paired Ig-like receptors (PIRs) and mouse gp49[3]. Interestingly, LAIRs, KIRs, LILRs, and FcαR are all localized to the leukocyte receptor complex (LRC) on human chromosome 19q13.4, suggesting that these molecules have evolved from a common ancestral gene (reviewed in [6;7]). *LAIR-1* consists of 10 exons and shows considerable homology to *LAIR-2* (CD306). The *LAIR-2* gene encodes a protein that is ~84% homologous to human LAIR-1, but lacks a transmembrane and intracellular domain suggesting it is a secreted protein[3;8]. The *LAIR* genes lie close together in the LRC and are transcribed in opposite directions, suggesting that one locus arose from the other by a large genomic inverse duplication event[9]

Several different splice variants of the LAIR-family have been cloned (Fig. 1). LAIR-1b and LAIR-2b lack 17 amino acids in the stalk region between the transmembrane and Ig-like domain as compared to the full-length LAIR-1a and LAIR-2a forms, which may affect their glycosylation[8]. In addition, LAIR-1c is identical to LAIR-1b except for a single amino acid change in the extracellular domain, and LAIR-1d lacks part of the intracellular tail[10].

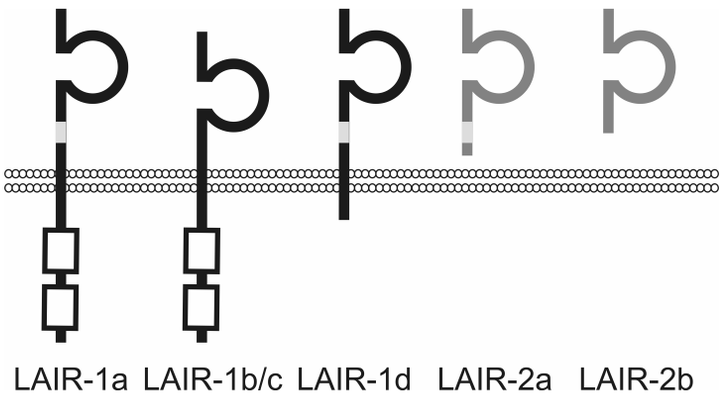


Figure 1. Schematic representation of the human LAIR family. Different isoforms of LAIR-1 (black) and LAIR-2 (dark grey) are shown. The ITIMs in LAIR-1 are represented by white boxes. The 17 amino acid region which is lacking in LAIR-1b, LAIR-1c and LAIR-2b is indicated in light grey. LAIR-1c is identical to LAIR-1b, with the exception of a single amino acid change (not depicted).

Distribution and function of LAIR-1

LAIR-1 is expressed on the majority of peripheral blood mononuclear cells, including NK cells, T cells, B cells, monocytes, eosinophils, and dendritic cells, as well as the majority of thymocytes and CD34+ hematopoietic progenitor cells[3;11-13]. Furthermore, the receptor is inducibly expressed on blood neutrophils[11] and differentially expressed on B[14] and T[15;16] cells. The receptor is absent on resting mature neutrophils, platelets or non-haematopoietic cells[3].

In accordance with the presence of two ITIMs in its cytoplasmic tail, LAIR-1 functions as an inhibitory immune receptor[3]. Upon cross linking of the receptor with monoclonal antibodies (mAbs), the tyrosines in the cytoplasmic ITIMs of LAIR-1 become phosphorylated by a yet unknown kinase[3;17] and these subsequently recruit the SH2 domain-containing phosphatases SHP-1, SHP-2 and the C-terminal Src kinase (Csk)[3;11;17] as downstream effectors that mediate the inhibition of cellular action. Mutation of the tyrosines in the LAIR-1 tail demonstrated that both ITIMs of LAIR-1 are simultaneously required for full inhibition of cellular responses[17]. Furthermore, LAIR-1 has phosphatase-independent function, since LAIR-1 can still inhibit immune responses in SHP-1, SHP-2 and SHIP-deficient cell lines[4]. The kinase Csk may be the key downstream effector of LAIR-1 in cells where phosphatase activity is limited[4].

LAIR-1 cross-linking via mAbs can inhibit the function of various immune cell types *in vitro*. Cross-linking of LAIR-1 on human NK cells delivers a potent inhibitory signal that is capable of inhibiting target cell lysis by both resting and activated NK cells[3;12]. Furthermore, LAIR-1 can inhibit the cytotoxic activity of effector T cells upon CD3 cross-linking[8;15;18]. Cross-linking LAIR-1 inhibits FcγRII induced calcium mobilization in U937 cells[19] and BCR induced calcium mobilization in B cells[14]. In addition, LAIR-1 inhibits the differentiation of peripheral blood precursors towards dendritic cells[20] and the receptor prevents proliferation and induces apoptosis in human myeloid leukemia cell

lines and primary leukemias[21;22]. Thus, LAIR-1 is a broadly expressed inhibitory immune receptor that potentially modulates many aspects of immune cell function.

Scope of this thesis

Although the inhibitory potential and mechanisms of LAIR-1 inhibition are well established, the actual function of the receptor *in vivo* remains unknown due the lack of animal models and the unknown identity of the LAIR-1 ligand.

This thesis first focuses on the identification and characterization of LAIR-1 orthologues in non-human species as a prerequisite to develop animal models that dissect the role of the LAIR-1 receptors in regulation of immune responses. In Chapter 2 and 3 we document the identification and initial characterization of the mouse and rat orthologues of human LAIR-1 respectively. For further study of LAIR-1 biology, identification of its ligand(s) is imperative. Chapter 4 describes the identification of collagens as high affinity, functional ligands for the LAIR-1 proteins. The collagen/LAIR-1 interaction is examined in more detail by using synthetic trimeric collagen peptides in chapter 5. Besides the identification of collagens as ligands for LAIR-1, recent years has also led to the discovery of ligands for other inhibitory immune receptors. These recent developments are reviewed in chapter 6. To support *in vivo* studies in mice, a detailed comparison of the expression profile and ligand binding of human and mouse LAIR-1 is given in chapter 7. In chapter 8 we study the biology of human LAIR-2, a putative soluble competitor of the collagen/LAIR-1 interaction. Finally, in chapter 9, the findings of this thesis are summarized and discussed.

REFERENCES

1. Lanier,L.L. 2005. NK Cell Recognition. *Annu. Rev. Immunol.*225-274.
2. Ravetch,J.V. and L.L.Lanier. 2000. Immune inhibitory receptors. *Science* 290:84-89.
3. Meyaard,L., G.J.Adema, C.Chang, E.Woollatt, G.R.Sutherland, L.L.Lanier, and J.H.Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
4. Verbrugge,A., E.S.Rijkers, T.de Ruiter, and L.Meyaard. 2006. Leukocyte-associated Ig-like receptor-1 has SH2 domain-containing phosphatase-independent function and recruits C-terminal Src kinase. *Eur. J. Immunol.* 36:190-198.
5. Sayos,J., A.Martinez-Barriocanal, F.Kitzig, T.Bellon, and M.Lopez-Botet. 2004. Recruitment of C-terminal Src kinase by the leukocyte inhibitory receptor CD85j. *Biochem. Biophys. Res. Commun.* 324:640-647.
6. Barten,R., M.Torkar, A.Haude, J.Trowsdale, and M.J.Wilson. 2001. Divergent and convergent evolution of NK-cell receptors. *Trends Immunol.* 22:52-57.
7. Martin,A.M., J.K.Kulski, C.Witt, P.Pontarotti, and F.T.Christiansen. 2002. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol.* 23:81-88.
8. Meyaard,L., J.Hurenkamp, H.Clevers, L.L.Lanier, and J.H.Phillips. 1999. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. *J. Immunol.* 162:5800-5804.
9. Wende,H., A.Volz, and A.Ziegler. 2000. Extensive gene duplications and a large inversion characterize the human leukocyte receptor cluster. *Immunogenetics* 51:703-713.
10. Xu,M., R.Zhao, and Z.J.Zhao. 2000. Identification and characterization of leukocyte-associated Ig-like receptor-1 as a major anchor protein of tyrosine phosphatase SHP-1 in hematopoietic cells. *J. Biol. Chem.* 275:17440-17446.
11. Verbrugge,A., T.de Ruiter, C.Geest, P.J.Coffer, and L.Meyaard. 2006. Differential expression of leukocyte-associated Ig-like receptor-1 during neutrophil differentiation and activation. *J. Leukoc. Biol.* 79:828-836.
12. Poggi,A., N.Pella, L.Morelli, F.Spada, V.Revello, S.Sivori, R.Augugliaro, L.Moretta, and A.Moretta. 1995. p40, a novel surface molecule involved in the regulation of the non-major histocompatibility complex-restricted cytolytic activity in humans. *Eur. J. Immunol.* 25:369-376.
13. Ouyang,W., D.Ma, D.Lin, Y.Sun, X.Liu, Q.Li, W.Jia, Y.Cao, Y.Zhu, and B.Jin. 2003. 9.1C3 is identical to LAIR-1, which is expressed on hematopoietic progenitors. *Biochem. Biophys. Res. Commun.* 310:1236-1240.
14. van der Vuurst de Vries AR, H.Clevers, T.Logtenberg, and L.Meyaard. 1999. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur. J. Immunol.* 29:3160-3167.
15. Maasho,K., M.Masilamani, R.Valas, S.Basu, J.E.Coligan, and F.Borrego. 2005. The inhibitory leukocyte-associated Ig-like receptor-1 (LAIR-1) is expressed at high levels by human naive T cells and inhibits TCR mediated activation. *Mol. Immunol.* 42:1521-1530.
16. Jansen, C. A., Cruijsen, C., de Ruiter, T., Nanlohy, N., Willens, N, Janssens-Korpela, P. L, and Meyaard, L. Regulated expression of the inhibitory receptor LAIR-1 during human T-cell activation and differentiation. 2006. Submitted for publication.
17. Verbrugge,A., T.de Ruiter, H.Clevers, and L.Meyaard. 2003. Differential contribution of the Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) of human Leukocyte Associated Ig-like Receptor (LAIR)-1 to inhibitory function and phosphatase recruitment. *Int. Immunol.* 15:1349-1358.
18. Poggi,A., E.Tomasello, V.Revello, L.Nanni, P.Costa, and L.Moretta. 1997. p40 molecule regulates NK cell activation mediated by NK receptors for HLA class I antigens and TCR-mediated triggering of T lymphocytes. *Int. Immunol.* 9:1271-1279.

19. Fournier,N., L.Chalus, I.Durand, E.Garcia, J.J.Pin, T.Churakova, S.Patel, C.Zlot, D.Gorman, S.Zurawski, J.Abrams, E.E.Bates, and P.Garrone. 2000. FDF03, a novel inhibitory receptor of the immunoglobulin superfamily, is expressed by human dendritic and myeloid cells. *J. Immunol.* 165:1197-1209.
20. Poggi,A., E.Tomasello, E.Ferrero, M.R.Zocchi, and L.Moretta. 1998. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. *Eur. J. Immunol.* 28:2086-2091.
21. Poggi,A., F.Pellegatta, B.E.Leone, L.Moretta, and M.R.Zocchi. 2000. Engagement of the leukocyte-associated Ig-like receptor-1 induces programmed cell death and prevents NF-kappaB nuclear translocation in human myeloid leukemias. *Eur. J. Immunol.* 30:2751-2758.
22. Zocchi,M.R., F.Pellegatta, I.Pierri, M.Gobbi, and A.Poggi. 2001. Leukocyte-associated Ig-like receptor-1 prevents granulocyte-monocyte colony stimulating factor-dependent proliferation and Akt1/PKB alpha activation in primary acute myeloid leukemia cells. *Eur. J. Immunol.* 31:3667-3675.

2
CHAPTER

The mouse homologue of LAIR-1 is an inhibitory receptor that recruits SHP-2, but not SHP-1

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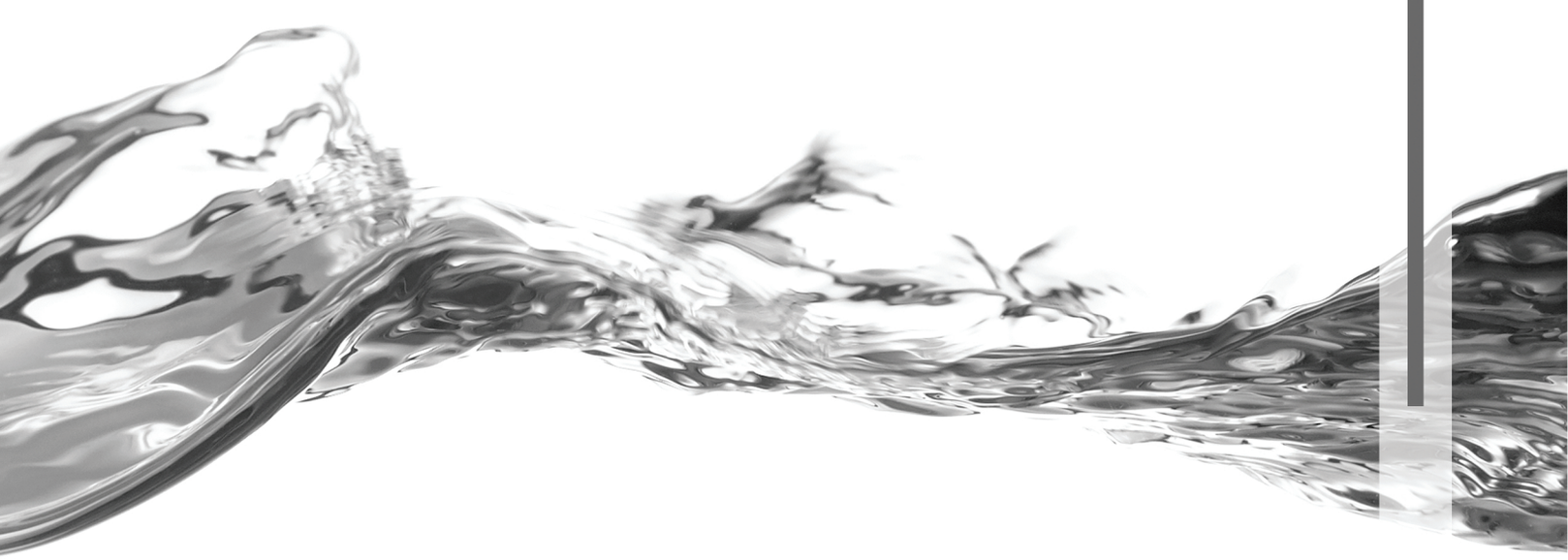

ABSTRACT

We report the molecular cloning and characterization of the first leukocyte-associated Ig-like Receptor (LAIR)-1 homologue in mice that we have named mouse LAIR-1 (mLAIR-1). The *mLAIR-1* gene maps to the proximal end of mouse chromosome 7 in a region syntenic with human chromosome 19q13.4 where the leukocyte receptor cluster is located. The protein shares 40% sequence identity with human LAIR-1, has a single Ig-like domain and contains two immunoreceptor tyrosine-based inhibitory motif-like structures in its cytoplasmic tail. Mouse LAIR-1 is broadly expressed on various immune cells, and cross-linking of the molecule on stably transfected RBL-2H3 and YT.2C2 cells results in strong inhibition of their degranulation and cytotoxic activity respectively. Upon pervanadate stimulation, the mLAIR-1 cytoplasmic tail becomes phosphorylated, thereby recruiting Src homology region 2-containing tyrosine phosphatase-2. Interestingly, unlike human LAIR-1, Src homology region 2-containing tyrosine phosphatase-1 is not recruited to the mLAIR-1 cytoplasmic tail. Screening human and mouse cell lines for mLAIR-1 and human LAIR-1 binding partners identified several lines expressing putative ligand(s) for both receptors.

INTRODUCTION

Immune responses are tightly controlled by the activities of both activating and inhibitory signals. Inhibitory receptors (reviewed in [1]) often bear immunoreceptor tyrosine-based inhibitory motifs (ITIMs)[3] in their cytoplasmic domain with a I/V/L/SxYxxL/V consensus sequence. Ligand-induced clustering of these inhibitory receptors results in tyrosine phosphorylation of the ITIM's central tyrosine residue, providing a docking site for the recruitment of cytoplasmic protein tyrosine phosphatases such as Src homology region 2-containing tyrosine phosphatase (SHP)-1, SHP-2 and SH2-containing inositol 5'-phosphatase (SHIP). These phosphatases abrogate signaling through activating receptors, thereby preventing cellular immune functions such as cytotoxicity or proliferation. In the last few years, many families of inhibitory receptors bearing ITIMs have been recognized and cloned both in humans and mice (reviewed in [2]).

The leukocyte-associated Ig-like receptor (LAIR)-1 is a member of the Ig superfamily (IgSF) that is expressed on the majority of peripheral blood mononuclear cells, including NK cells, T cells, B cells, monocytes, and dendritic cells, as well as the majority of thymocytes[3]. Cross-linking of LAIR-1 by mAb *in vitro* delivers a potent inhibitory signal that is capable of inhibiting cellular functions of NK cells, effector T cells, B cells, and dendritic cell precursors[3-6]. In agreement with the observed inhibitory capacity of LAIR-1, the molecule bears two ITIMs in its cytoplasmic tail and selectively recruits the tyrosine phosphatases SHP-1[3;7-10] en SHP-2[3;8;10] upon activation.

LAIR-1 is structurally related to several other inhibitory IgSF members, including human killer cell Ig-like receptors (KIRs), human FcαR, human leukocyte Ig-like receptors (LILRs, also known as Ig-like transcripts (ILTs), leukocyte Ig-like receptors (LIRs), monocyte-macrophage inhibitory receptors (MIRs) and CD85), mouse gp91 or paired Ig-like receptors (PIRs) and mouse gp49[3]. Interestingly, LAIRs, KIRs, LILRs, and FcαR are all localized to the Leukocyte Receptor Complex (LRC) on human chromosome 19q13.4, suggesting that these molecules have evolved from a common ancestral gene (reviewed in [11;12]). It is generally believed that the LRC is only partially conserved between humans and mice, as illustrated by the absence of some family members in rodents, such as the KIRs and FcαR. As homologues of the LRC-encoded LAIR genes have not yet been identified, they were long believed not to be present in mice.

In this study, we identified the first LAIR family member in mice, which we have named mouse LAIR-1 (mLAIR-1). The *mLAIR-1* gene maps to the proximal end of mouse chromosome 7 in a region syntenic with human chromosome 19q13.4 where the LRC is located. The protein is broadly expressed on various immune cells and is capable of inhibiting immune responses. The mLAIR-1 cytoplasmic tail can become phosphorylated thereby recruiting SH2-containing tyrosine phosphatase-2 (SHP-2). Interestingly, unlike human LAIR-1, SHP-1 is not recruited to the mLAIR-1 cytoplasmic tail. Soluble human (h) LAIR-1 and mLAIR-1 fusion proteins bind to both human and mouse cell lines, indicating that they might bind the same ligand(s) on these cells. Identification of a mouse

homologue of LAIR-1 allows for *in vivo* studies on the function of LAIR molecules in regulation of different immune responses.

MATERIALS AND METHODS

Cells

Cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured using standard techniques. Mouse B cell lines IIA1.6 (BALB/cAnN), Sp2/0 (BALB/c), and NS-1 (BALB/c); mouse T cell lines D011.10 (BALB/c) and EL4 (C57BL/6); mouse melanoma B16 (C57BL/6); mouse sarcoma CMS7 (BALB/c); mouse fibroblasts: L929 (C3H/An) and 3T6 (Swiss albino); mouse brain-derived endothelioma bEND3; human colon carcinomas HT29 and LS174; human embryonic kidney 293T cells Jurkat human T cells; and rat basophilic leukemia cell line RBL-2H3[13] were used. The human NK-like tumor cell line YT.2C2 was kindly provided by Dr. K. Smith[14]. YT.2C2 stably transfected with hLAIR-1 and the Epstein-Barr virus (EBV)-transformed human B cell line 721.221 stably transfected with human FcγRIIa (CD32)[15] were generated at the DNAX Research Institute (Palo Alto, CA) and have been described previously[3;5]. The Armenian hamster fibroblast line ARHO12 was kindly provided by Dr. J. Hamann (Academic Medical Center, Amsterdam, The Netherlands).

Bone-marrow derived dendritic cells were obtained as described by Inaba *et al.*[16]. Briefly, bone marrow was flushed from mouse femurs (BALB/c), erythrocytes were lysed, and cells were grown at 1×10^6/ml RPMI 1640 medium supplemented with 10% FBS, 50 IU/ml penicillin, and 50 μg/ml streptomycin in the presence of 10 ng/ml granulocyte/macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA). Non-adherent cells were replated on day 1, and non-adherent cells were removed on days 2 and 4 from the cultures, with concomitant refreshment of culture media. Non-adherent and loosely adherent DC were harvested on day 7.

Antibodies

The DX26 IgG1 mAb directed against hLAIR-1 has been described previously[3]. The 8A8 (IgG1)-producing hybridoma was generated by fusing the SP2/0 myeloma cell line with splenocytes from a BALB/c mouse immunized with purified hLAIR-1 protein. Rat and mouse IgG isotype controls and phycoerythrin-conjugated streptavidin were purchased from BD Biosciences (San Diego, CA). Rat anti-mouse CD16/CD32 (Mouse Fc Block™) was obtained from PharMingen (San Diego, CA). Anti-phosphotyrosine mAb 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal rabbit anti-SHP-1 (C19) and anti-SHP-2 (C18) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Biotin-conjugated, goat anti–human IgG1 mAbs were obtained from Caltag Laboratories (Burlingame, CA). Biotin-conjugated, goat anti-rabbit Abs were purchased from Vector Laboratories, Inc. (Burlingame, CA). Goat anti-mouse $F(ab)_2$ fragments were purchased from Southern Biotechnology Associates (Birmingham, AL). For Western blot

analysis, Horse Radish Peroxidase (HRP)-conjugated rabbit anti-mouse Ab (DAKO (Glostrup, Denmark)) and HRP-conjugated goat anti-rabbit Ab (Pierce Biotechnology (Rockford, IL)) were used. Monoclonal IgE anti-TNP was generously provided by Prof. Dr. L. Aarden (Sanquin Research, Amsterdam, The Netherlands). For mouse fusion-protein FACS-stainings, cells were pretreated with 10% BSA, 20% FCS, 10% normal mouse serum and 20 μg/ml anti-mouse CD32/16 (FcγR) mAbs to block mouse Fcγ receptors.

Generation of mLAIR-1 polyclonal antibodies

The extracellular domain of mLAIR-1 was fused to a six-histidine tag in a pET21a expression vector (Novagen Inc., Madison, WI) and subsequently overexpressed in *Escherichia. coli* BL21(DE3) cells. The histidine-tagged protein was purified by Ni^{2+}-chelate affinity chromatography in the presence of 8 M urea according to the manufacturer's protocol (Qiagen, Hilden, Germany). Antisera were produced in two rabbits by a standard immunization protocol (Eurogentec, Seraing, Belgium).

cDNA constructs and transfectants

cDNA encoding hLAIR-1 was cloned into the pcDNA3.1/zeo$^+$ vector (Invitrogen, Breda, The Netherlands) and the pMX puro retroviral vector. Chimeric hLAIR-1/mLAIR-1 proteins were constructed in the same vectors by fusing the extracellular part of hLAIR-1 (hLAIR-1a aa. 1-160) to the transmembrane region and cytoplasmic domains of mLAIR-1 (mLAIR-1a aa. 140-263) by means of a linker sequence encoding the amino acids leucine and glutamic acid. The chimeric protein allows detection and triggering using anti-hLAIR-1 antibodies. Myc-tagged mLAIR-1 was constructed by fusing a Myc-epitope (EQKLISEEDL) to the C-terminal part of mLAIR-1 and subsequently cloning in the pcDNA3.1/zeo$^+$ vector. The DNA sequences were confirmed by automated DNA sequencing. To generate stable transfectants expressing either hLAIR-1 or hLAIR-1/mLAIR-1 chimeric proteins, RBL-2H3 cells were transfected by electroporation. Stable transfectants were selected in 50 μg/ml Zeocin™ (Invitrogen, Breda, The Netherlands) and subsequently cloned by the limiting dilution method. Stable YT.2C2 transfectants were generated by retroviral transfection as previously described[5]. Stable ARHO12 transfectants of Myc-tagged mLAIR-1 were generated by the FuGENE™ 6 transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) according manufacturer's instructions and subsequent cloning by the limiting dilution method in presence of 500 μg/ml Zeocin™ (Invitrogen, Breda, The Netherlands). Expression levels of the various LAIR-expressing clones were assessed by standard flow cytometry methods.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from several mouse cell lines and mouse (C57BL/6) organs using the RNeasy® method (QIAGEN GmbH, Hilden, Germany). Total RNA was converted to first-strand cDNA with oligo$(dT)_{18}$ primers and murine leukemia virus (MuLV) reverse transcriptase using the GeneAmp® RNA PCR kit (Applied Biosystems, Foster City, CA).

The cDNA-mixtures were amplified by PCR using mLAIR-1-specific forward (5'-GCTCTGACCAGACCTGGTAAGG-3') and reverse (5'-CCATGTGTGTCTCCAGGTGTGC-3') primers and the AmpliTaq® Gold DNA Polymerase system (Applied Biosystems, Foster City, CA). These primers correspond to the 5' and 3' untranslated region adjacent to the mLAIR-1 coding region. Each amplification reaction underwent 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 64°C, and elongation at 72°C for 50 s. As a control, GAPDH transcripts were amplified using GAPDH-specific primers (5'- ATCAACGACCCCTTCAT-3' and 5'-CACACCCATCACAAACAT-3'). Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.
mLAIR-1 isoforms present in the bone marrow RT-PCR sample were cloned into pGEM®-T Easy vectors using the pGEM®-T Easy vector system (Promega, Madison, WI) and subsequently sequenced on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA) using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). The sequences obtained were analyzed by Lasergene software (DNASTAR, London, UK)

Northern blot analysis

Total RNA from several mouse cell lines and organs was separated on a 1.6% formaldehyde agarose gel and blotted to a Zeta-Probe® GT blotting membrane (Bio-Rad, Hercules, CA) by capillary transfer in 10 x SSC as described by Sambrook *et al.*[17]. Radiolabeled DNA probes were generated using the RadPrime DNA Labeling System (Invitrogen, Breda, The Netherlands) of a DNA probe of the intracellular domain of mLAIR-1 and subsequently used for hybridization under stringent conditions using the ExpressHyb™ Hybridization Solution (CLONTECH Laboratories, Inc., Palo Alto, CA), according to the manufacturer's prescriptions. After washing the blots, they were exposed to phosphor screens and analyzed on a STORM PhosphorImager® (Molecular Dynamics, Wokingham, UK).

Degranulation assay

The degranulation assay of RBL-2H3 clones has previously been described[10]. Measurements were performed using triplicate cultures. The percentage of inhibition of degranulation by hLAIR-1- and chimeric hLAIR-1/mLAIR-1-transfected RBL-2H3-clones was calculated as: percentage of inhibition = 100*[(OD_{405} without LAIR-1 cross-linking – OD_{405} with LAIR-1 cross-linking)/(OD_{405} without LAIR-1 cross-linking - OD_{405} spontaneous release)].

Cytotoxicity assay

721.221 target cells stably expressing FcγRIIa were labeled with ^{51}Cr and used in a 4-hour cytotoxicity assay using transfected YT.2C2 cells as effector cells as described previously[18]. To engage hLAIR-1 and the hLAIR-1/mLAIR-1 chimeric molecules, 10 μg/ml 8A8 anti-LAIR antibody was added. The maximum release was determined by

lysing target cells with 5% Triton X-100. The percentage of specific lysis was calculated as: [(cpm specific ^{51}Cr release – cpm spontaneous ^{51}Cr release)/(cpm maximum ^{51}Cr release – cpm spontaneous ^{51}Cr release)]*100. Data are expressed as the mean of triplicate cultures.

Tyrosine phosphorylation and phosphatase recruitment

RBL-2H3 cells stably transfected with hLAIR-1 and the hLAIR-1/mLAIR-1 chimeric proteins were treated with pervanadate and subsequently subjected to immunoprecipitation using DX26 anti-human LAIR-1 antibody as previously described[10].

Detection of mLAIR-1 and hLAIR-1 ligand(s)

Chimeric proteins composed of the leader sequence and the extracellular parts of mLAIR-1 (aa 1–139) or hLAIR-1 (aa 1-162) fused to the Fc region of human IgG1 were inserted into the pcDNA3.1/zeo$^+$ vector (Invitrogen, Breda, The Netherlands). The proteins, designated mLAIR-1-hIg and hLAIR-1-hIg respectively, were produced by stable expression in 293T cells and subsequent purified by affinity chromatography on protein A-Sepharose columns (Amersham, Freiburg, Germany). Cell lines were screened for the presence of putative mLAIR-1 and hLAIR-1 ligand(s) by assaying for binding of the fusion proteins. Approximately $2.5*10^5$ cells were incubated at room temperature (RT) for 30 min with 20 μl PBS containing approximately 1 μg mLAIR-1-hIg or hLAIR-1-hIg, 5% normal mouse serum, 5% BSA, 10% FCS, and 20 μg/ml Mouse Fc Block™. After washing, 10 μg/ml (15 μl) biotin-conjugated goat anti–human-IgG1 was added for 30 min at RT, followed by washing and 30 min incubation with 10 μg/ml phycoerythrin-conjugated streptavidin. Cells were assayed on a FACSCalibur™ with the addition of propidium iodide to exclude dead cells. As isotype controls, 1 μg hIgG1 or irrelevant hIgG1 fusion protein was used. For fusion protein blocking studies, hLAIR-1-hIgs or mLAIR-1-hIgs were incubated for 30 minutes at RT with anti hLAIR-1 antibodies (8A8) or polyclonal anti mLAIR-1 antibodies respectively prior the above described procedure. As a control mLAIR-1-hIgs were incubated with preimmune serum from the same rabbits that served to generate polyclonal antibodies against mLAIR-1.

Computer-assisted analysis

Identification of mLAIR-1 was achieved by homology search on the Celera mouse genome database (www.celera.com). Comparison of the human and mouse LRC was performed by comparing both regions on the genomic databases of Celera and the National Center of Biotechnology Information (NCBI; http://www.ncbi.nih.gov/Genomes/). The protein sequence alignment was generated by the Clustal method, using Lasergene analysis software (DNASTAR, Inc., Madison, WI).

RESULTS

Identification of mLAIR-1

To identify a mouse homologue of human LAIR-1, the Celera mouse genome database was searched with a BLASTN algorithm for sequences bearing homology with the human LAIR-1 cDNA sequence. As a result, a mouse LAIR-locus was identified on chromosome 7, the syntenic chromosome of human chromosome 19q13.4 where both LAIR-1 and LAIR-2 loci are located. Specific primers were generated to PCR amplify putative LAIR-homologue transcripts using C57BL/6J mouse bone marrow-derived cDNA as template, resulting in amplification of at least six different LAIR transcripts (Fig. 2A). One transcript contained the full-length open reading frame of 792 nt with the first ATG start codon contained in a consensus Kozak sequence. The deduced polypeptide conformed to a type I transmembrane protein composed of 263 aa, including a 21 aa signal peptide, a 121 aa extracellular domain, a hydrophobic transmembrane segment of 22 aa, and a 99 aa cytoplasmic tail (Fig. 1, A and B). The putative human LAIR-1 homologue had a predicted relative molecular mass of 29.8 kDa and two potential sites for *N*-linked glycosylation at positions N34 and N90, indicated by a circle in Fig. 1A.

The presence of one pair of cysteines in the extracellular domain, generating a single Ig-like domain, classifies the LAIR-1 homologue as a member of the IgSF. Both residues are conserved between the human and mouse LAIR molecules (Fig. 1A). The cytoplasmic domain contains two tyrosines at residues Y228 and Y257, both contained in ITIM-like sequences. The N-terminal sequence (VTYIQL) fits the I/V/L/SxYxxL/V-consensus sequence for ITIMs[1], whereas the C-terminal sequence (STYAAI) resembles, but does not fit, the consensus.

Aligning the protein sequence of hLAIR-1a to its mouse homologue (Fig. 1A) showed that the molecules are moderately conserved, with overall sequence identity and homology of 40 and 50%, respectively. In particular, the cytoplasmic domains of both LAIR molecules were more conserved than their extracellular domains. The N-terminal ITIM-sequences were highly similar between both receptors; only a single aa substitution was observed, whereas the C-terminal ITIM-sequences were less alike. Based on the sequence identity between human LAIR-1 and the newly identified mouse LAIR molecule, the protein was named mouse LAIR-1 (mLAIR-1).

The mLAIR-1 gene maps to a LRC-like region on chromosome 7

The *mLAIR-1* gene spans an area of approximately 53.6 kb of genomic sequence (Fig. 1B) and closely resembles the human *LAIR-1* gene. Although human *LAIR-1* contains 10 exons, and its mouse homologue only eight (*mouse LAIR-1* misses human exons 4 and 5), the shared exons are highly similar in size, have almost identical intron-exon boundaries (data not shown), and encode similar regions of the protein (Fig. 1B).

The *mLAIR-1* gene maps to the proximal end of mouse chromosome 7 in a region syntenic with human chromosome 19q13.4 where the LRC is located (Fig. 1C). Based on sequence similarity, the region located near the *mLAIR-1* gene resembles that of the

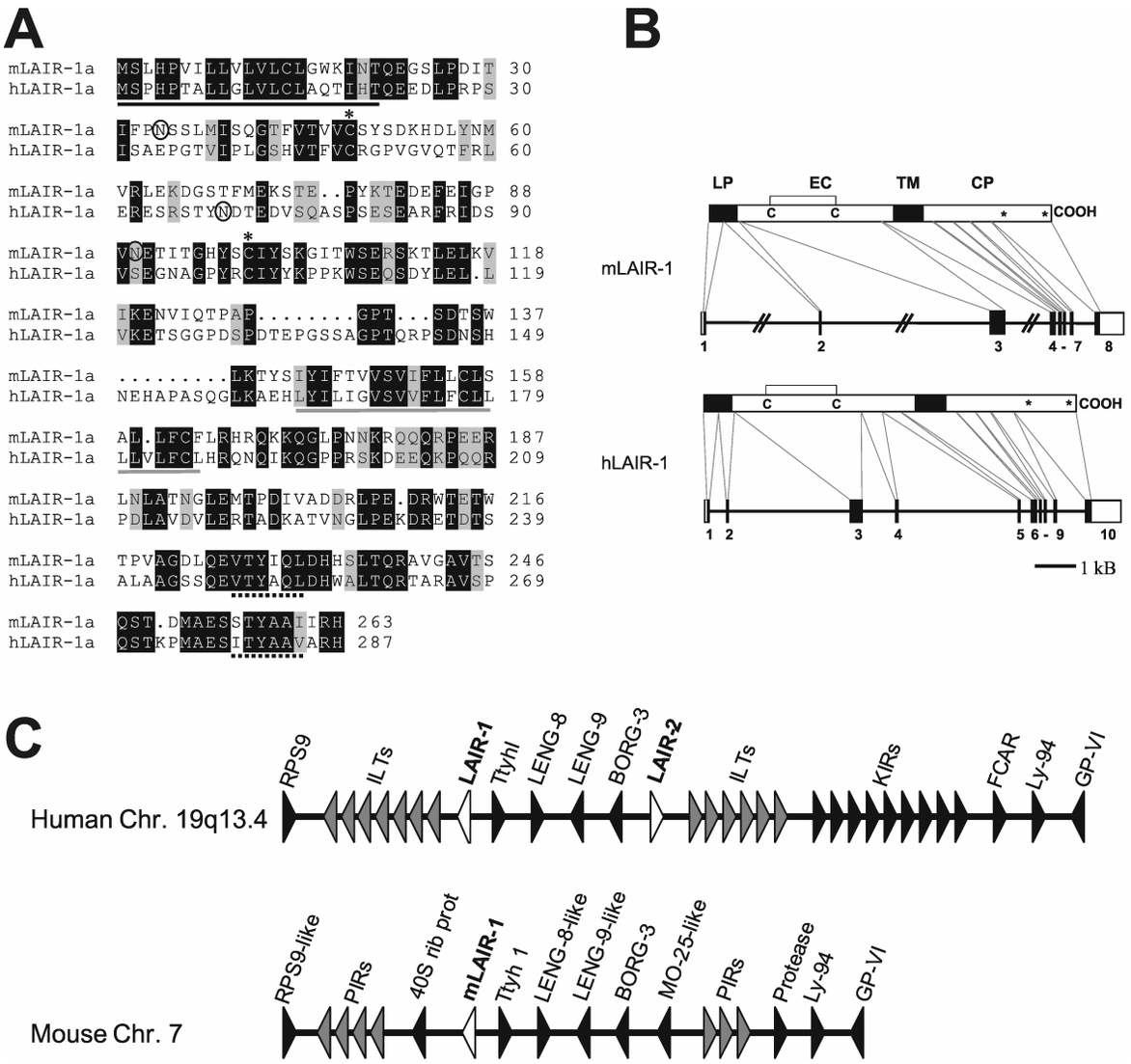

Figure 1. Human LAIR-1 has a mouse homologue. (A) Protein sequence alignment of mLAIR-1a and hLAIR-1a. The leader sequence and putative transmembrane domain are respectively black and gray underlined; the putative ITIM sequences in the cytoplasmic domain are underlined (dotted). Conserved residues are enclosed by darkly shaded boxes and residues showing conservative substitutions are enclosed in lightly shaded boxes. Conserved cysteines involved in intradomain disulfide bond formation are indicated by asterisks. Potential N-linked glycosylation sites of both receptors are circled. Gaps introduced to optimize the sequence alignment are indicated by dots. The mLAIR-1a sequence data is available from Genbank/EMBL/DDBJ under accession no. (AY392763). (B) Schematic overview of the genes and respective proteins of mLAIR and hLAIR-1. The protein-coding sequences are denoted as closed boxes, and the non-coding sequences as open boxes. The protein structure is subdivided into the leader peptide (LP), the extracellular domain (EC), the transmembrane domain (TM), and cytoplasmic domains (CP). The two cysteines forming the putative disulfide bonds (C-C) in the extracellular domain are shown. Putative ITIM-sequences are indicated by asterisks. Introns 1,2, and 3 of the mLAIR-1 gene are shown at 15% of their relative size. (C) Schematic organization of the human LRC on chromosome 19q13.4 (top panel) compared to its syntenic region on mouse chromosome 7 (bottom panel). The genes include platelet glycoprotein VI (GP-VI), lymphocyte antigen 94 (Ly-94 or natural cytotoxicity triggering receptor 1 (NCR-1)), FcαR, KIR genes, leukocyte Ig-like receptors (LILRs), LAIR genes, paired Ig-like receptors (PIRs) and genes not belonging to the Ig-like superfamily: LRC-encoded novel genes (LENGs), ribosomal protein S9 (RBS9), Tweety homologue 1 (TtyhI), Binder of Rho GTPase 3 (BORG-3), and a protein showing similarity to MO-25. Genes are represented by arrows, indicating their direction of transcription. The maps are not to scale.

human LRC indicating it is conserved through evolution. Genes homologous to the Ig-like protein platelet glycoprotein VI (GP-VI), lymphocyte antigen 94 (or natural cytotoxicity-triggering receptor 1), and LILRs appear to be conserved between the species. Furthermore, several non-Ig molecules, such as the LRC-encoded novel genes (LENGs), ribosomal protein S9 (RBS9), Tweety homologue 1 (TtyhI), Binder of Rho GTPase 3 (BORG-3) seem conserved. Significantly, the mouse LRC lacks genes encoding LAIR-2, KIRs and FcαR. Furthermore, there appear to be fewer PIR genes present compared with genes encoding its human homologues (LILRs). Only mLRC contained putative genes encoding proteins with homology to a protease, 40S ribosomal protein and MO-25. MO25 (CAB39) is a gene transcribed during early mouse development encoding a putative Ca2+ binding protein[19].

mLAIR-1 is expressed in hematopoietic cells

To asses the cellular distribution of mLAIR-1, RT-PCR analysis on various mouse tissues (C57BL/6) and cell lines using mLAIR-1-specific primers was performed (Fig. 2A). Transcripts were detected in lymphoid organs (bone marrow, spleen, lymph nodes, and thymus), but not in nonlymphoid organs such as muscle and skin. Furthermore, cell lines of hematopoietic origin of various mouse strains contained mLAIR-1 transcripts (IIA1.6, Sp2/0, NS-1, D011.10, and EL4), whereas nonhematopoietic cell lines (CT26, B16, CMS7, L929, and 3T6) did not.

Several mLAIR-1 transcripts were detected by RT-PCR (Fig. 2A). Cloning, sequencing and subsequent sequence alignment indicated that the different transcripts were all transcribed from the same gene and were characteristic of alternative RNA splicing (data not shown). The two major transcripts encoded the full-length mLAIR-1a and a splice variant missing the entire Ig-like domain, which is encoded by exon 3 (mLAIR-1b). Two minor forms, which we designated mLAIR-1d and mLAIR-1e, encoded isoforms missing exon 3 and 4 or exon 4 alone, respectively. Furthermore, two minor transcripts were detected similar to mLAIR-1a and mLAIR-1b, with a 115-nt intronic sequence adjacent exon 2 resulting in a transcript encoding a nonsense protein. The DNA sequences of mLAIR-1a, b, d, and e were deposited in the GenBank™ database under accession numbers AY392763, AY392764, AY392765, and AY392766, respectively.

Northern blot analysis of bone marrow-derived DCs (BALB/c) showed two predominant transcripts of approximately 1.7 and 3.3 kb (Fig. 2B). Hybridization transcripts were not detected in other RNA samples (CMS7, Fig. 2B; EL4, SP2/0, NS-1, CT26, IIA1.6, and D011.10, data not shown), and only faintly in lymphoid tissues (spleen, bone marrow and lymph nodes; data not shown), indicating that mLAIR-1 transcripts are relatively low abundant transcripts in immune-associated tissues other than DCs. Taken together, these data indicate that, like human LAIR-1, mLAIR-1 is exclusively expressed by cells of hematopoietic origin.

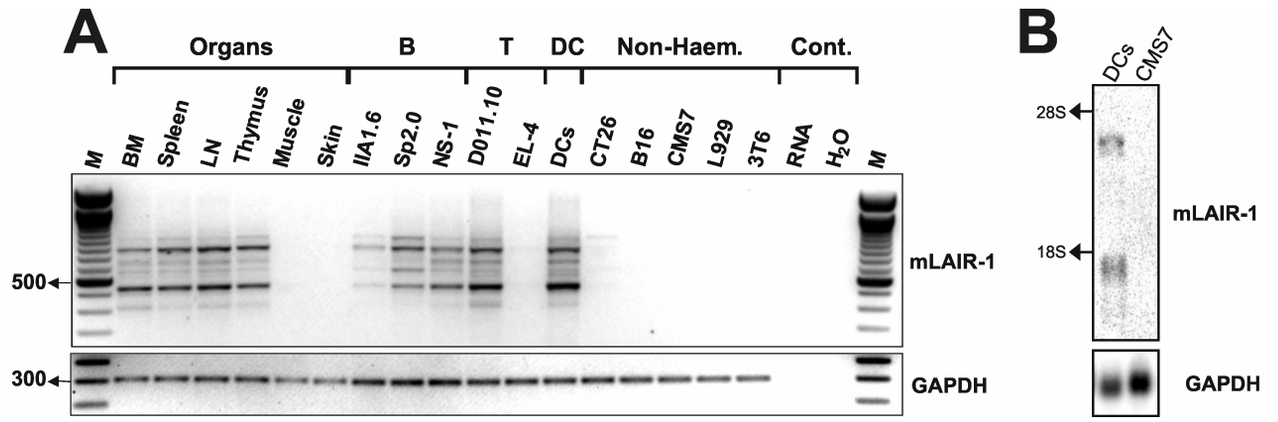

Figure 2. mLAIR-1 is expressed in hematopoietic cells. (A) Semi-quantitative RT-PCR amplification of mLAIR-1 and GAPDH cDNA fragments in various mouse cell populations. mLAIR-1 cDNA fragments were selectively amplified by RT-PCR from total RNA samples obtained from various mouse tissues (C57BL/6) or cell lines, and subsequently visualized by ethidium bromide staining. Bottom panel, control amplifications of a GAPDH cDNA fragment in the same samples. RNA samples were prepared from the following tissues and cell lines: (from left to right) bone marrow (BM); spleen; lymph nodes (LN); thymus; muscle (leg); skin; B cell lines IIA1.6 (BALB/cAnN), Sp2/0 (BALB/c) and NS-1 (BALB/c); T cell lines D011.10 (BALB/c) and EL4 (C57BL/6); bone marrow-derived DCs (BALB/c); colon carcinoma CT26 (BALB/c); melanoma B16 (C57BL/6); sarcoma CMS7 (BALB/c); and fibroblasts L929 (C3H/An) and 3T6 (Swiss albino). Negative controls using total RNA from bone marrow-derived DCs and water as templates are shown. (B) Northern blot analysis of mLAIR-1 transcript expression in bone marrow-derived DCs and the sarcoma cell line CMS7 (BALB/c). Upper panel represents a Northern blot probed with an mLAIR-1 probe; bottom panel shows the same blot probed with a GAPDH-probe as relative loading control. The migration of the 28S (4.7 kb) and 18S (1.9 kb) ribosomal RNAs are indicated.

mLAIR-1 is able to inhibit degranulation of RBL-2H3 cells and cytotoxic activity of NK cells

To investigate the potential inhibitory capacity of mLAIR-1, we generated a chimeric molecule composed of the extracellular domain of human LAIR-1 fused to the transmembrane and cytoplasmic domain of mLAIR-1. Stable transfectants of the rat basophilic leukemia cell line (RBL-2H3) and the human NK cell line YT.2C2, were generated using this chimeric protein and hLAIR-1 as a control. Cell surface immunofluorescence analysis (Fig. 3A) indicated that the transfected RBL cells expressed both proteins at comparable levels in two independent transfectants. We investigated whether these chimeric molecules could inhibit signaling mediated by the endogenous ITAM-bearing IgE receptor FcεRI expressed on RBL cells in a β-glucuronidase release assay (degranulation assay). Incubation of the transfectants with TNP-specific IgE and subsequent triggering with TNP-conjugated BSA, led to degranulation of the cells and release of β-glucuronidase. Simultaneous cross-linking of the stably transfected chimeric LAIR molecules or hLAIR-1 with anti human-LAIR-1 mAb (8A8) and anti-mouse Ig led to an inhibition of IgE-induced β-glucuronidase release for the LAIR-molecules, whereas no effect was observed for nontransfected RBL cells (Fig. 3B). Furthermore, triggering of the chimeric protein by mAbs on transfectants of the human NK cell line YT.2C2 (Fig.

4A) inhibited its spontaneous cytotoxic activity towards 721.221 FcγRIIa-bearing target cells (Fig. 4B). The inhibitory effect observed in the cytotoxicity assay was completely due to triggering of the chimeric LAIR molecules alone, as previous experiments showed that mutating both tyrosines of hLAIR-1 to phenylalanine abolished the inhibition of cytotoxicity of the NK cell line towards its target cells in an identical experimental set-up[10]. Taken together, these data indicate that, like human LAIR-1, cross-linking of the human LAIR-1/mLAIR-1 chimeric protein results in inhibition of ITAM-dependent signals initiated via the FcεRI complex on RBL-2H3 cells and cytotoxic activity of NK cells towards FcγR-bearing target cells.

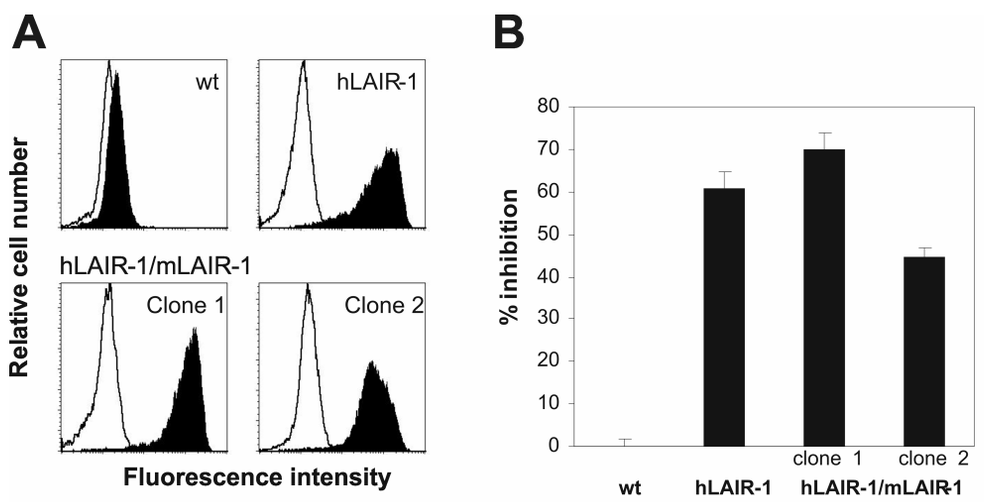

Figure 3. The mLAIR-1 cytoplasmic tail is able to inhibit degranulation of RBL-2H3 cells. (A) Expression of hLAIR-1/mLAIR-1 chimeric proteins by transfected Rat Basophilic Leukemia (RBL) cells. The chimeric proteins consist of the extracellular part of hLAIR-1a (aa. 1-160) and the transmembrane region and cytoplasmic tail of mLAIR-1a (aa. 140-263). Expression of the hLAIR-1 and hLAIR-1/mLAIR-1 chimeric proteins on the cell surface was confirmed by flow cytometry after staining with DX26 anti-hLAIR-1 Ab and phycoerythrin (PE)-conjugated goat anti-mouse IgG (*solid histogram*) or PE-conjugated goat anti-mouse IgG alone (*open histogram*). Clones 1 and 2 were derived from independent transfections. Non-transfected RBL-2H3 (wild-type (wt)) cells were used as a control. (B), Inhibition of Fcε-mediated degranulation by human LAIR-1 and hLAIR-1/mLAIR-1 chimeric protein. RBL clones were primed with IgE anti-TNP and triggered by adding 10 ng/ml BSA-TNP. For cross-linking LAIR-1 on the cell surface, cells were coated with both IgE and 8A8 anti-LAIR IgG and triggered in the presence of 10 μg/ml goat anti-mouse $F(ab)_2$. The inhibitory capacity of human LAIR-1 and hLAIR-1/mLAIR-1 chimeric protein was calculated by comparing the degranulation of the cells with and without LAIR-1 cross-linking, as described in *Materials and methods*. Typical degranulation values ranged from 10 to 25% of the amount observed when all RBL cells were lysed by addition of 10% Triton, depending on the clone tested. Although degranulation values varied between experiments, the percentage of inhibition remained constant. Data are expressed as mean values of three independent experiments plus standard deviation.

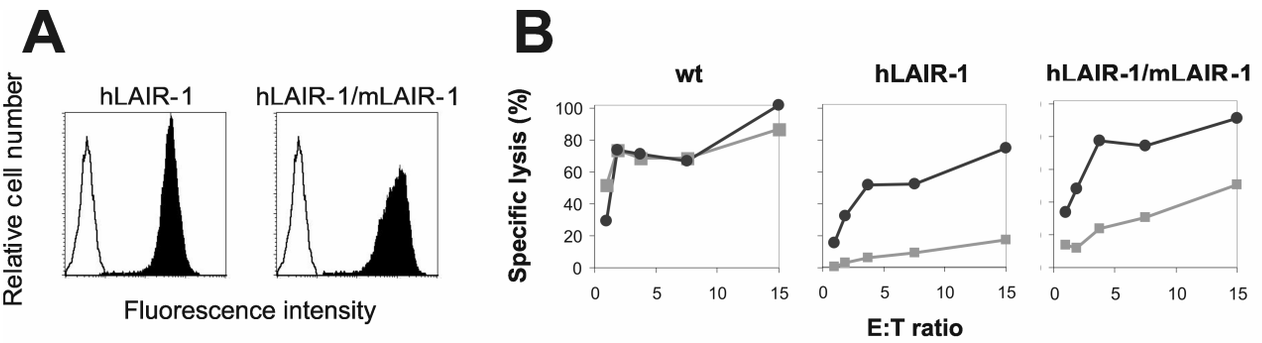

Figure 4. The mLAIR-1 cytoplasmic tail is able to inhibit cytotoxic activity of YT.2C2 cells. (A) Expression of hLAIR-1/mLAIR-1 chimeric proteins by human NK cell line YT.2C2. Expression of the hLAIR-1 and hLAIR-1/mLAIR-1 chimeric proteins on the cell surface was confirmed by flow cytometry after staining with DX26 anti-hLAIR-1 Ab and phycoerythrin (PE)-conjugated goat anti-mouse IgG (*solid histogram*). Non-transfected YT.2C2 cells are indicated as an open histogram. (B) YT.2C2 cells (wt) and YT.2C2 cells expressing hLAIR-1 and mLAIR-1 were assayed for lysis of the human FcγRIIa (CD32) transfected Epstein-Barr virus (EBV)-transformed B cell line, 721.221/CD32 at different effector-to-target (E:T) ratios in the absence (•) or presence (■) of 10 μg/ml 8A8 anti-LAIR-1 antibody. Data are representative of four independent experiments.

The mLAIR-1 cytoplasmic tail is phosphorylated upon pervanadate stimulation, recruits SHP-2, but does not associate with SHP-1

The existence of two potential ITIM sequences within the cytoplasmic domain of mLAIR-1 (Fig. 1A) suggested that the generation of inhibitory signals in the degranulation and cytotoxicity assay (Fig. 3B and 4B) was manifested by the recruitment of SHP-1 and/or SHP-2. To determine whether the chimeric proteins were capable of binding protein tyrosine phosphatases, the previously described RBL-2H3 transfectants were stimulated with pervanadate (an inhibitor of protein tyrosine phosphatases inducing tyrosine phosphorylation[20]), lysed, and immunoprecipitated with anti-hLAIR-1 mAbs. Immunoprecipitates were subsequently analyzed by Western blotting using Abs specific for phosporylated tyrosine residues, hLAIR-1, SHP-1 and SHP-2. As shown in Fig. 5A, the chimeric proteins became phosphorylated upon pervanadate treatment. Unexpectedly, unlike hLAIR-1, the chimeric protein did not recruit SHP-1 upon phosphorylation. Analogous to human LAIR-1, SHP-2 was recruited after pervanadate stimulation (Fig. 5B), albeit to a much lesser extent. These results suggest that the negative signal transduced via engagement of the mLAIR-1 molecule might be mediated through recruitment of SHP-2, whereas SHP-1 does not seem to play a role.

mLAIR-1 and hLAIR-1 bind ligand(s) expressed on the same cell lines

To identify the natural ligand(s) for mLAIR-1, we constructed a fusion protein consisting of the extracellular domain of mLAIR-1 fused to the Fc portion of human IgG1 (mLAIR-1-hIg). The protein was used as staining reagent to screen mouse cell lines for presence of putative ligands. The mouse fibroblast cell lines 3T6 and L929 and the mouse brain-derived endothelial cell line bEND3 bound the protein whereas several other cell

lines did not (NS-1, IIA1.6, Sp2/0, D011.10, and EL4 cells; Fig 6A and data not shown). To examine whether human LAIR-1 also binds ligand(s) on these cells, a hLAIR-1-hIg fusion protein was generated. This fusion protein stained the same cells, indicating that hLAIR-1 reacts with a ligand on mouse cells. This observation led us to expand the screening for putative ligands to human cell lines. Both mLAIR-1 and hLAIR-1 fusion proteins stained the same human cells (human embryonic kidney 293T, colon carcinoma cell lines HT29 and LS174) whereas the human Jurkat T cell line appeared not to express a ligand for these receptors. This could indicate that both LAIR molecules recognize the same ligand(s) on these cells, confirming that the proteins are true homologues. However, the receptors might also bind to different cell surface molecules expressed on these cells. Binding of hLAIR-1-hIg and mLAIR-1-hIg to all cell lines was abolished by prior incubation with anti-hLAIR-1 Abs (8A8) or polyclonal anti-mLAIR-1 Abs respectively, demonstrating the specificity of these interactions (Fig. 6B). The mLAIR-1-hIg binding could not be abolished by prior incubation of the human LAIR-1 recognizing Ab (8A8, data not shown). Interestingly, hLAIR-1-hIg binding was not abolished by prior incubation of another anti hLAIR-1 Ab (DX26, Fig. 6B), indicating that 8A8 antibodies recognize a hLAIR-1 epitope that is involved in ligand binding, whereas DX26 antibodies do not. Specific binding of the polyclonal antiserum to mLAIR-1 was confimed using flow cytometry by staining ARHO12 cells stably transfected with or without mLAIR-1 (Fig. 6C).

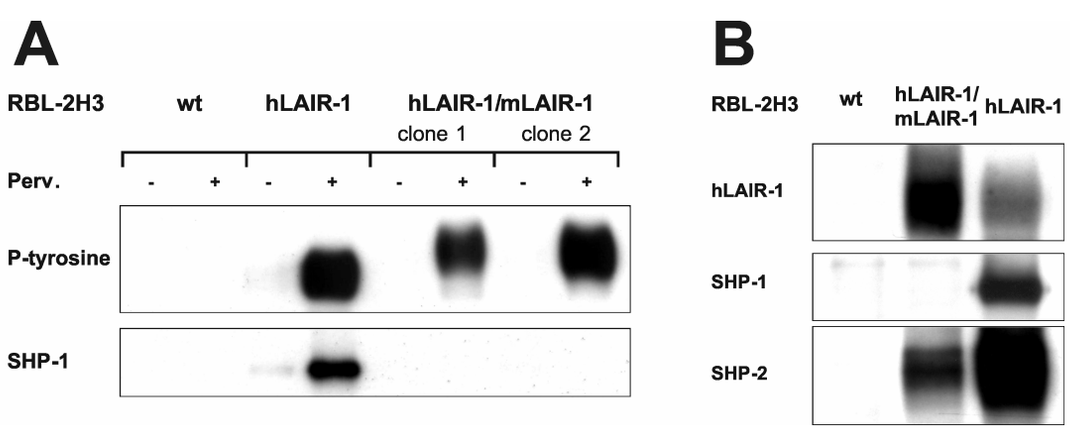

Figure 5. The mLAIR-1 cytoplasmic tail is phosphorylated upon pervanadate treatment and recruits SHP-2, but does not associate with SHP-1. (A) RBL-2H3 cells (wt) or RBL-2H3 cells stably transfected with either hLAIR-1 or chimeric hLAIR-1/mLAIR-1, were treated with 50 μM pervanadate in PBS or with PBS alone for 15 minutes at 37°C and immediately lysed in Triton lysisbuffer. Cell lysates were subjected to immunoprecipitations with anti-hLAIR-1 Abs coupled to protein-A/G beads. Proteins were separated by non-reducing SDS-PAGE, transferred to Immobilon-P membranes and Western blotted using anti-phosphotyrosine (upper panel) and anti-SHP-1 (lower panel), or (B) anti-human LAIR-1, anti-SHP-1, anti-SHP2. The results are representative of four independent experiments

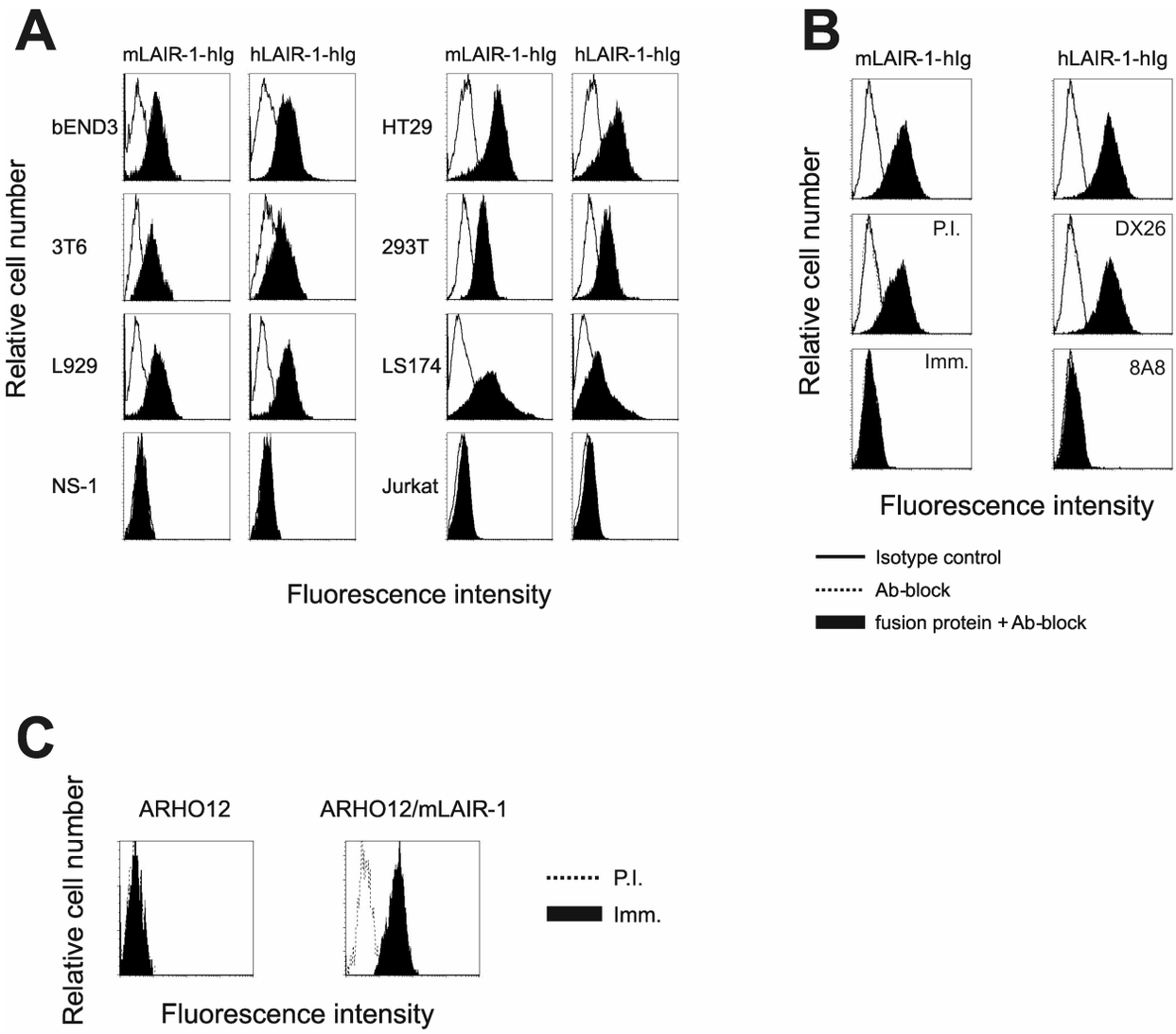

Figure 6. mLAIR-1 and hLAIR-1 bind a ligand(s) on the same cells (A) mLAIR-1-hIg and hLAIR-1-hIg binding to several human and mouse cell lines, demonstrating the presence of a putative ligand(s). Filled histograms represent staining with mLAIR-1-hIg or hLAIR-1-hIg. Open histograms represent staining with hIgG isotype control. (B) Anti hLAIR Abs and polyclonal anti mLAIR-1 immune serum inhibits binding of hLAIR-1-hIg and mLAIR-1-hIg, respectively, to HT29 cells. hLAIR-1-hIgs or mLAIR-1-hIgs were pre-incubated for 30 minutes at RT with indicated blocking antibodies, as represented in the upper right corner of the histogram. Subsequently, HT29 cells were incubated with the mixture in the presence of 5% normal mouse serum, 5% BSA, 10% FCS, and 20 μg/ml Mouse Fc Block™. Specific binding of the fusion proteins were assessed by detection of the human IgG1 tail present in the fusion protein, as described in Materials and Methods. DX26 and 8A8 are both mouse anti-hLAIR-1 mAbs recognizing different epitopes of the extracellular domain of the receptor. (C) Rabbit polyclonal anti-mLAIR-1 immune serum specifically recognizes mLAIR-1 stably transfected in ARHO12 cells. ARHO12 cells transfected with or without mLAIR-1 were incubated with preimmune rabbit serum (*open histograms*) or anti-mLAIR-1 immune serum (*solid histograms*). Specific binding of the antibodies was assessed by incubating with biotinilated anti-rabbit Abs and subsequent detection with PE-conjugated streptavidin. P.I., rabbit polyclonal preimmune serum; Imm., rabbit polyclonal anti-mLAIR-1 immune serum.

DISCUSSION

In this report we describe the identification and characterization of the mouse homologue of hLAIR-1, an inhibitory receptor believed to function in the down-regulation of immune responses[3-6;21;22].

The *mLAIR-1* gene maps to the proximal end of mouse chromosome 7 in a region similar to the hLRC (Fig. 1C). Like hLAIR-1, the mLAIR-1 molecule is structurally related to several other inhibitory IgSF members located on the same region of the chromosome, suggesting that these molecules could have evolved from a common ancestral gene. Comparison of the hLRC to its syntenic region in mice reveals remarkable similarities as well as some striking differences. Besides encoding many members of the IgSF, both regions appear to show a considerable degree of genetic polymorphism and are characterized by extensive gene duplications[12;23]. Furthermore, the orientation of several homologous genes in the human LRC indicate that this region arose as a result of a large inverse duplication[23]; this same pattern is observed in the mouse. The mouse LRC however, is smaller in size than the human variant (~530 kb vs ~900 kb respectively) and appears to encode fewer IgSF members. A direct homologue for the human FcαR (CD89) for instance, is absent in the mLRC. Furthermore, whereas humans encode ~ 13 KIR family members[24], mice completely lack KIR genes on mouse chromosome 7. Recently however, a KIR-like locus was identified outside the LRC on mouse chromosome X, suggesting that the KIR family did evolve from a primordial gene already present in the common rodent/primate ancestor[25;26]. The mouse PIR gene family, comprised of both activating and inhibitory receptors with six Ig domains, is the closest in sequence homology and gene structure to the human LRC-encoded LILRs (reviewed in [27]). Although both gene families are encoded in the LRC and share a moderate level of sequence identity, their relatedness was long subject of debate since the PIRs possess six Ig-like domains whereas the LILRs contain only 2 or 4. Recently however, studies suggested that PIR-B interacts with the HLA class Ib molecule HLA-G[28], a molecule that also serves as a binding partner of several members of the LILR family (reviewed in [29]).

In contrast to the human LAIR family, which consists of the transmembrane protein LAIR-1 and the putatively secreted protein LAIR-2 which shares 84% sequence homology to hLAIR-1[3], the mouse genome appears to encode only the membrane-bound variant. Searching mouse EST and genome databases did not retrieve a LAIR-2 homologue. It seems that the LAIR-1 gene appeared prior to the rodent/primate split in mammalian evolution and that human LAIR-2 might originate as a result of a *LAIR-1* gene duplication-event in primates. The similar architecture and high sequence identity between the human *LAIR-1* and *LAIR-2* genes and the absence of a mouse *LAIR-2* gene supports this hypothesis. Altogether, although the human and mouse LRC show obvious similarities, it is apparent that the region is highly dynamic and that its members have probably evolved by a series of duplications, deletions and rearrangements from an ancient common gene.

Like hLAIR-1[3], its mouse homologue is broadly expressed on immune cells. Interestingly, several different mLAIR-1 splice variants were detected by RT-PCR analysis, among these a high abundant splice variant lacking the entire Ig domain encoded by exon 3 (mLAIR-1b). To our knowledge, there are no other immune receptors with similar splice variants, it would therefore be interesting to determine whether this protein is also expressed on mouse immune cells, and explore what function it might have.

The presence of two ITIM-like structures in the mLAIR-1 cytoplasmic tail corresponds with the observed inhibition of immune responses by YT.2C2 and RBL cells. However, although the N-terminal sequence fits the consensus sequence for ITIMs, the C-terminal sequence does not completely. This raises the question whether the C-terminal ITIM-like structure of mLAIR-1 can function as such. Studies using human KIR molecules have indicated that two intact ITIMs are required for SHP-1 recruitment[30;31]. However, whereas a KIR mutant containing only the C-terminal ITIM is no longer effective, a KIR mutant containing only the N-terminal ITIM still recruits SHP-2[32] and has inhibitory capacity[30;31]. These studies are in agreement with our recent study[10], in which we showed that mutating either ITIM of hLAIR-1 resulted in loss of SHP-1 recruitment upon Ab triggering, whereas mutating the C-terminal ITIM still allowed SHP-2 recruitment. It appears that mutating any ITIM of inhibitory receptors bearing two ITIM sequences, results in abolishing of SHP-1 recruitment[10;30;31]. This suggests that receptors bearing a single ITIM are not able to recruit SHP-1. C-type lectin inhibitory receptors like LLIR and Ly49 however, often bear a single ITIM in their cytoplasmic tail and do recruit SHP-1 upon tyrosine phosphorylation[33;34]. The latter class of molecules however, can be expressed as a disulfide-linked homodimer[35;36], allowing one receptor to carry two identical ITIMs. The mast cell function-associated antigen (MAFA) is an exception to this, the receptor bears a single ITIM, can be expressed on the cell membrane as a dimer but does not recruit SHP-1, whereas SHP-2 and SHIP are recruited to this molecule upon phosphorylation (reviewed in [37]). As mLAIR-1 does not recruit SHP-1 upon pervanadate treatment, and the C-terminal ITIM-like sequence does not completely match the ITIM consensus, it is possible that this motif might not function as such. This would correspond with the hypothesis that two bonafide ITIMs are required for SHP-1 recruitment. However, mutational studies are required to determine if this motif completely lacks inhibitory signaling capacities or if other, as yet unknown, factors play a role in the inhibitory capacities of mLAIR-1.

Studies determining the basis for specific binding of SHP-2 to tyrosine motifs have indicated that position +1 (relative to pY) of the ITIM contributes to specificity. The N-terminal SH2 domain of SHP-2 has a preference for I/V/T at this position. Presence of an I at position pY + 1 in the N-terminal ITIM of mLAIR-1 suggests that SHP-2 is recruited to this ITIM. Although human LAIR-1 contains an alanine at this position, this N-terminal ITIM proved responsible for SHP-2 recruitment[10].

Unlike previously described, human LAIR-1 does not interact with the epithelial cell adhesion molecule (Ep-CAM) as a ligand[38], therefore we used mLAIR-1-hIg and hLAIR-1-hIg fusion proteins to screen a panel of human and mouse cell lines for presence of

putative LAIR ligands. As both hLAIR-1-hIg and mLAIR-1-hIg fusion proteins bound to the same human and mouse cell lines, it is tempting to speculate that both receptors cross-react with the same ligand(s) expressed on these cells. However, both receptors could potentially bind to different cell surface molecules expressed on these cells. It is imperative for understanding LAIR biology to identify the molecules that bind these fusion proteins, and whether ligation can stimulate the inhibitory function of both human and mouse LAIR-1.

The here presented data show that mLAIR-1 is a genuine homologue of human LAIR-1. Despite the moderate level of sequence-identity, the genes encoding the receptors are both located in the LRC, their exons are highly similar in size and have almost identical intron-exon boundaries. Furthermore, both proteins have similar expression patterns, share a potent inhibitory capacity, and potentially bind the same ligand(s) on both human and mouse cells. Although both receptors recruit SHP-2 upon phosphorylation of their cytoplasmic tails, their difference in SHP-1 recruitment is intriguing.

To conclude, identification of the mouse homologue of LAIR-1 could facilitate *in vivo* studies on the role of this receptor in regulation of immune responses and broaden the general knowledge on the function of inhibitory receptors in immune surveillance.

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REFERENCES

1. Ravetch,J.V. and L.L.Lanier. 2000. Immune inhibitory receptors. *Science* 290:84-89.
2. Long,E.O. 1999. Regulation of immune responses through inhibitory receptors. *Annu. Rev. Immunol.* 17:875-904.
3. Meyaard,L., G.J.Adema, C.Chang, E.Woollatt, G.R.Sutherland, L.L.Lanier, and J.H.Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
4. van der Vuurst de Vries AR, H.Clevers, T.Logtenberg, and L.Meyaard. 1999. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur. J. Immunol.* 29:3160-3167.
5. Meyaard,L., J.Hurenkamp, H.Clevers, L.L.Lanier, and J.H.Phillips. 1999. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. *J. Immunol.* 162:5800-5804.
6. Poggi,A., E.Tomasello, E.Ferrero, M.R.Zocchi, and L.Moretta. 1998. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. *Eur. J. Immunol.* 28:2086-2091.
7. Sathish,J.G., K.G.Johnson, K.J.Fuller, F.G.LeRoy, L.Meyaard, M.J.Sims, and R.J.Matthews. 2001. Constitutive association of SHP-1 with leukocyte-associated Ig-like receptor-1 in human T cells. *J. Immunol.* 166:1763-1770.
8. Fournier,N., L.Chalus, I.Durand, E.Garcia, J.J.Pin, T.Churakova, S.Patel, C.Zlot, D.Gorman, S.Zurawski, J.Abrams, E.E.Bates, and P.Garrone. 2000. FDF03, a novel inhibitory receptor of the immunoglobulin superfamily, is expressed by human dendritic and myeloid cells. *J. Immunol.* 165:1197-1209.
9. Xu,M., R.Zhao, and Z.J.Zhao. 2000. Identification and characterization of leukocyte-associated Ig-like receptor-1 as a major anchor protein of tyrosine phosphatase SHP-1 in hematopoietic cells. *J. Biol. Chem.* 275:17440-17446.
10. Verbrugge,A., T.de Ruiter, H.Clevers, and L.Meyaard. 2003. Differential contribution of the Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) of human Leukocyte Associated Ig-like Receptor (LAIR)-1 to inhibitory function and phosphatase recruitment. *Int. Immunol.* 15:1349-1358.
11. Barten,R., M.Torkar, A.Haude, J.Trowsdale, and M.J.Wilson. 2001. Divergent and convergent evolution of NK-cell receptors. *Trends Immunol.* 22:52-57.
12. Martin,A.M., J.K.Kulski, C.Witt, P.Pontarotti, and F.T.Christiansen. 2002. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol.* 23:81-88.
13. Barsumian,E.L., C.Isersky, M.G.Petrino, and R.P.Siraganian. 1981. IgE-induced histamine release from rat basophilic leukemia cell lines: isolation of releasing and nonreleasing clones. *Eur. J. Immunol.* 11:317-323.
14. Yodoi,J., K.Teshigawara, T.Nikaido, K.Fukui, T.Noma, T.Honjo, M.Takigawa, M.Sasaki, N.Minato, and M.Tsudo. 1985. TCGF (IL 2)-receptor inducing factor(s). I. Regulation of IL 2 receptor on a natural killer-like cell line (YT cells). *J. Immunol.* 134:1623-1630.
15. Stuart,S.G., M.L.Trounstine, D.J.Vaux, T.Koch, C.L.Martens, I.Mellman, and K.W.Moore. 1987. Isolation and expression of cDNA clones encoding a human receptor for IgG (Fc gamma RII). *J. Exp. Med.* 166:1668-1684.
16. Inaba,K., M.Inaba, N.Romani, H.Aya, M.Deguchi, S.Ikehara, S.Muramatsu, and R.M.Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693-1702.
17. Sambrook J., E.F.Fritsch, and T.Maniatis. 1989. Molecular cloning: A laboratory manual, 2nd edition. Cold Spring Harbor Laboratory, New York.
18. Lanier,L.L., A.M.Le, J.H.Phillips, N.L.Warner, and G.F.Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. *J. Immunol.* 131:1789-1796.

19. Miyamoto,H., A.Matsushiro, and M.Nozaki. 1993. Molecular cloning of a novel mRNA sequence expressed in cleavage stage mouse embryos. *Mol. Reprod. Dev.* 34:1-7.
20. O'Shea,J.J., D.W.McVicar, T.L.Bailey, C.Burns, and M.J.Smyth. 1992. Activation of human peripheral blood T lymphocytes by pharmacological induction of protein-tyrosine phosphorylation. *Proc. Natl. Acad. Sci. U. S. A* 89:10306-10310.
21. Meyaard,L. 1999. LAIR-1, a widely distributed human ITIM-bearing receptor on hematopoietic cells. *Curr. Top. Microbiol. Immunol.* 244:151-157.
22. Poggi,A., F.Pellegatta, B.E.Leone, L.Moretta, and M.R.Zocchi. 2000. Engagement of the leukocyte-associated Ig-like receptor-1 induces programmed cell death and prevents NF-kappaB nuclear translocation in human myeloid leukemias. *Eur. J. Immunol.* 30:2751-2758.
23. Wende,H., A.Volz, and A.Ziegler. 2000. Extensive gene duplications and a large inversion characterize the human leukocyte receptor cluster. *Immunogenetics* 51:703-713.
24. Vilches,C. and P.Parham. 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu. Rev. Immunol.* 20:217-251.
25. Welch,A.Y., M.Kasahara, and L.M.Spain. 2003. Identification of the mouse killer immunoglobulin-like receptor-like (Kirl) gene family mapping to chromosome X. *Immunogenetics* 54:782-790.
26. Hoelsbrekken,S.E., O.Nylenna, P.C.Saether, I.O.Slettedal, J.C.Ryan, S.Fossum, and E.Dissen. 2003. Cutting edge: molecular cloning of a killer cell Ig-like receptor in the mouse and rat. *J. Immunol.* 170:2259-2263.
27. Takai,T. and M.Ono. 2001. Activating and inhibitory nature of the murine paired immunoglobulin-like receptor family. *Immunol. Rev.* 181:215-222.
28. Liang,S., B.Baibakov, and A.Horuzsko. 2002. HLA-G inhibits the functions of murine dendritic cells via the PIR-B immune inhibitory receptor. *Eur. J. Immunol.* 32:2418-2426.
29. Borges,L. and D.Cosman. 2000. LIRs/ILTs/MIRs, inhibitory and stimulatory Ig-superfamily receptors expressed in myeloid and lymphoid cells. *Cytokine Growth Factor Rev.* 11:209-217.
30. Bruhns,P., P.Marchetti, W.H.Fridman, E.Vivier, and M.Daeron. 1999. Differential roles of N- and C-terminal immunoreceptor tyrosine-based inhibition motifs during inhibition of cell activation by killer cell inhibitory receptors. *J. Immunol.* 162:3168-3175.
31. Burshtyn,D.N., A.S.Lam, M.Weston, N.Gupta, P.A.Warmerdam, and E.O.Long. 1999. Conserved residues amino-terminal of cytoplasmic tyrosines contribute to the SHP-1-mediated inhibitory function of killer cell Ig-like receptors. *J. Immunol.* 162:897-902.
32. Yusa,S., T.L.Catina, and K.S.Campbell. 2002. SHP-1- and phosphotyrosine-independent inhibitory signaling by a killer cell Ig-like receptor cytoplasmic domain in human NK cells. *J. Immunol.* 168:5047-5057.
33. Huang,X., Z.Yuan, G.Chen, M.Zhang, W.Zhang, Y.Yu, and X.Cao. 2001. Cloning and characterization of a novel ITIM containing lectin-like immunoreceptor LLIR and its two transmembrane region deletion variants. *Biochem. Biophys. Res. Commun.* 281:131-140.
34. Olcese,L., P.Lang, F.Vely, A.Cambiaggi, D.Marguet, M.Blery, K.L.Hippen, R.Biassoni, A.Moretta, L.Moretta, J.C.Cambier, and E.Vivier. 1996. Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. *J. Immunol.* 156:4531-4534.
35. Yokoyama,W.M., L.B.Jacobs, O.Kanagawa, E.M.Shevach, and D.I.Cohen. 1989. A murine T lymphocyte antigen belongs to a supergene family of type II integral membrane proteins. *J. Immunol.* 143:1379-1386.
36. Adachi,T., H.Flaswinkel, H.Yakura, M.Reth, and T.Tsubata. 1998. The B cell surface protein CD72 recruits the tyrosine phosphatase SHP-1 upon tyrosine phosphorylation. *J. Immunol.* 160:4662-4665.
37. Abramson,J., R.Xu, and I.Pecht. 2002. An unusual inhibitory receptor--the mast cell function-associated antigen (MAFA). *Mol. Immunol.* 38:1307-1313.
38. Meyaard,L., van der Vuurst de Vries AR, T.de Ruiter, L.L.Lanier, J.H.Phillips, and H.Clevers. 2003. Retraction. *J. Exp. Med.* 198:1129.

3
CHAPTER

Identification and characterization of the rat homologue of LAIR-1

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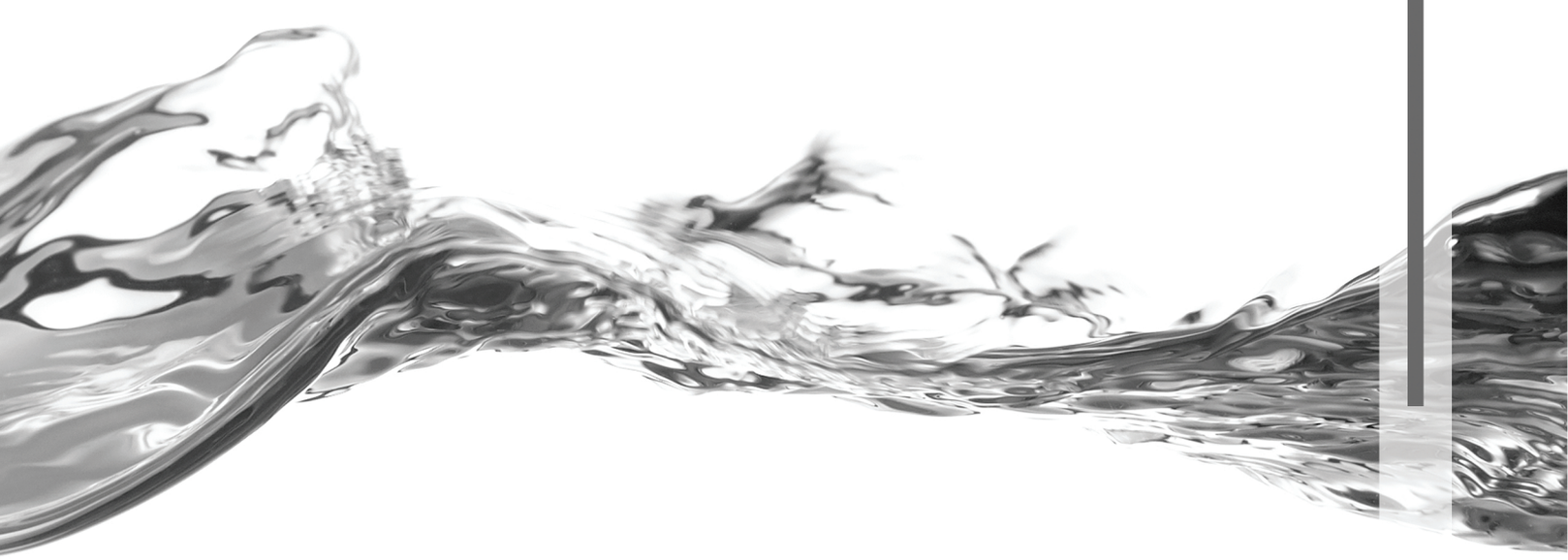

ABSTRACT

Leukocyte-associated Ig-like receptor-1 (LAIR-1) is a cell-surface molecule that functions as an inhibitory receptor on various immune cells in both humans and mice. We have cloned a LAIR-1 homologue from the rat that we have named rat LAIR-1. The *LAIR-1* gene maps to rat chromosome 1q12 in a region showing conserved synteny with human chromosome 19q13.4 and mouse chromosome 7 where the leukocyte receptor cluster is located. Rat LAIR-1 shows 40% and 71% protein sequence identity with human LAIR-1 and mouse LAIR-1 respectively, has a single Ig-like domain and contains two immunoreceptor tyrosine-based inhibitory motif-like sequences in its cytoplasmic tail. Soluble rat LAIR-1 fusion proteins bind to the same adherent cell lines as human LAIR-1 and mouse LAIR-1, indicating that a putative ligand for all the LAIR-1 molecules is expressed on these cells. Furthermore, we show that rat and mouse LAIR-1 bind the same molecule expressed on human HT29 cells. Since many autoimmune diseases are studied in rat models, identification of rat LAIR-1 allows for *in vivo* studies on the function of LAIR molecules in these systems.

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. Inhibitory receptors often bear immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domain with a I/V/L/SxYxxL/V consensus sequence (reviewed by [1]. The ITIMs become tyrosine phosphorylated upon ligand-induced clustering and subsequently recruit cytoplasmic phosphatases such as Src homology region 2-containing tyrosine phosphatase (SHP)-1, SHP-2 and SH2-containing inositol 5'-phosphatase (SHIP). These phosphatases abrogate signaling through activating receptors, thereby preventing cellular immune functions such as cytotoxicity or proliferation. In the last few years, many families of ITIM-bearing receptors have been recognized and cloned both in humans and rodents (reviewed by [2;3]). All mice described so far with targeted deletions in these inhibitory receptors display an autoimmune phenotype[4].

The human leukocyte-associated immunoglobulin-like receptor (LAIR)-1 is a member of the immunoglobulin superfamily (IgSF) that is expressed on the majority of peripheral blood mononuclear cells and thymocytes[5;6]. Cross-linking of human LAIR-1 by monoclonal antibodies (mAb) *in vitro* delivers a potent inhibitory signal that is capable of inhibiting cellular functions of several immune cell types[5;7-9]. In agreement with the observed inhibitory capacity of LAIR-1, the molecule has two ITIMs in its cytoplasmic tail and recruits the tyrosine phosphatases SHP-1[5;10-13] and SHP-2[5;11;13] upon activation. LAIR-1 is structurally related to several other inhibitory IgSF members, including human killer cell Ig-like receptors (KIRs), human FcαR, human leukocyte Ig-like receptors (LILRs, also known as Ig-like transcripts (ILTs) and CD85)[5]. Interestingly, LAIRs, KIRs, LILRs, and FcαR are all localized to the Leukocyte Receptor Complex (LRC) on human chromosome 19q13.4, suggesting that these molecules have evolved from a common ancestral gene (reviewed in [14;15]). Recently, we identified the mouse LAIR-1 homologue sharing a potent inhibitory capacity and similar expression profile with its human counterpart [16]. Although soluble human and mouse LAIR-1 molecules specifically interact with a panel of human and mouse adherent cell lines, the molecule mediating this binding has not yet been identified[16].

We identified a novel member of the LAIR family present in the rat, which we have named rat LAIR-1. The molecule has two ITIM-like sequences in its cytoplasmic tail, suggesting an inhibitory potential. Rat and mouse LAIR-1 bind the same ligand expressed on a variety of adherent cell lines. Identification of the rat homologue of LAIR-1 allows for *in vivo* studies in the rat on the function of LAIR molecules in regulation of different immune responses and could facilitate the search for a ligand of the LAIR-1 receptors.

MATERIALS AND METHODS

Cells

Cell lines were obtained from American Type Culture Collection (Manassas, VA) and cultured using standard techniques. Cell lines used in these studies were: human colon carcinomas HT29 and LS174, human embryonic kidney 293T cells, and human Jurkat T cells. Primary mouse and rat ear fibroblasts were obtained as follows: mouse and rat ears were rinsed twice in PBS, minced carefully, and subsequently incubated for 6 hours at 37°C with 30% FCS, 300 IU/ml penicillin/streptomycin, and 1 mg/ml collagenase type IA (Sigma Chemicals, St. Louis, MO) in RPMI 1640 medium. The treated tissues were dissociated by gentle pipetting, pelleted by centrifugation, resuspended in growth medium (RPMI supplemented with 30% FCS, 300 IU/ml penicillin/streptomycin) and subsequently cultured at 37°C for 10 days.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The rat LAIR-1 transcript was amplified by PCR from Wistar U:WU (CpB) rat spleen-derived cDNA using rat LAIR-1-specific forward (5'-CAGACCTGGTAAGGTTGCTGG-3') and reverse (5'-GTGACCATTTGTGTCTCCAGG -3') primers and the AmpliTaq® Gold DNA Polymerase system (Applied Biosystems, Foster City, CA). These primers correspond to the 5' and 3' untranslated region adjacent to the rat LAIR-1 coding region. The amplification reaction underwent 35 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 64°C, and elongation at 72°C for 45 s. The amplified RT-PCR product was cloned into the pGEM®-T Easy vector using the pGEM®-T Easy vector system (Promega, Madison, WI) and subsequently sequenced on an ABI 3100 sequencer (Applied Biosystems, Foster City, CA) using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA). The obtained sequence was analyzed by Lasergene software (DNASTAR, London, UK).

For analysis of the expression pattern of rat LAIR-1, total RNA was isolated from several rat organs (Wistar U:WU (CpB)) using the TRIZOL® Reagent (Invitrogen, Paisley, UK) according to the manufacturer's prescriptions. Total RNA was converted to first-strand cDNA with oligo(dT) primers and the SuperScript™ III Reverse Transcriptase Kit (Invitrogen, Paisley, UK). The cDNA-mixtures were amplified by PCR using rat LAIR-1-specific forward (5'-GCTTGTACTGTGCCTGGGATGG-3') and reverse (5'-TTCAGCCAAGATGTATCTGAGGTC-3') primers and the AmpliTaq® Gold DNA Polymerase system (Applied Biosystems, Foster City, CA). These primers correspond to exon 2 and 4 of rat *LAIR-1* respectively. Each amplification reaction underwent 31 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 62°C, and elongation at 72°C for 20 s. As a control, beta-2-microglobulin (B2M) transcripts were amplified using B2M-specific primers (forward: 5'-GACCGATGTATATGCTTGCAGAGT-3' and reverse: 5'-GGATCTGGAGTTAAACTGGTCCAG-3'). B2M primers were designed spanning several exons to ensure that products of expected size were derived from mRNA only.

Each amplification reaction underwent 32 cycles of denaturation at 95°C for 30 s, annealing for 30 s at 60°C, and elongation at 72°C for 10 s. Equal amounts of PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

Detection of LAIR ligand

Chimeric proteins composed of the leader sequence and the extracellular parts of rat LAIR-1 (aa 1-138), mouse LAIR-1 (aa 1–139) or human LAIR-1 (aa 1-162) fused to the Fc region of human IgG1 were inserted into the pcDNA3.1/zeo$^+$ vector (Invitrogen, Paisley, UK). The proteins, designated rLAIR-1-hIg, mLAIR-1-hIg and hLAIR-1-hIg respectively, were produced by stable expression in 293T cells and subsequent purification by affinity chromatography on protein A-Sepharose columns (Amersham, Freiburg, Germany). Cell lines were screened for the presence of a putative rat LAIR-1, mouse LAIR-1 and human LAIR-1 ligand as described before[16].

For fusion protein blocking studies, HT29 cells were incubated with unlabeled rLAIR-1-hIg, mLAIR-1-hIgs or hLAIR-1-hIgs for 30 minutes at RT and subsequently incubated with biotinylated rLAIR-1-hIg, mLAIR-1-hIgs or hLAIR-1-hIgs. For all fusion protein stainings, cells were pretreated with 10% BSA, 20% FCS, 10% normal mouse serum to block aspecific interactions. Binding of biotinylated fusion proteins was detected with PE-conjugated streptavidin. Biotin-conjugated, goat anti–human IgG1 mAbs were obtained from Caltag Laboratories (Burlingame, CA). Phycoerythrin (PE)-conjugated streptavidin was purchased from BD Biosciences (San Diego, CA).

Computer-assisted analysis

Identification of rat LAIR-1 was achieved by homology search on the National Center of Biotechnology Information (NCBI) rat genome database. Comparison of the rat, mouse and human LRC was performed using the public NCBI genomic database. The protein sequence alignment was generated by the Clustal method, using Lasergene analysis software (DNASTAR, Inc., Madison, WI) and with the aid of the BioEdit Sequence alignment editor (Hall, 1999).

Results

Identification of rat LAIR-1

To investigate whether LAIR-1 has a rat homologue, the NCBI rat genomic database was searched with a BLASTN algorithm for sequences bearing homology with the mouse LAIR-1a cDNA sequence. As a result, a rat LAIR-locus was identified on chromosome 1 in a region showing conserved synteny with human chromosome 19q13.4 and mouse chromosome 7 where the LAIR family members are encoded. Using specific primers, a single rat LAIR transcript was amplified from Wistar U:WU (CpB) rat spleen-derived

cDNA. The rat transcript contained the full-length open reading frame of 792 nt encoding a type I transmembrane protein of 263 amino acids (aa) with a predicted relative molecular mass of 29.7 kDa (Fig. 1, A and B). The cDNA sequence of the coding region of rat LAIR transcript derived from the Wistar U:WU (CpB) strain were identical to the genomic sequence of the BN/SsNHsdMCW-strain. The extracellular part of the rat LAIR molecule harbors one pair of cysteines (Fig 1A), allowing the protein to form a single Ig-like domain and classifying it as a member of the IgSF. The cytoplasmic domain contains two tyrosines at residues Y227 and Y257, both in ITIM-like sequences. The N-terminal sequence (VTYAQL) fits the I/V/L/SxYxxL/V-consensus sequence for ITIMs[1] and is identical to the human N-terminal ITIM. The C-terminal sequence (STYAAI) is identical to mouse LAIR-1, and does not exactly fit the ITIM consensus sequence.
Based on the moderate protein sequence identity to human LAIR-1 and mouse LAIR-1 of 40% and 71% respectively (Fig. 1A), and the similar protein architecture among the molecules, the newly identified protein was named rat LAIR-1. The DNA sequence of rat LAIR-1 was deposited in the GenBank™ database under the accession number AY863023.

The rat LAIR-1 gene maps to a LRC-like region on chromosome 1

The rat *LAIR-1* gene spans an area of approximately 28.2 kB of genomic sequence (Fig. 1B) and its intron-exon boundaries closely resemble the human and mouse *LAIR-1* gene (data not shown). Unlike human *LAIR-1* that consists of 10 exons, both mouse *LAIR-1* and rat *LAIR-1* contain 8 exons.
The rat *LAIR-1* gene maps to chromosome 1 in a region showing conserved synteny with human chromosome 19q13.4 and mouse chromosome 7 where the LRC is located (Fig. 1C). Based on sequence similarity, the region located near the rat *LAIR-1* gene resembles that of the human and mouse LRC indicating it is well conserved through evolution. Genes homologous to the lymphocyte antigen 94 (or natural cytotoxicity-triggering receptor 1), and LILRs appear to be conserved between the species. Furthermore, several non-Ig molecules, such as the LRC-encoded novel genes (LENGs), Tweety homologue 1 (TtyhI), CDC42 effector protein (Rho GTPase binding) 5 (CDC42EP5) seem conserved. In contrast, whereas the human LRC encodes a LAIR-2 gene[5], the rat and mouse LRC lack this gene. In addition, although the mouse LRC lacks a gene encoding the FcαR (CD89), this molecule is encoded in both humans and rats[17;18]. Furthermore, rats and mice appear to encode fewer PIRs located on this region of the chromosome as compared with genes encoding its human homologues (LILRs). Centromeric to rat Ly-94, the rat annotation is incomplete. Interestingly, the centromeric ILT cluster in man and the corresponding homologous PIR cluster in mice are missing in the rat LRC. This region, however, appears to be located several Mb centromeric of the rat NKp46 gene in an inverse orientation, other genes located in the vicinity of the human and mouse LRC (e.g. LENG5, LENG4, TMC4 and LENG1) lie here as well (data not shown). Only the mLRC contained putative genes encoding proteins with homology to a protease, 40S ribosomal protein and MO-25 (CAB39), these putative genes are absent in the rat and human LRC.

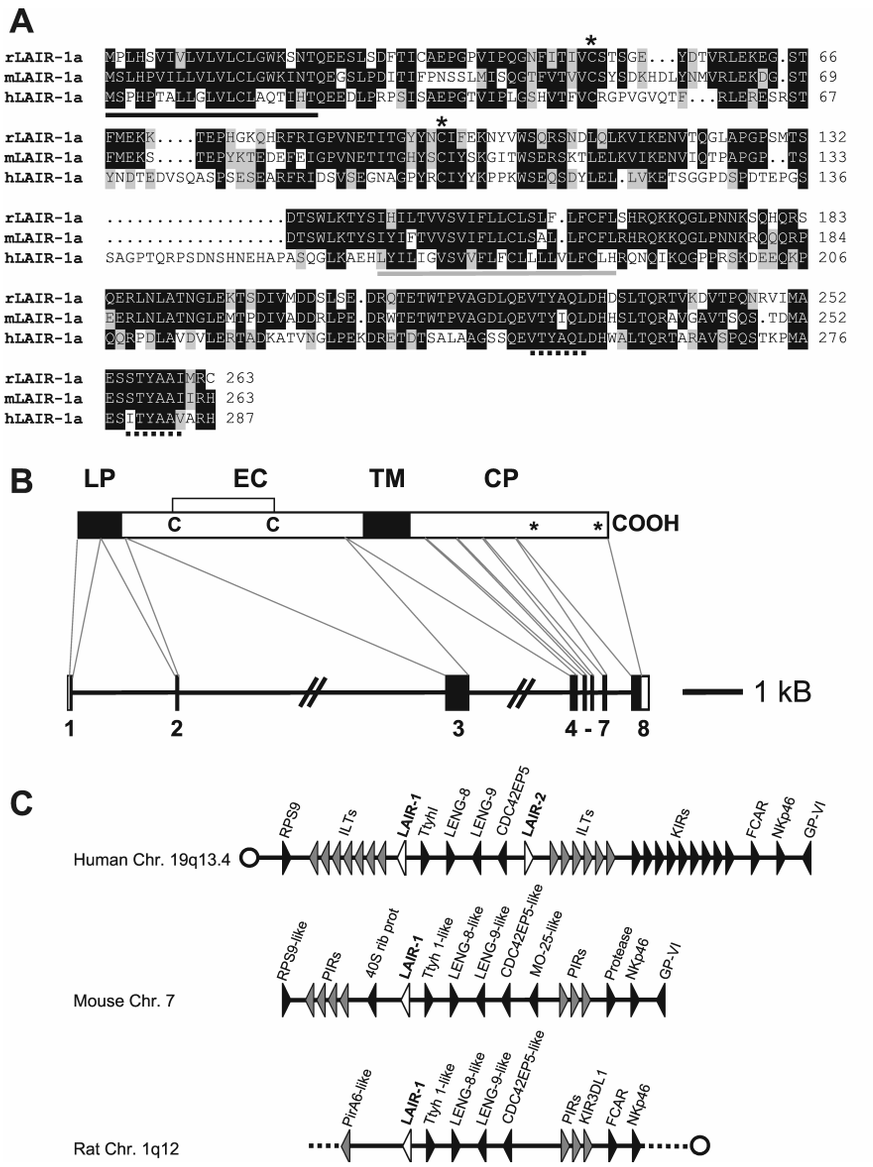

Figure 1. LAIR-1 has a rat homologue. (A) Protein sequence alignment of rat LAIR-1, mouse LAIR-1a and human LAIR-1a. The leader sequence and putative transmembrane domain are respectively black and gray underlined; the putative ITIM sequences in the cytoplasmic domain are underlined (dotted). Conserved residues are enclosed by darkly shaded boxes and residues showing conservative substitutions are enclosed in lightly shaded boxes. Conserved cysteines involved in intradomain disulfide bond formation are indicated by asterisks. Gaps introduced to optimize the sequence alignment are indicated by dots. The rat LAIR-1 sequence data is available from Genbank/EMBL/DDBJ under accession no. AY863023. (B) Schematic overview of the rat LAIR-1 gene and protein. The protein-coding sequences are denoted as closed boxes, and the non-coding sequences as open boxes. The protein structure is subdivided into the leader peptide (LP), the extracellular domain (EC), the transmembrane domain (TM), and a cytoplasmic domain (CP). The two cysteines forming the putative disulfide bonds (C-C) in the extracellular domain are shown. Putative ITIM-sequences are indicated by asterisks. Introns 2 and 3 are shown at 25% of their relative size. (C) Schematic organization of the human LRC on chromosome 19q13.4 (top panel) compared to mouse chromosome 7 (middle panel) and rat chromosome 1q12 (bottom panel). The genes include platelet glycoprotein VI (GP-VI), lymphocyte antigen 94 (Ly-94 or natural cytotoxicity triggering receptor 1 (NCR-1)), FcαR, KIR genes, leukocyte Ig-like receptors (LILRs), LAIR genes, paired Ig-like receptors (PIRs) and genes not belonging to the Ig-like superfamily: LRC-encoded novel genes (LENGs), ribosomal protein S9 (RBS9), Tweety homologue 1 (TtyhI), CDC42 effector protein (Rho GTPase binding) 5 (CDC42EP5), and a protein showing similarity to MO-25. Genes are represented by arrows, indicating their direction of transcription. The orientation of the rat and human LRC is indicated compared to the centromere (open circles), for the mouse LRC this is not known. Gaps and uncertainties in the rat genome assembly are represented by dotted lines. The maps are not to scale.

rat LAIR-1 is expressed in immune and non-immune organs

To assess rat LAIR-1 expression in various cell-types, RT-PCR analysis on various rat organs (Wistar U:WU (CpB)) using rat LAIR-1-specific primers was performed (Fig. 2). Unexpectedly, besides expression in immune-related organs (thymus, spleen and lymph nodes), rat LAIR-1 transcripts were also detected in non-lymphoid organs (e.g. brain, spinal cord, pituitary gland, adrenal gland and testis). In contrast, human and mouse LAIR-1 expression is restricted to immune cells[5;16]. Control amplifications of the same cDNA samples using beta-2-microglobulin (B2M)-specific primers indicated that all samples contained cDNA.

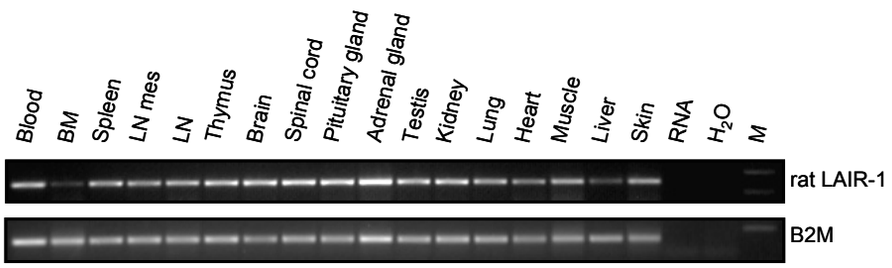

Figure 2. rat LAIR-1 is expressed in immune and non-immune organs. Semi-quantitative RT-PCR amplification of rat LAIR-1 and β2M cDNA fragments in various rat cell populations. rat LAIR-1 cDNA fragments were selectively amplified by RT-PCR from total RNA samples obtained from various rat tissues (Wistar U:WU (CpB)) or rat-derived primary cells and subsequently visualized by ethidium bromide staining. Bottom panel, control amplifications of a β2M cDNA fragment in the same cDNA samples. RNA samples were prepared from the following tissues: (from left to right) blood; bone marrow (BM); spleen; mesenteric lymph nodes (LNmes); additional lymph nodes (LN); thymus; brain; spinal cord; pituitary gland; adrenal gland; testis; kidney; lung; heart; muscle; liver; and skin. Negative controls using total RNA from spleen and water as templates are shown.

rat LAIR-1, mouse LAIR-1 and human LAIR-1 bind a ligand expressed on the same cell lines

To identify the natural ligand for the LAIR molecules, we constructed fusion proteins consisting of the extracellular domains of rat LAIR-1, mouse LAIR-1 or human LAIR-1 fused to the Fc portion of human IgG1 (r/m/hLAIR-1-hIg). The proteins were used as staining reagent to screen human cell lines for presence of a putative LAIR-ligand. Like mouse LAIR-1 and human LAIR-1[16], rat LAIR-1 fusion proteins bound to adherent human cells (human embryonic kidney 293T, colon carcinoma cell lines HT29 and LS174) whereas the human Jurkat T cell line appeared not to express a ligand for the receptor (Fig 3A). Furthermore, primary rat and mouse ear fibroblasts interacted with all three LAIR fusion proteins (Fig 3B). Binding of mLAIR-1-hIg to all cell lines was abolished after prior incubation with polyclonal anti-mouse LAIR-1 Abs, indicating the specificity of the observed interactions (as shown in [16]), this was also observed on the ear-derived fibroblasts (data not shown) suggesting that rat fibroblasts express a ligand for the LAIR-molecules.

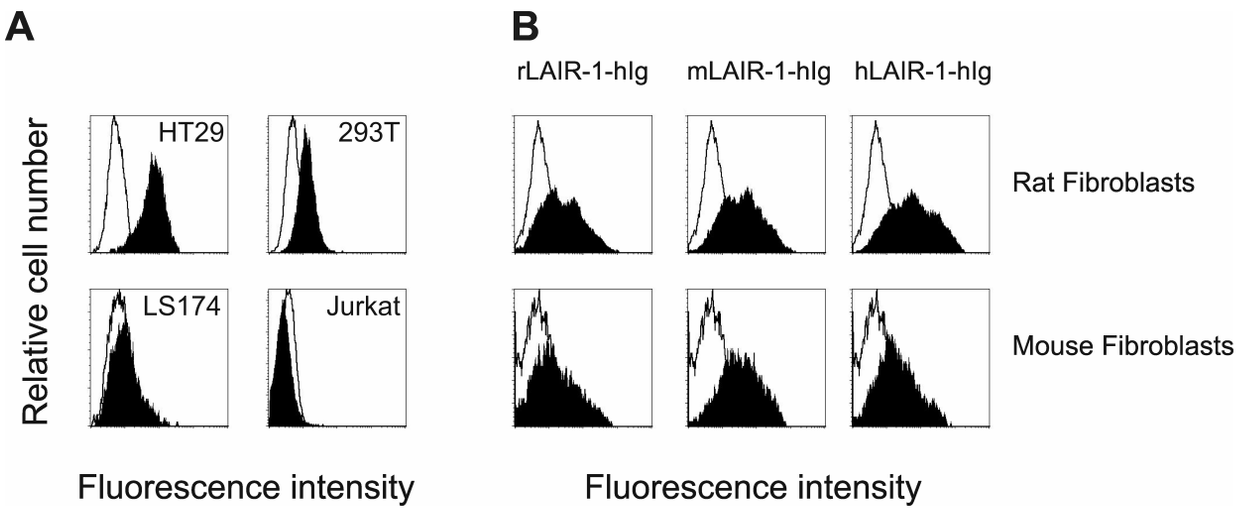

Figure 3. rat LAIR-1, mouse LAIR-1 and human LAIR-1 bind a ligand on the same cells (A) rLAIR-1-hIg binding to several human cell lines, demonstrating the presence of a putative ligand. Filled histograms represent staining with rLAIR-1-hIg. Open histograms represent staining with hIgG isotype control. (B) rLAIR-1-hIg, mLAIR-1-hIg and hLAIR-1-hIg binding to primary rat and mouse ear-derived fibroblasts. Staining conditions were identical as in Figure 3a.

rat LAIR-1 and mouse LAIR-1 bind the same ligand

The finding that all three LAIR-molecules bind a ligand on the same cells could indicate that they recognize the same molecule on these cells. To investigate this, we performed cross-species blocking experiments to determine whether LAIR-molecules from one species can block the interaction to that of another species. The human colon carcinoma cell line HT29 was pre-incubated with unlabeled rat LAIR-1-hIg and subsequently stained with biotinylated mouse or rat LAIR-fusion proteins. The biotinylated fusion protein was detected with PE-conjugated streptavidin. HT29 cells pre-incubated with mLAIR-1-hIg, showed abrogated binding of biotinylated rLAIR-1-hIg (cross-species block, 4B left panel) and mLAIR-1-hIg (intra-species block, 4A left panel) indicating that both rat and mouse LAIR bind the same molecule expressed on HT29 cells. In contrast, pre-incubation of HT29 cells with rLAIR-1-hIg (4B, right panel) did not abrogate subsequent biotinylated mLAIR-1-hIg binding. The rLAIR-1-hIg pre-incubation resulted in abrogation of the binding of biotinylated rLAIR-1-hIg, indicating that pre-incubation with rLAIR-hIg does lead to ligand occupation (Fig 4A, right panel). This could indicate that, although mouse and rat LAIR-1 bind the same molecule expressed on HT29 cells, mouse LAIR-1 interacts with a higher affinity to the unknown molecule.

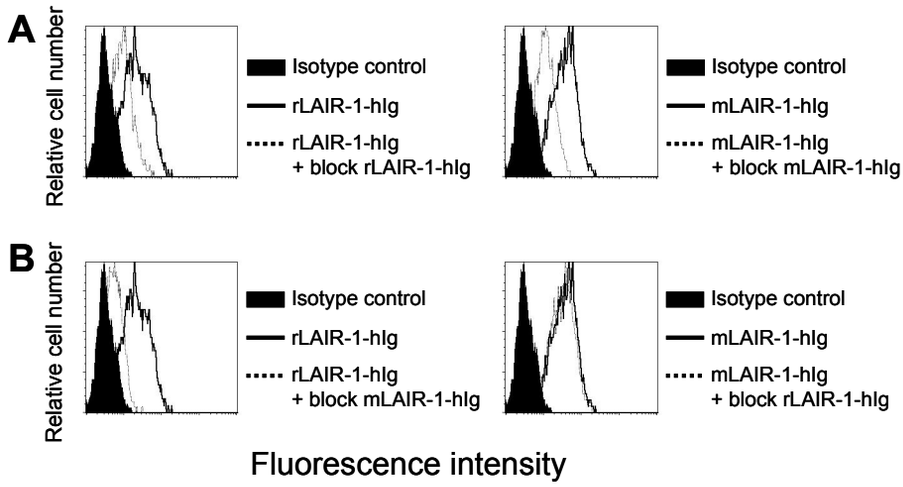

Figure 4. rat LAIR-1 and mouse LAIR-1 bind the same ligand expressed on HT29 cells (A) HT29 cells were pre-incubated for 30 minutes at RT with unlabeled rLAIR-1-hIg, mLAIR-1-hIg or control hIg (as indicated) and subsequently stained with biotin-conjugated versions of the same fusion protein (intra-species block). Specific binding of the biotinylated fusion proteins was detected with PE-conjugated streptavidin. (B) Same conditions as for Fig 4A, however the staining is performed with biotin-conjugated versions of the fusion protein of the other indicated species (cross-species block).

DISCUSSION

In this report we describe the identification of a rat homologue of the inhibitory receptors human LAIR-1 and mouse LAIR-1 and study the expression pattern of its putative, yet unknown, ligand.

The rat *LAIR-1* gene maps to rat chromosome 1 in a region similar to the LRC in humans and mice (Fig. 1C). This region encodes many members of the IgSF and is well conserved between humans and rodents. The orientation of several homologous genes in the human LRC indicate that this region arose as a result of a large inverse duplication and extensive gene duplications[15;19]. This same pattern is observed in the mouse[16] and rat LRC (Figure 1C). Nevertheless, there are some inter-species differences. Humans encode a soluble member of the LAIR family (LAIR-2)[5], while the encoding gene appears to be absent in mice [16] and rats. The similar architecture and high sequence identity between the human *LAIR-1* and *LAIR-2* genes suggest that the LAIR-1 gene appeared prior to the rodent/primate split in mammalian evolution and that human LAIR-2 might originate as a result of a *LAIR-1* gene duplication-event after the rodent-primate split. In addition, while both the human[17] and rat[18] LRC encode a copy of the FcαR (CD89), this gene appears to be absent from the mouse genome. Furthermore, whereas humans encode ~ 13 KIR family members[20], mice completely lack KIR genes on mouse chromosome 7. Although the rat LRC does encode a member of the KIR family (KIR3DL1), it remains unclear whether this gene can really be expressed since it has features of a pseudogene[21]. In addition, the centromeric ILT cluster in man and the corresponding homologous PIR cluster in mice is lacking in the rat LRC. Nevertheless, this region appears to be located several Mb centromeric of the rat NKp46 gene in an inverse orientation. This difference

could be caused by a large inverse translocation of that part of the genome in rats after the rat/mouse split in evolution. However, since the annotation of the rat genome at the NCBI-database is not yet completed and several gaps in the assembly remain, the comparison of this part of the chromosome between the three species should be interpreted with caution.

The presence of two ITIM-like structures in the rat LAIR-1 cytoplasmic tail suggests that this molecule can function as an inhibitory receptor on immune cells, as described for its human and mouse homologues[5;16]. While the rat N-terminal ITIM sequence is identical to the human N-terminal ITIM, the C-terminal ITIM-like sequence is identical to that of mouse LAIR-1. The latter sequence however, does not completely fit the ITIM consensus, and its contribution to signaling is not yet known. Cross-linking of chimeric mouse LAIR-1 molecules resulted in inhibition of ITAM-dependent signals initiated via the FcεRI complex on RBL-2H3 cells and cytotoxic activity of NK cells towards FcγR-bearing target cells. Furthermore, mouse LAIR-1 is able to recruit SHP-2 upon receptor triggering, but not SHP-1[16]. Considering the similarities between the rat and mouse LAIR-1 ITIM sequences, it is likely that rat LAIR-1 functions as an inhibitory receptor and is able to recruit SHP2 but not SHP-1.

Semi-quantitive RT-PCR studies with human and mouse LAIR showed expression of LAIR-1 predominantly in immune cell lines and immune-related organs[5;16], while rat LAIR-1 appears to be expressed in all organs. We also performed Northern blot analysis on total and poly-A^+ RNA samples from various rat organs (data not shown). Unfortunately, we were not able to detect a clear specific signal using two different rat LAIR-1 probes. This could indicate that the presence of rat LAIR-1-transcripts in whole-organ RNA samples is too low for detection using Northern blot analysis. Likewise, Northern blot analysis of mouse LAIR-1[16] and human LAIR-1[5] proved very difficult, suggesting that LAIR-1 mRNA is not abundantly present. The observed presence of rat LAIR-1 transcripts in non-immune organs by RT-PCR could be due to presence of rat LAIR-1 positive immune cells in these tissues. Unfortunately, because of the unavailability of rat cell lines, we were not able to compare expression in purified cells. Furthermore, since as yet no antibodies against the molecule are available, we are not able to investigate whether the presence of transcripts actually leads to expression of the protein in non-immune organs. Nevertheless, the result indicates that rat LAIR-1 expression is not restricted to a specific organ, but is likely to be more broadly expressed in the body. Whereas mouse LAIR-1 and human LAIR-1 give rise to several splice variants of the same gene[8;12;16], rat LAIR-1 appears to lead to a single transcript. The biological significance of the observed splice variants in humans and mice are still unknown.

We used LAIR-1-hIgG fusion proteins to screen a panel of human cell lines for presence of putative LAIR ligands. We showed that all LAIR-1 molecules interact with one or several putative ligand(s) expressed on the same adherent human cell lines. Furthermore, since pre-incubation of human HT29 cells with soluble mLAIR-1-hIg abolished the binding of rat LAIR-1 fusion proteins, it appears that mouse and rat LAIR-1 bind the

same molecule expressed on these cells. This cross-species ligand binding is also observed for several other members of the IgSF. Mouse ALCAM (CD166), for example, is capable of binding both human and mouse CD6[22]. Furthermore, human CD2 interacts with sheep CD58, providing the molecular basis for the aggregate formation (rosetting) between human T cells and sheep erythrocytes[23]. The cross-species ligand binding suggests that the ligand for LAIR-1 is a protein that is well conserved between humans and rodents. It is imperative for our understanding of LAIR biology to identify the molecule that binds the LAIR-1 fusion proteins, to explore whether ligation can stimulate the inhibitory function of the LAIR-1 molecules, and to confirm whether the observed cross-species interaction truly exists.

Previously, we published[24] and retracted[25] the finding that human LAIR-1 interacts with the epithelial cell adhesion molecule (Ep-CAM) as a ligand. The prior experiments showing that LAIR-1–hIg fusion proteins bind to EpCAM transfectants were an artifact resulting from contamination of the fusion proteins with an anti–human EpCAM monoclonal antibody, which was affinity purified using the same protein A columns. In the current experiments, we used a new Prot-A column for the purification of each fusion-protein, ruling out the possibility of co-purification of additional antibodies from previous experiments. Additionally, we showed that mLAIR-1-hIg binding to HT29 cells was abolished after prior incubation of the fusion proteins with polyclonal rabbit anti-mouse LAIR-1 antibodies, whereas irrelevant polyclonal antibodies had no influence on the observed interaction[16].

Inhibitory immune receptors are believed to play an important role in the prevention of excessive immune reactions and autoimmune disease. Since several autoimmune diseases are studied using rat models (e.g. experimental autoimmune encephalomyelitis (EAE), diabetes, and arthritis), identification of rat LAIR-1 could allows for *in vivo* studies on the function of LAIR molecules in these systems.

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REFERENCES

1. Ravetch,J.V. and L.L.Lanier. 2000. Immune inhibitory receptors. *Science* 290:84-89.
2. Katz,H.R. 2002. Inhibitory receptors and allergy. *Curr. Opin. Immunol.* 14:698-704.
3. Lanier,L.L. 2004. NK Cell Recognition. *Annu. Rev. Immunol.*225-274.
4. Pritchard,N.R. and K.G.Smith. 2003. B cell inhibitory receptors and autoimmunity. *Immunology* 108:263-273.
5. Meyaard,L., G.J.Adema, C.Chang, E.Woollatt, G.R.Sutherland, L.L.Lanier, and J.H.Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
6. Poggi,A., N.Pella, L.Morelli, F.Spada, V.Revello, S.Sivori, R.Augugliaro, L.Moretta, and A.Moretta. 1995. p40, a novel surface molecule involved in the regulation of the non-major histocompatibility complex-restricted cytolytic activity in humans. *Eur. J. Immunol.* 25:369-376.
7. van der Vuurst de Vries AR, H.Clevers, T.Logtenberg, and L.Meyaard. 1999. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur. J. Immunol.* 29:3160-3167.
8. Meyaard,L., J.Hurenkamp, H.Clevers, L.L.Lanier, and J.H.Phillips. 1999. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. *J. Immunol.* 162:5800-5804.
9. Poggi,A., E.Tomasello, E.Ferrero, M.R.Zocchi, and L.Moretta. 1998. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. *Eur. J. Immunol.* 28:2086-2091.
10. Sathish,J.G., K.G.Johnson, K.J.Fuller, F.G.LeRoy, L.Meyaard, M.J.Sims, and R.J.Matthews. 2001. Constitutive association of SHP-1 with leukocyte-associated Ig-like receptor-1 in human T cells. *J. Immunol.* 166:1763-1770.
11. Fournier,N., L.Chalus, I.Durand, E.Garcia, J.J.Pin, T.Churakova, S.Patel, C.Zlot, D.Gorman, S.Zurawski, J.Abrams, E.E.Bates, and P.Garrone. 2000. FDF03, a novel inhibitory receptor of the immunoglobulin superfamily, is expressed by human dendritic and myeloid cells. *J. Immunol.* 165:1197-1209.
12. Xu,M., R.Zhao, and Z.J.Zhao. 2000. Identification and characterization of leukocyte-associated Ig-like receptor-1 as a major anchor protein of tyrosine phosphatase SHP-1 in hematopoietic cells. *J. Biol. Chem.* 275:17440-17446.
13. Verbrugge,A., T.de Ruiter, H.Clevers, and L.Meyaard. 2003. Differential contribution of the Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) of human Leukocyte Associated Ig-like Receptor (LAIR)-1 to inhibitory function and phosphatase recruitment. *Int. Immunol.* 15:1349-1358.
14. Barten,R., M.Torkar, A.Haude, J.Trowsdale, and M.J.Wilson. 2001. Divergent and convergent evolution of NK-cell receptors. *Trends Immunol.* 22:52-57.
15. Martin,A.M., J.K.Kulski, C.Witt, P.Pontarotti, and F.T.Christiansen. 2002. Leukocyte Ig-like receptor complex (LRC) in mice and men. *Trends Immunol.* 23:81-88.
16. Lebbink,R.J., T.de Ruiter, A.Verbrugge, W.S.Bril, and L.Meyaard. 2004. The mouse homologue of the leukocyte-associated Ig-like receptor-1 is an inhibitory receptor that recruits Src homology region 2-containing protein tyrosine phosphatase (SHP)-2, but not SHP-1. *J. Immunol.* 172:5535-5543.
17. Maliszewski,C.R., C.J.March, M.A.Schoenborn, S.Gimpel, and L.Shen. 1990. Expression cloning of a human Fc receptor for IgA. *J. Exp. Med.* 172:1665-1672.
18. Maruoka,T., T.Nagata, and M.Kasahara. 2004. Identification of the rat IgA Fc receptor encoded in the leukocyte receptor complex. *Immunogenetics* 55:712-716.
19. Wende,H., A.Volz, and A.Ziegler. 2000. Extensive gene duplications and a large inversion characterize the human leukocyte receptor cluster. *Immunogenetics* 51:703-713.

20. Vilches,C. and P.Parham. 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu. Rev. Immunol.* 20:217-251.
21. Hoelsbrekken,S.E., O.Nylenna, P.C.Saether, I.O.Slettedal, J.C.Ryan, S.Fossum, and E.Dissen. 2003. Cutting edge: molecular cloning of a killer cell Ig-like receptor in the mouse and rat. *J. Immunol.* 170:2259-2263.
22. Bowen,M.A., J.Bajorath, M.D'Egidio, G.S.Whitney, D.Palmer, J.Kobarg, G.C.Starling, A.W.Siadak, and A.Aruffo. 1997. Characterization of mouse ALCAM (CD166): the CD6-binding domain is conserved in different homologs and mediates cross-species binding. *Eur. J. Immunol.* 27:1469-1478.
23. Arulanandam,A.R., A.Kister, M.J.McGregor, D.F.Wyss, G.Wagner, and E.L.Reinherz. 1994. Interaction between human CD2 and CD58 involves the major beta sheet surface of each of their respective adhesion domains. *J. Exp. Med.* 180:1861-1871.
24. Meyaard,L., van der Vuurst de Vries AR, T.de Ruiter, L.L.Lanier, J.H.Phillips, and H.Clevers. 2001. The epithelial cellular adhesion molecule (Ep-CAM) is a ligand for the leukocyte-associated immunoglobulin-like receptor (LAIR). *J. Exp. Med.* 194:107-112.
25. Meyaard,L., van der Vuurst de Vries AR, T.de Ruiter, L.L.Lanier, J.H.Phillips, and H.Clevers. 2003. Retraction. *J. Exp. Med.* 198:1129.

4

CHAPTER

Collagens are functional, high-affinity ligands for the inhibitory immune receptor LAIR-1

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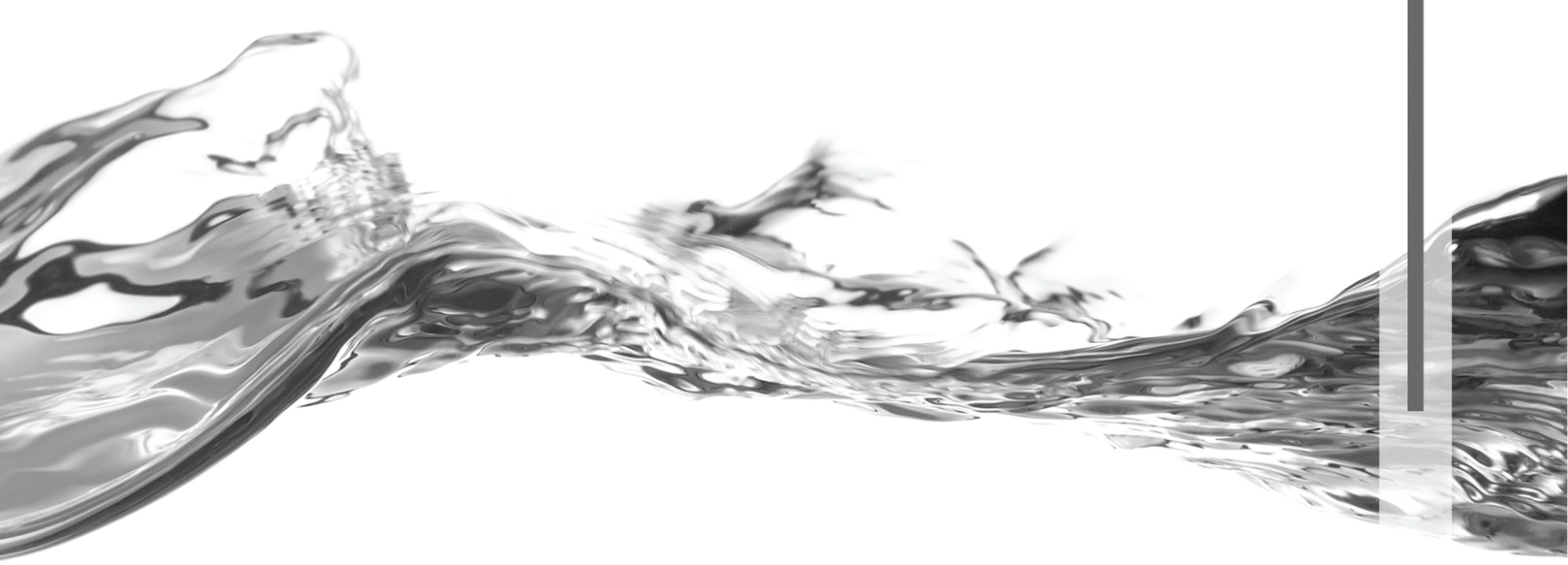

ABSTRACT

Collagens are the most abundant proteins in the human body, important in maintenance of tissue structure and hemostasis. Here we report that collagens are high affinity ligands for the broadly expressed inhibitory Leukocyte Associated Ig-like Receptor-1 (LAIR-1). The interaction is dependent on the conserved Gly-Pro-Hyp collagen repeats. Antibody cross-linking of LAIR-1 is known to inhibit immune cell function in vitro. We now show that collagens are functional ligands for LAIR-1 and directly inhibit immune cell activation in vitro. Thus far, all documented ligands for immune inhibitory receptors are membrane molecules, implying a regulatory role in cell-cell interaction. Our data reveal a novel mechanism of peripheral immune regulation by inhibitory immune receptors binding to extracellular matrix collagens.

INTRODUCTION

Collagens represent the most abundant type of proteins in vertebrates and play crucial roles in the development, morphogenesis, and growth of many tissues[1]. Besides their mechanical properties, collagens serve as substrates for cell attachment, migration, coagulation and mediate signaling events by binding to several cell surface receptors such as integrins, discoidin domain receptors (DDRs), glycoprotein VI, and proteoglycan receptors[2].

LAIR-1 is a member of the immunoglobulin superfamily (IgSF), which is expressed on the majority of peripheral blood mononuclear cells (PBMC) and thymocytes[3]. Antibody-induced cross-linking of the receptor in vitro delivers a potent inhibitory signal that is capable of inhibiting cellular functions of NK cells, effector T cells, B cells, and dendritic cell precursors[3-6]. This inhibitory signal is dependent on phosphorylation of tyrosine-residues located in immunoreceptor tyrosine based inhibitory motifs (ITIMs) present in the cytoplasmic tail of LAIR-1[7].

ITIM bearing receptors are important for an appropriate immune response which needs to be tightly controlled by the opposing action of activating and inhibitory signals. Immune cells are potentially exposed to multiple activating signals in the tissues, and inhibitory receptors are required to set a threshold for cell activation and thus prevent unwanted immune reactions[8]. Although all immune cells express multiple inhibitory receptors, these receptors have crucial, non-redundant functions, as underlined by receptor knock-out mice that demonstrate enhanced sensitivity to autoimmune-like diseases caused by an over-activated immune system[9]. The expression pattern of the receptors and the identity of their ligand determine at what stage of an immune response they are effective. Thus far, all documented ligands for immune ITIM-bearing receptors are membrane molecules, implying a regulatory role in cell-cell interaction. Our finding that collagens are ligands for an ITIM-bearing receptor reveals a novel mechanism of peripheral immune regulation by extracellular matrix proteins.

MATERIALS AND METHODS

Cells, transfectants, and cDNA

All cells were obtained from American Type Culture Collection. cDNA encoding hLAIR-1a and mLAIR-1a were cloned into the pMX-neo retroviral vector. Full-length mouse collagen XXIII was amplified by PCR from mouse lung cDNA and cloned in-frame with a C-terminal His_6- and FLAG-tag in the pCEP4-vector. Full-length hcollagen XVII, HIS-tagged hcollagen XIII, and hKIR3DL1 were kindly provided by PA Khavari (Stanford University, USA), T Väisänen (University of Oulu, Finland) and LL Lanier (UCSF, USA) respectively. The chimeric reporter construct was generated by fusing the extracellular domain of hLAIR-1a to the transmembrane and intracellular domain of hCD3ζ.

Retroviral-based constructs were packaged by the pCL-eco or pCL-ampho system[29], and virus was used to infect Ba/F3, K562 or 2B4 NFAT-GFP T cell hybridoma reporter cells (kindly provided by LL Lanier (UCSF, USA). Three days after transduction, transfectants expressing either hLAIR-1, mLAIR-1, hcollagen XVII, hKIR3DL1, or hLAIR-1-CD3ζ were sorted for high expression on the cell surface by using a flow cytometer (FACSAria; BD Biosciences). hCollagen XVII expression was assessed by using the 233 mAb (a kind gift from K.Owaribe, Nagoya University, Japan). mLAIR-1 was detected by using a biotinylated anti-mLAIR-1 monoclonal antibody.

Detection of LAIR ligand

Chimeric proteins of the extracellular domain of rat, mouse, and hLAIR-1 or hLAIR-2 fused to the Fc region of human IgG1 were prepared, and cell lines were stained with these reagents in the absence or presence of blocking antibodies, as described previously[11]. When indicated, cells were incubated for 1 h with 100 units/ml chromatography purified *C.histolyticum* collagenase Type VII (Sigma) prior to staining with the fusion proteins.

Identification of LAIR-ligand

To identify the natural ligand for LAIR-1 we employed expression cloning by using a mouse retroviral cDNA library (day 14 whole embryo, a gift from GQ Daley, Harvard Medical School, Boston, MA, USA). Viral supernatant was produced[29] and used to infect LAIR-ligand-negative Ba/F3 cells. Three days after transduction, LAIR-ligand positive cells were sorted in the presence of 20% normal mouse serum using magnetic beads (Dynal, Oslo, Norway) coated with mLAIR-1-IgG. The positive cells were expanded and subsequently single-cell cloned by limiting dilution. We obtained 57 LAIR-ligand positive Ba/F3 clones from two independent transductions. PCR amplification of the retroviral inserts by using primers specific to the retroviral vector sequences flanking the cDNA inserts was not successful, most likely due to the large size of the insert. We took two alternative approaches to reveal the identity of LAIR-ligand in these clones. First, we compared the mRNA expression of two independent LAIR-ligand-expressing Ba/F3 clones with the parental Ba/F3 line by using microarrays. Briefly: RNA was extracted by using the TRIzol reagent according the procedure recommended by the supplier (Invitrogen). RNA amplification and labeling of cRNA was performed as described[27], and the cRNA was hybridized onto Corning UltraGAPS slides containing the Operon Mouse Genome Oligo Set V3[30]. After washing, scanning, and data-extraction, analysis was performed with SAM 1.2.1[31] and/or ANOVA (R/MAANOVA version 0.95-3) software. This screen revealed two mRNAs that were significantly upregulated in two independently derived mouse LAIR-ligand positive Ba/F3 clones compared to the parental Ba/F3 cell line. These cRNAs encoded the transmembrane collagen XVII and an unknown putative protein.

In parallel, we performed immunoprecipitation, by using mLAIR-1-IgG on surface biotinylated LAIR-ligand-expressing cells and the parent Ba/F3 cells. Briefly: protein

A/G PLUS–agarose beads (Santa Cruz Biotechnology) were coated with mLAIR-1-IgG fusion proteins. $2x10^9$ Ba/F3 cells were surface-biotinylated for 30 min on ice using 2 mg/ml Biotin 3-sulfo-N-hydroxysuccinimide ester sodium salt (Sigma) in PBS and were washed three times with 10 mM ammonium chloride in PBS. Cells were lysed in Triton lysis buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 and 0.02% sodium azide) supplemented with protease inhibitors (Complete Mini EDTA-free protease inhibitor cocktail tablets; Roche) and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were cleared by centrifugation and used for immunoprecipitation for 2 h in the presence of 0.5% BSA. Beads were washed 5 times with Triton wash buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 0.02% sodium azide). Interacting proteins were subsequently dissolved in electric focusing (IEF)-buffer (8M urea, 2M Thiourea, 4% CHAPS, 20 mM DTT, 0.2% Biolythe pH3-10 and 0.2% bromophenolblue) and subjected to 2D-electrophoresis by using Immobiline dry strips (pH 3–10; 11 cm) on an Ettan™ IPGphor™ system (Amersham, Little Chalfont, UK) for separation in the first dimension. Subsequently, separation in the second dimension was performed on 10% polyacrylamide gels by SDS-PAGE using a Sturdier Vertical Slab Gel Unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The gels were transferred to Immobilon-P membranes (Millipore, Bedford, MA) or subjected to silver staining by using standard techniques. Western blot analysis was performed with HRP-linked streptavidin (Invitrogen). Proteins were detected by enhanced chemiluminescence (Amersham). One specific biotinylated spot of ~140kDa was visible in the LAIR-ligand positive clone that was absent in the wild type Ba/F3 cells. The corresponding spot on the silver stained 2D-gel was subjected to mass spectrometry. In-gel proteolytic digestion of the silver-stained spot using trypsin (Roche) was performed essentially as described previously[32]. Samples were subjected to nanoflow liquid (LC) chromatography (Agilent 1100 series) and concentrated on a C18 precolumn (100um ID, 2cm). Peptides were separated on an analytical column (100μM ID, 20 cm) at a flow rate of 200 nl/min with a 60 min linear acetonitrile gradient from 0 to 80%. The LC system was directly coupled to a QTOF Micro tandem mass spectrometer (Micromass Waters, UK). A survey scan was performed from 400-1200 amu s^{-1}, and precursor ions were sequenced in MS/MS mode at a threshold of 150 counts. Data were processed and subjected to database searches by using Proteinlynx Global Server version 2.1 (Micromass, UK) or MASCOT software (Matrixscience) against the SWISSPROT and the NCBI nonredundant database, with a 0.3 Da mass tolerance for both precursor ion and fragment ion. The identified peptide (DGFTGDLDYNK) was confirmed by manual interpretation of the spectra and proved identical to mouse collagen XVII.

Conjugate-analysis

K562 transfectants were labeled for 5 min at room temperature with either PKH67 (green) or PKH26 (red) (Sigma) according the manufacturer's protocol. Cells were mixed at a ratio of 1:1 and incubated at 37°C for 1.5 h for mLAIR-1-expressing cells and 5 h for

hLAIR-1-expressing cells. Cells were gently resuspended before flow cytometric analysis or analysis by light microscopy.

Analysis of the binding of K562 transfectants to plate-bound collagens

96-well MAXIsorp (Nunc) flat-bottom plates were coated overnight at 4°C with purified collagens I, III (Sigma), II (Chemicon International), or BSA (100 µl/well, 20 µg/ml in PBS, 2 mM acetic acid). After washings, wells were blocked with 1% (w/v) BSA. Meanwhile, $5x10^6$ cells/ml wild type K562 cells or K562 transfectants were fluorescently labeled for 30 min at 37 °C with 5 µM calceine AM (Molecular Probes) in PBS. Cells were washed twice with RPMI-1640 containing 1% FCS, $1.5x10^6$ cells/ml in 100 µl medium were added to each well, and plates were incubated at 37°C for 2 h. Where indicated, cells were pre-incubated with 50 µg/ml anti-hLAIR-1 $F(ab')_2$ (8A8) fragments for 15 minutes at room temperature prior to addition to the wells. Input fluorescence was determined by using a fluorescence plate reader (Fluoroskan Ascent, Thermo Labsystems). After incubation, the plates were firmly flicked and washed four times in culture medium. The retained fluorescence was determined for each well as a percentage of input fluorescence.

Precipitation studies

For immunoprecipitation using LAIR-1-IgG: Protein A/G PLUS–agarose beads (Santa Cruz Biotechnology) were coated with either mLAIR-1-IgG, hLAIR-1-IgG or control protein. Immunoprecipitation was performed for 2 h in the presence of 10 µg purified human collagen III (Sigma) and 1% BSA in Triton lysis buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 % Triton X-100 and 0.02% sodium azide) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitors (Complete Mini EDTA-free protease inhibitor cocktail tablets; Roche, Mannheim, Germany). Immune complexes were washed with Triton wash buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 % Triton X-100 and 0.02% sodium azide) supplemented with 1 mM phenylmethylsulfonyl fluoride, and boiled in Laemmli sample buffer supplemented with 2-mercaptoethanol. Proteins were resolved by SDS–PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blot analysis was performed with goat anti-human collagen III antibodies (SBA) followed by HRP-linked secondary antibodies. Proteins were detected by enhanced chemiluminescence (Amersham, Little Chalfont, UK).

For precipitations using insoluble collagen I fibrils: Collagen fibrils were prepared by dialysis of a nonfibrillar collagen I solution (1 mg/ml in 50 mM acetic acid) against 0.02 M sodium phosphate buffer, pH 7.4, for 24 h at 4°C. Collagen fibrils were separated from nonpolymerized collagen by centrifugation (5 min, 13,000 rpm, 4°C). Human PBMC or K562 cells transfected with hLAIR-1 were pre-incubated with blocking anti-human LAIR-1 F(ab')2 fragments or left untreated, lysed in Triton lysis buffer and cleared by centrifugation (5 min, 13,000 rpm, 4°C). Cell-lysates were incubated with insoluble collagen I fibrils for 1 hour at 4°C. Collagen fibrils and interacting proteins were centrifuged, washed three times with Triton wash buffer and boiled in Laemmli sample buffer supplemented with 2-mercaptoethanol. Proteins were resolved by SDS–PAGE and

transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blot analysis was performed with mouse anti-hLAIR-1 mAbs (8A8) followed by HRP-linked secondary antibodies. Proteins were detected by enhanced chemiluminescence (Amersham, Little Chalfont, UK).

Human skin tissue section staining

Frozen tissue sections from healthy human skin were fixed in 10 % acetone in the presence of 1.5% H_2O_2 and free biotin in the sections was blocked using the avidin/biotin blocking kit (Vector, Burlingame, CA, USA). Tissue sections were stained with biotinylated mLAIR-1-IgG, hLAIR-1-IgG or isotype-matched control Ig in the presence of 2% Fetal calf serum and, when indicated, 100 μg/ml purified human collagen I (Sigma). After washing with PBS, sections were treated with streptABComplex/HRP (DAKO, Glostrup, Denmark) according to the manufacturer's instructions, counterstained with hematoxylin, dehydrated and mounted in DePeX mounting medium (British Drug House Ltd, Pool, UK). Sections were analyzed by light microscopy.

Reporter cell assay

2B4 T cell hybridoma cells stably transduced with an NFAT-GFP reporter and hLAIR-1-CD3ζ were analyzed as described[17]. Briefly, 96-well MAXIsorp flat-bottom plates (Nunc) were coated overnight at 4°C with purified collagens I, III, BSA, or anti-hLAIR-1 Abs (8A8) (100 μl/well, 10 μg/ml in PBS, 2 mM acetic acid). After washings, $3.5x10^5$ cells/ml in 200 μl medium were added to each well, and plates were incubated at 37°C for 20 hours and then analyzed for GFP expression by flow cytometry.

Surface plasmon resonance experiments

Surface plasmon resonance (BIAcore) binding studies were performed by using a BIAcore2000 system (BIAcore AB, Uppsala, Sweden). Approximately 2000-3000 response units (RU) 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 $GCO(GPO)_{10}GCOG\text{-}NH_2$ ($(GPO)_{10}$, also known as collagen-related peptide, CRP[16]) and $GCP(GPP)_{10}GCPG\text{-}NH2$ ($(GPP)_{10}$) were described previously[16]. 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 for collagen I and III interaction-studies and 5 μl/min for the immobilized peptides. Binding of hLAIR-1-IgG to collagen I and III was specific, because nonspecific binding to an uncoated control channel was less than 2% compared to collagen-coated channels. In addition, an irrelevant IgG-fusion protein did not bind to the collagen-coated surface. hLAIR-1-IgG dimer concentration was calculated based on a theoretical mass of 85.2 kDa (corrected for removal of leader peptide). Increasing concentrations of hLAIR-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).
Dissociation constants (Kd) and the number of binding sites expressed as the response at infinite hLAIR-1-IgG concentration (Bmax) were calculated as follows. First, the response at equilibrium (Req) was calculated for each association curve. Subsequently, Kd and Bmax were determined from the binding isotherms (Req plotted against hLAIR-1-IgG concentration) by the fitting equation Req = Bmax*[hLAIR-1-IgG]/(Kd + [hLAIR-1-IgG]). The fit was calculated by using GraphPad Prism (GraphPad Prism version 3.00 for Windows; GraphPad Software, San Diego, CA, USA). Bmax values were converted to number of hLAIR-1-IgG molecules interacting with a single collagen trimer by using the theoretical mass of hLAIR-1-IgG (85.2 kDa) versus collagen I and III (416.7 and 415.7 kDa, respectively).
The dissociation of hLAIR-1-IgG in the presence of buffer was followed for at least 13 h and Koff-values were calculated by using the Biaevaluation software version 3.0.1.

Perfusion studies

Perfusions were carried out in a single-pass perfusion chamber as described previously[33]. Briefly, collagen type III was solubilized in 50 mM acetic acid and sprayed onto glass coverslips by using a retouching airbrush (Badger model 100; Badger Brush) at a density of 6.5 μg/cm^2. Afterwards, coverslips were blocked for 1 h at room temperature with 1% human albumin in PBS. Subsequently, wild type or transfected K562 cells were perfused for 5 min at a shear rate of 0.75 dyne/cm^2 at 37^{o}C. After perfusion, slides were washed with HEPES buffer (10 mm HEPES, 150 mm NaCl, pH 7.35), fixed in 0.5% glutaraldehyde in PBS, dehydrated in methanol, and stained with May-Grünwald and Giemsa. Adhered K562 cells were counted by using light microscopy and presented as number of cells per mm^2.

RESULTS AND DISCUSSION

Collagen XVII is a ligand for LAIR-1.

By expression cloning, immunoprecipitation and subsequent protein sequencing, we identified transmembrane collagen XVII as a ligand for LAIR-1. The interaction was confirmed by specific binding of human (h) LAIR-1-IgG to Ba/F3 cells stably transfected with hcollagen XVII (Fig. 1A). Furthermore, rat (r) and mouse (m) LAIR-1-IgG bound to hcollagen XVII transfected cells but not to the untransfected parental cell line. Binding of hLAIR-1-IgG and mLAIR-1-IgG to hcollagen XVII was blocked by anti-hLAIR-1 Abs (8A8) or polyclonal anti-mLAIR-1 Abs respectively, demonstrating the specificity of these interactions (Fig. 1B and 1C). The association was divalent cation-independent; EDTA did not affect LAIR-1 fusion-protein binding (data not shown). In addition, human LAIR-2, a putatively secreted protein that is 84% homologous to hLAIR-1[10], interacted

with hcollagen XVII (Fig. 1A). Thus, collagen XVII is a ligand for LAIR-1 and LAIR-2 and, as we observed previously, ligand recognition occurs cross-species[11;12].

To confirm that LAIR-1 expressed on cells can bind to collagen XVII, we measured formation of conjugates between LAIR-1 and collagen XVII transfected K562 cells by flow cytometry. We observed profound aggregation between mLAIR-1 and hcollagen XVII-expressing cells, an interaction that was formed within minutes and remained stable for at least 24 h (Fig. 2A and 2B). mLAIR-1 transfected cells were more efficient in forming conjugates with hcollagen XVII-transfected cells than hLAIR-1 transfected cells (Fig. 2A). This difference was evident both in the percentage of cells present in a conjugate (Fig. 2A) as in the time after which optimal conjugate formation was observed (data not shown). This may indicate an intrinsic difference between mouse and hLAIR-1 in affinity to the collagen XVII trimer.

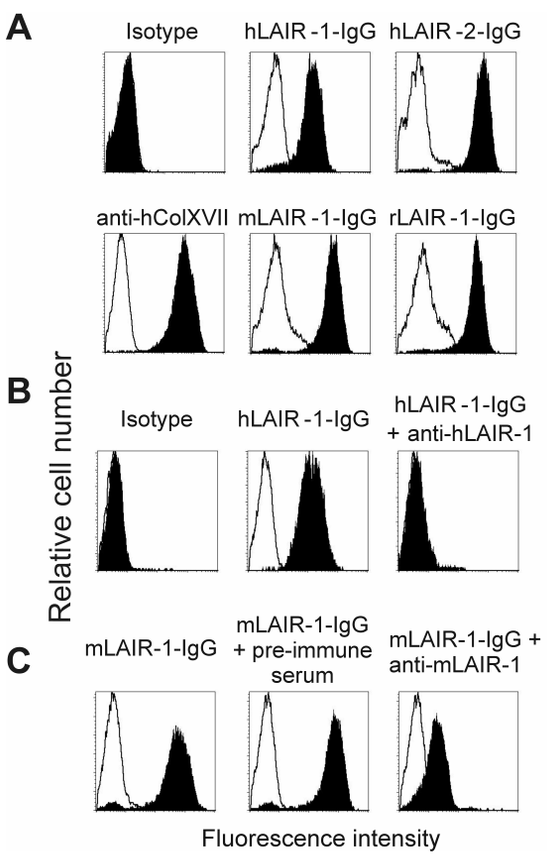

Figure 1. Collagen XVII is a ligand for LAIR-1. (A) Ba/F3 cells transfected with hcollagen XVII (filled histograms) or the parental cell line (open histograms) were stained with the indicated LAIR-fusion proteins (LAIR-IgGs), hIgG isotype control (isotype), or anti-collagen XVII antibodies (anti-hColXVII). (B) Anti-hLAIR-1 mAb (8A8) completely abrogated the hcollagen XVII/hLAIR-1-IgG-interaction. (C) Polyclonal anti-mLAIR-1 antibodies abrogated hcollagen XVII/mLAIR-1-IgG-interaction, whereas control serum does not. Experiments are representative of three independent experiments.

LAIR-1 is a general collagen receptor

We investigated whether the previously observed binding of LAIR-1 to human tumor cell lines[11] correlated with collagen XVII expression. LAIR-1-ligand-positive[11] HT29 colon carcinoma cells expressed collagen XVII and pre-treatment of these cells with *C.histolyticum* collagenase abrogated both LAIR-1-IgG and anti-collagen XVII mAb binding (Fig. 3A). The collagen XVII-negative breast carcinoma cell line SK-BR-3 however, also expressed a ligand for LAIR-1 that was removed after collagenase

treatment, suggesting that LAIR-1 may bind to another collagen family member on these cells (Fig. 3A). Indeed, transient expression of transmembrane collagens XIII and XXIII in 293T cells resulted in binding of LAIR-1-IgG (Fig. 3B). In addition, immobilized non-transmembrane collagens I, II and III were ligands for LAIR-1 (Fig. 3C). LAIR-1-transfected K562 cells firmly adhered to collagens, which coincided with cell spreading (Fig. 3D). Both mouse and human LAIR-1-IgG immunoprecipitated hcollagen III from solution (Fig. 3E). Furthermore, insoluble collagen I fibrils specifically precipitated LAIR-1 from hLAIR-1-transfected K562 cell-lysate as well as from human PBMC-lysate expressing endogenous LAIR-1 (Fig. 3F). Additionally, mouse and human LAIR-1-IgG bound specifically to human skin tissue sections (Fig. 3G). The collagen I and III-rich dermis stained brightly with LAIR-1-IgG, which was completely blocked by pre-incubation of the fusion proteins with hcollagen I. We conclude that LAIR-1 is a receptor for multiple transmembrane and extracellular matrix collagens.

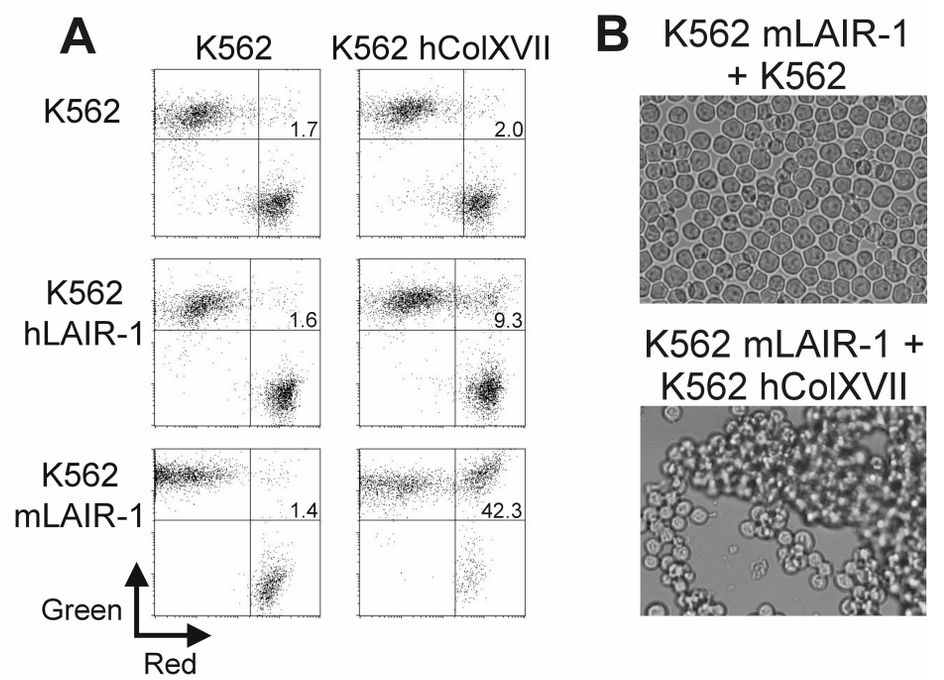

Figure 2. Collagen XVII and LAIR-1 transfected cells form aggregates. K562 cells transfected with hLAIR-1, mLAIR-1 or hcollagen XVII were either red or green fluorescently labeled, co-incubated in various indicated combinations at 37°C and analyzed by flow cytometry (percentage of double-positive cell conjugates is indicated) (A) or by visual inspection for cell clustering (B). Experiments are representative of three independent experiments.

LAIR-1 is a high-affinity collagen receptor

We measured affinity of the collagen-LAIR interaction by surface plasmon resonance (BIAcore). hLAIR-1-IgG bound with high affinity to collagen I and III (Fig. 4A), and its relatively slow dissociation was characterized by a rapid initial phase and a slower secondary phase (Fig. 4B). LAIR-1 bound to collagen with approximately 40-times higher affinity than the well-studied collagen receptor glycoprotein VI (GpVI)[13]. Furthermore,

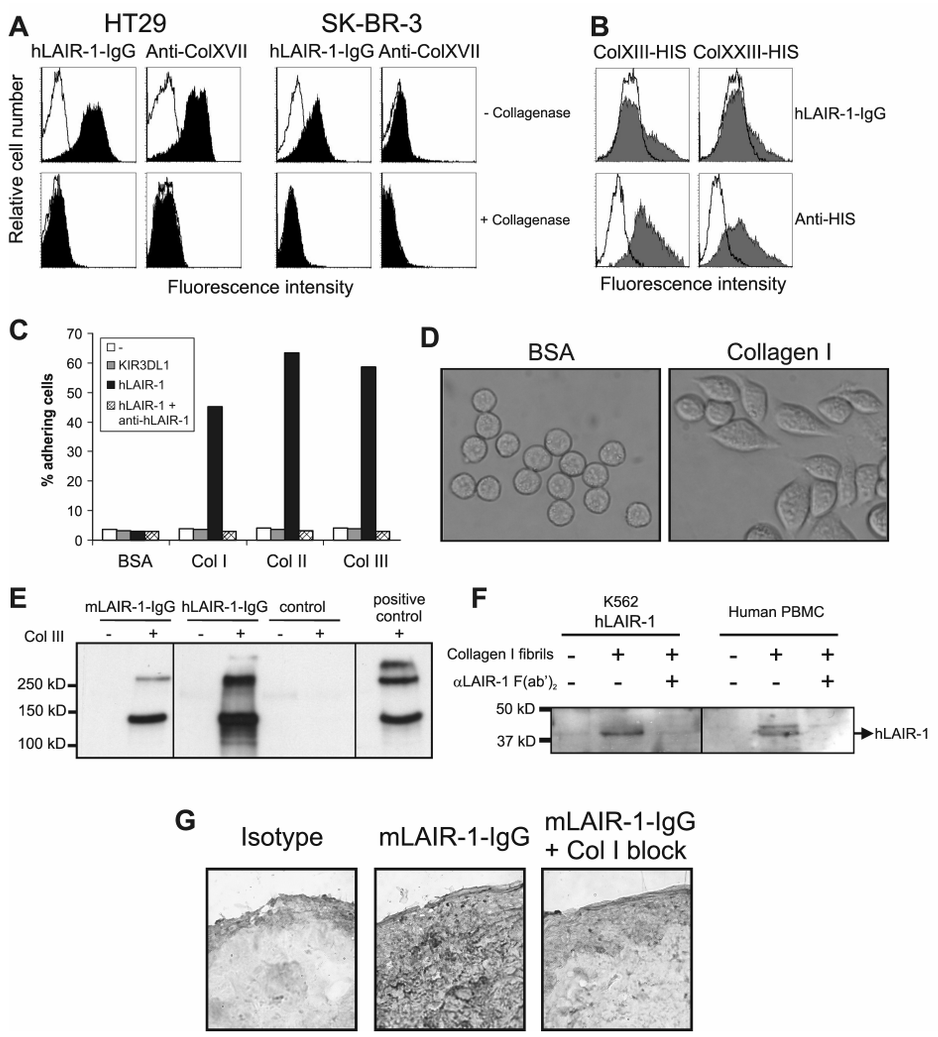

Figure 3. LAIR-1 is a collagen receptor. (A) SK-BR3 cells express a collagen ligand for LAIR-1 that is not collagen XVII. HT29 or SK-BR3 cells were stained with hLAIR-1-IgG or anti-collagen XVII mAb (filled histograms) with or without pre-treatment with collagenase; open histograms display staining with isotype-matched control Ig. (B) 293T cells were transiently transfected with control vector (open histograms) or indicated HIS-tagged collagens (gray histograms) and stained with hLAIR-1-IgG (top panels) or anti-HIS-antibody (bottom panels). Similar results were obtained using hLAIR-2-IgG, mLAIR-1-IgG, and rLAIR-1-IgG. (C) Fluorescently labeled wt K562 cells (open bars) or K562 cells expressing hLAIR-1 (black bars) or KIR3DL1 (gray bars) were monitored for their capacity to bind immobilized collagens I, II and III. Where indicated, cells were pre-incubated with anti-hLAIR-1 F(ab')$_2$ (8A8) fragments (hatched bars). Two other members of the IgSF (CD48 and CD80 transfected in K562 cells) did not associate with collagens (data not shown). Percentage of adhering cells relative to input is shown. One of three independent experiments is shown. (D) mLAIR-1-transfected K562 cells spread upon interaction with immobilized collagen I. Spreading was also observed upon interaction with immobilized $(GPO)_{10}$, but not $(GPP)_{10}$ (data not shown). Non-transfected cells did not adhere to collagen I or $(GPO)_{10}$ (data not shown). (E) Immunoprecipitation using mLAIR-1-IgG, hLAIR-1-IgG or control protein with (+) or without (-) purified hcollagen III. Interacting proteins were Western blotted using anti-hcollagen III specific mAbs. Positive control represents purified collagen III. (F) Cell-lysates from human PBMCs or hLAIR-1-transfected K562 were pre-incubated with or without anti-hLAIR-1 F(ab')2 fragments before incubation with insoluble collagen I fibrils. Collagen fibrils and interacting proteins were centrifuged, washed and Western blotted using anti-hLAIR-1 mAbs. (G) Tissue sections of human skin stained with biotinylated mLAIR-1-IgG (middle panel) or control Ig (left panel) followed by streptavidin-HRP and counterstaining with hematoxylin. Staining was blocked by pre-incubation of mLAIR-1-IgG with purified collagen I (right panel). Staining with hLAIR-Ig gave similar results.

the interaction was of 20-1000-fold higher affinity as compared to most IgSF-members interacting with their ligands[14]. Although purified LAIR-1-IgG bound directly to collagen I and III (Fig. 4A), we cannot exclude that additional proteins might modulate the binding in vivo. A single triple-helical collagen I or III molecule interacted with approximately 10.4 and 9.7 LAIR-1 proteins respectively (Fig. 4A), and this high affinity interaction was sufficient to arrest K562 cells expressing hLAIR-1 on collagen III-coated coverslips under flow conditions (Fig. 4C).

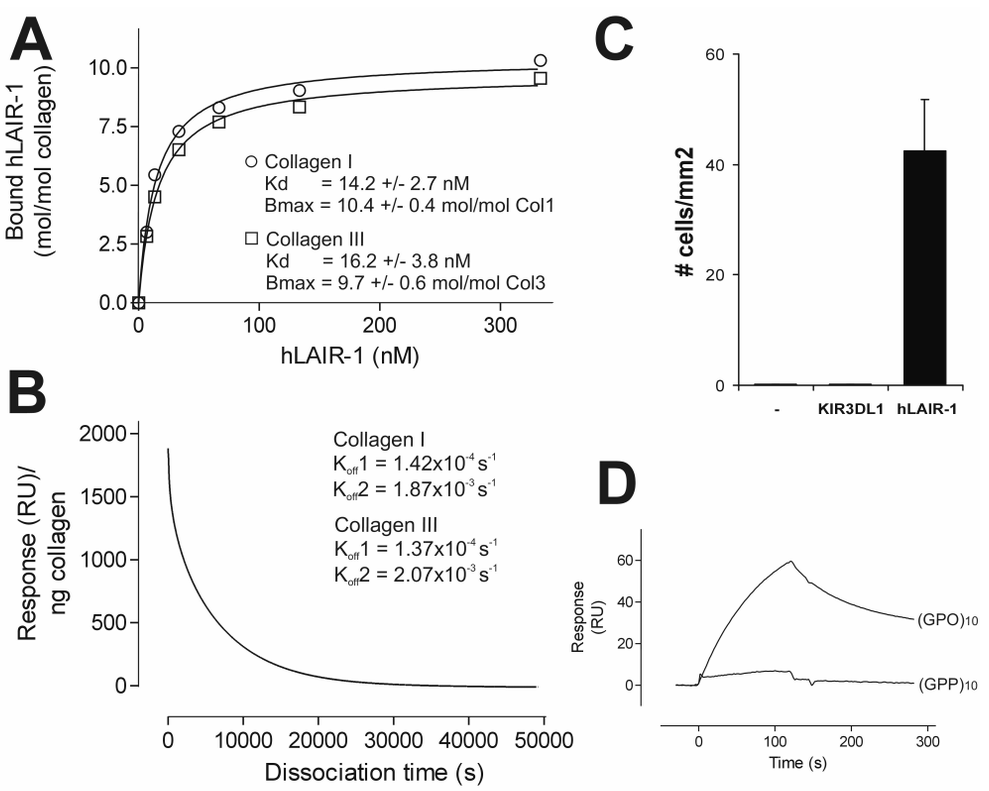

Figure 4. LAIR-1 is a high-affinity collagen receptor. (A) Indicated concentrations hLAIR-1-IgG were injected at 20 μl/min sequentially through a BIAcore flow cell containing ~2000-3000 RU of directly immobilized collagen I (○), III (□) or nothing. Each symbol represents the resonance unit at equilibrium and the corresponding concentration of the fusion protein; these data were used to determine the indicated Kd-values. Control fusion-proteins did not associate with collagen. (B) Rate of dissociation of hLAIR-1-IgG from collagen I and III as monitored by surface plasmon resonance. (C) K562 cells transfected with hLAIR-1 bind collagen III under flow conditions. The indicated transfectants were perfused at wall shear rates of 0.76 dynes/cm^2 over collagen III-coated coverslips. The number of binding cells was counted after 5 min (mean ± SEM, n = 6). (D) hLAIR-1 binds immobilized $(GPO)_{10}$, but not $(GPP)_{10}$. hLAIR-1-IgG (780 nM) was injected at 5 μl/min through a BIAcore flow cell containing ~250 RU of immobilized $(GPO)_{10}$ or $(GPP)_{10}$.

LAIR-1 binds Gly-Pro-Hyp collagen repeats

Because LAIR-1 interacted cross-species with multiple collagen molecules, we hypothesized that LAIR-1 bound to a common collagen-restricted structure. The collagens are a large family of trimeric molecules composed of three polypeptide α chains,

which contain the sequence repeat $(Gly\text{-}X\text{-}Y)_n$, X being frequently proline (P) and, after post-translational modification, Y being hydroxyproline (O). The GPO triplet is almost exclusively present in collagenous molecules and allows the formation of a triple helix, which is the main characteristic feature of collagens[15]. Immobilized triple-helical peptides composed of 10 repeated GPO triplets ($(GPO)_{10}$, also known as collagen-related peptide, CRP[16]) bound hLAIR-1-IgG (Fig. 4D), whereas a corresponding triple-helical $(GPP)_{10}$ peptide did not. Thus, LAIR-1 binds a common collagen motif in a hydroxyproline-dependent manner. hLAIR-1-IgG bound less efficiently to $(GPO)_{10}$ peptide as compared to collagen, suggesting that, apart from the GPO-sequence, additional structural components are required for optimal interaction. Interestingly, $(GPO)_{10}$, but not $(GPP)_{10}$, is also a selective ligand for GpVI[16], a major player in platelet-collagen adhesive interactions leading to thrombus formation. Like LAIR-1, GPVI is a member of the IgSF and is encoded in the leukocyte receptor cluster on human chromosome 19q13.4[11].

Collagens directly cross-link LAIR-1 and inhibit degranulation of RBL-2H3 cells

To analyze whether collagen induces functional cross-linking of LAIR-1, we generated 2B4 NFAT-GFP reporter cells[17] expressing a chimeric protein consisting of the extracellular domain of hLAIR-1 and the transmembrane and intracellular domain of CD3ζ. Receptor engagement of cells expressing the hLAIR-1-CD3ζ chimera, but not the parental cells, via plate-bound collagens I, III, or anti-hLAIR-1 mAbs resulted in expression of GFP (Fig. 5A). Pre-treatment of reporter cells with anti-hLAIR-1 $F(ab')_2$ fragments abrogated the NFAT-activation (data not shown). Collagens I and III are thus capable of cross-linking hLAIR-1.

We next investigated whether cross-linking of LAIR-1 by extracellular matrix collagens leads to inhibition of immune cell function in vitro. As a model, we used LAIR-1 transfected RBL-2H3 cells, which express endogenous IgE receptor FcεRI[7]. Incubation of RBL-2H3 hLAIR-1 transfectants with TNP-specific IgE and subsequent triggering with plate-bound TNP-conjugated BSA resulted in degranulation of the cells and release of β-glucuronidase (Fig. 5B). Simultaneous cross-linking of hLAIR-1 using plate-bound anti hLAIR-1 mAb, collagen I or III caused marked inhibition of degranulation (Fig. 5B). A LAIR-1 mutant that is unable to signal, because the tyrosine residues in the ITIM were changed to phenylalanine (LAIR-1-FF)[7], could not inhibit the degranulation upon collagen interaction (Fig. 5C). Furthermore, plate-bound triple-helical $(GPO)_{10}$ alone, but not $(GPP)_{10}$, was capable of specifically inhibiting the degranulation of RBL cells (Fig. 5D), suggesting that other GPO-repeat-bearing collagens also can inhibit immune cell function by binding to LAIR-1. The effect was specifically due to the LAIR-1/collagen interaction since pre-incubation of the cells with blocking anti-hLAIR-1 $F(ab')_2$ fragments completely abolished the inhibition (Fig. 5B and 5D). Thus, extracellular matrix collagens are functional ligands for the inhibitory LAIR-1 that can directly down regulate immune responses.

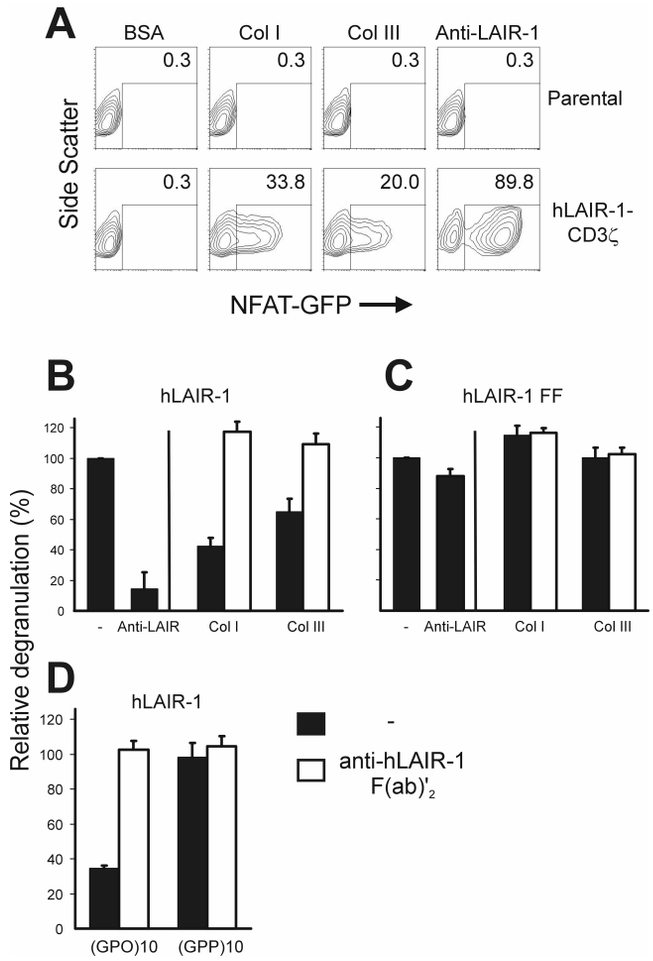

Figure 5. Collagens directly cross-link LAIR-1 and inhibit degranulation of RBL-2H3 cells. (A) NFAT-GFP reporter cells[17] transfected with hLAIR-1-CD3ζ chimera (bottom panels) or not (top panels) were incubated with immobilized collagens I, III, BSA, or anti-hLAIR-1 mAbs for 20 hours and GFP expression was analyzed by flow cytometry. Percentage of GFP-positive cells is indicated. (B) hLAIR-1 transfected RBL-2H3[7] cells were sensitized with IgE anti-TNP, incubated at 37°C in plates coated with TNP conjugated to BSA (0.8 μg/ml) in the absence or presence of plate bound collagen I, III, or anti-hLAIR-1 mAb (3.3 μg/ml, closed bars). Where indicated cells were pre-treated with 50 μg/ml anti-hLAIR-1 F(ab')$_2$ fragments (8A8, open bars). (C) The same experiment was performed using RBL-2H3 cells transfected with a LAIR-1 mutant in which both tyrosines in the intracellular tail are mutated to fenylalanines (hLAIR-1 FF). (D) hLAIR-1 transfected RBL-2H3 cells were sensitized with IgE anti-TNP, incubated at 37°C in plates coated with TNP conjugated to BSA (0.8 μg/ml) in the presence of plate-bound trimeric $(GPO)_{10}$ or $(GPP)_{10}$ peptides (coated at 3.3 μg/ml) (closed bars). Where indicated cells were pre-treated with 50 μg/ml anti-hLAIR-1 F(ab')$_2$ fragments (8A8, open bars).Degranulation was measured as described before. Typical degranulation values ranged from 9 to 26% of the amount observed when all RBL cells were lysed by addition of 10% Triton. The relative percentage of degranulation was calculated as: 100*[(OD_{405} TNP+collagen - OD_{405} spontaneous release)/(OD_{405} TNP alone - OD_{405} spontaneous release)]. Spontaneous release was measured after coating with BSA alone. Mean values ± SEM of three independent experiments are shown.

When immune cells migrate into the tissues, they are potentially exposed to multiple activating signals. To ensure that they respond appropriately to these stimuli, inhibitory receptors are required to set a threshold for cell activation[8]. Our results show that collagen/LAIR-1 interactions can inhibit cell activation and, as such, may contribute to a

dampening of the response. Under physiological conditions, immune cells present in the blood are not exposed to collagens[18]. Their extravasation, however, results in interaction with collagen-rich sub-endothelial structures, which may increase the threshold for activation needed to keep these potentially dangerous cells in check. When immune cells reach an inflammatory locus, the presence of specific and strong activating stimuli given by antigen-presenting cells, cytokines or pathogens, will override the threshold and allow cells to become activated and mediate their function. Indeed, we observed that sub-optimal activation via the FcεR was efficiently downregulated via the LAIR-1/collagen interaction, whereas maximal activation was not (data not shown). Regulation of the LAIR-1/collagen interaction can also occur by modulating LAIR-1 expression at different stages of differentiation or activation of immune cells, as was previously demonstrated for B cells[4], T cells[6], neutrophils[19], and dendritic cells (our own unpublished observations). In addition, secreted LAIR-2 might serve as a regulator of LAIR-1 function by binding collagen thereby circumventing the inhibitory potential of LAIR-1.

Loss of inhibitory immune receptors or down regulation of ligands for these receptors can result in a hyper-activated immune system, leading to chronic inflammation and autoimmunity[9]. Collagens are implied in several human autoimmune diseases. Collagen XVII, which we initially identified as a LAIR-ligand, is an auto-antigen in acquired blistering disorders e.g. bullous pemphigoid[20]. Collagen II is an autoantigen in rheumatoid arthritis and systemic lupus erythematosus[21], and collagen VII is an autoantigen in epidermolysis bullosa acquisita[22]. Potentially, autoantibodies targeting the various collagen molecules could interfere with the collagen-LAIR-1 interaction and thereby play a role in the pathology of these diseases.

Inhibitory receptors can be used by tumors and viruses to evade immune responses[23]. Expression of several members of the collagen family, including collagen I, III, V, VI, XIII, XVII, XVIII, XXIII, by neoplastic cells is associated with tumor progression[24-28]. It is tempting to speculate that overexpression of collagens by tumor cells may enable these cells to suppress anti-tumor responses via the inhibitor LAIR-1.

All previously documented ligands for ITIM-bearing receptors are membrane molecules, implying a regulatory role in cell-cell interaction. The functional interaction between extracellular matrix collagens and an inhibitory immune receptor presents a novel mechanism of immune regulation.

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REFERENCES

1. Myllyharju,J. and K.I.Kivirikko. 2004. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet.* 20:33-43.
2. Gelse,K., E.Poschl, and T.Aigner. 2003. Collagens--structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* 55:1531-1546.
3. Meyaard,L., G.J.Adema, C.Chang, E.Woollatt, G.R.Sutherland, L.L.Lanier, and J.H.Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
4. van der Vuurst de Vries AR, H.Clevers, T.Logtenberg, and L.Meyaard. 1999. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur. J. Immunol.* 29:3160-3167.
5. Poggi,A., E.Tomasello, E.Ferrero, M.R.Zocchi, and L.Moretta. 1998. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. *Eur. J. Immunol.* 28:2086-2091.
6. Maasho,K., M.Masilamani, R.Valas, S.Basu, J.E.Coligan, and F.Borrego. 2005. The inhibitory leukocyte-associated Ig-like receptor-1 (LAIR-1) is expressed at high levels by human naive T cells and inhibits TCR mediated activation. *Mol. Immunol.* 42:1521-1530.
7. Verbrugge,A., T.de Ruiter, H.Clevers, and L.Meyaard. 2003. Differential contribution of the Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) of human Leukocyte Associated Ig-like Receptor (LAIR)-1 to inhibitory function and phosphatase recruitment. *Int. Immunol.* 15:1349-1358.
8. Ravetch,J.V. and L.L.Lanier. 2000. Immune inhibitory receptors. *Science* 290:84-89.
9. Pritchard,N.R. and K.G.Smith. 2003. B cell inhibitory receptors and autoimmunity. *Immunology* 108:263-273.
10. Meyaard,L., J.Hurenkamp, H.Clevers, L.L.Lanier, and J.H.Phillips. 1999. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. *J. Immunol.* 162:5800-5804.
11. Lebbink,R.J., T.de Ruiter, A.Verbrugge, W.S.Bril, and L.Meyaard. 2004. The mouse homologue of the leukocyte-associated Ig-like receptor-1 is an inhibitory receptor that recruits Src homology region 2-containing protein tyrosine phosphatase (SHP)-2, but not SHP-1. *J. Immunol.* 172:5535-5543.
12. Lebbink,R.J., T.de Ruiter, G.J.Kaptijn, and L.Meyaard. 2005. Identification and characterization of the rat homologue of LAIR-1. *Immunogenetics* 57:344-351.
13. Miura,Y., T.Takahashi, S.M.Jung, and M.Moroi. 2002. Analysis of the interaction of platelet collagen receptor glycoprotein VI (GPVI) with collagen. A dimeric form of GPVI, but not the monomeric form, shows affinity to fibrous collagen. *J. Biol. Chem.* 277:46197-46204.
14. Maenaka,K., T.Juji, T.Nakayama, J.R.Wyer, G.F.Gao, T.Maenaka, N.R.Zaccai, A.Kikuchi, T.Yabe, K.Tokunaga, K.Tadokoro, D.I.Stuart, E.Y.Jones, and P.A.van der Merwe. 1999. Killer cell immunoglobulin receptors and T cell receptors bind peptide-major histocompatibility complex class I with distinct thermodynamic and kinetic properties. *J. Biol. Chem.* 274:28329-28334.
15. Ricard-Blum,S. and F.Ruggiero. 2005. The collagen superfamily: from the extracellular matrix to the cell membrane. *Pathol. Biol. (Paris)* 53:430-442.
16. Knight,C.G., L.F.Morton, D.J.Onley, A.R.Peachey, T.Ichinohe, M.Okuma, R.W.Farndale, and M.J.Barnes. 1999. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. *Cardiovasc. Res.* 41:450-457.
17. Voehringer,D., D.B.Rosen, L.L.Lanier, and R.M.Locksley. 2004. CD200 receptor family members represent novel DAP12-associated activating receptors on basophils and mast cells. *J. Biol. Chem.* 279:54117-54123.
18. Farndale,R.W., J.J.Sixma, M.J.Barnes, and P.G.de Groot. 2004. The role of collagen in thrombosis and hemostasis. *J. Thromb. Haemost.* 2:561-573.

19. Verbrugge,A., T.de Ruiter, C.Geest, P.J.Coffer, and L.Meyaard. 2006. Differential expression of Leukocyte Associated Ig-like Receptor-1 during neutrophil differentiation and activation. *J. Leukoc. Biol.* 79:828-836.
20. Franzke,C.W., P.Bruckner, and L.Bruckner-Tuderman. 2005. Collagenous transmembrane proteins: recent insights into biology and pathology. *J. Biol. Chem.* 280:4005-4008.
21. Gioud,M., A.Meghlaoui, O.Costa, and J.C.Monier. 1982. Antibodies to native type I and II collagens detected by an enzyme linked immunosorbent assay (ELISA) in rheumatoid arthritis and systemic lupus erythematosus. *Coll. Relat Res.* 2:557-564.
22. Schmidt,E. and D.Zillikens. 2000. Autoimmune and inherited subepidermal blistering diseases: advances in the clinic and the laboratory. *Adv. Dermatol.* 16:113-157.
23. Lanier,L.L. 2005. NK Cell Recognition. *Annu. Rev. Immunol.*225-274.
24. Parikka,M., T.Kainulainen, K.Tasanen, A.Vaananen, L.Bruckner-Tuderman, and T.Salo. 2003. Alterations of collagen XVII expression during transformation of oral epithelium to dysplasia and carcinoma. *J. Histochem. Cytochem.* 51:921-929.
25. Iizasa,T., H.Chang, M.Suzuki, M.Otsuji, S.Yokoi, M.Chiyo, S.Motohashi, K.Yasufuku, Y.Sekine, A.Iyoda, K.Shibuya, K.Hiroshima, and T.Fujisawa. 2004. Overexpression of collagen XVIII is associated with poor outcome and elevated levels of circulating serum endostatin in non-small cell lung cancer. *Clin. Cancer Res.* 10:5361-5366.
26. Banyard,J., L.Bao, and B.R.Zetter. 2003. Type XXIII collagen, a new transmembrane collagen identified in metastatic tumor cells. *J. Biol. Chem.* 278:20989-20994.
27. Roepman,P., L.F.Wessels, N.Kettelarij, P.Kemmeren, A.J.Miles, P.Lijnzaad, M.G.Tilanus, R.Koole, G.J.Hordijk, P.C.van der Vliet, M.J.Reinders, P.J.Slootweg, and F.C.Holstege. 2005. An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nat. Genet.* 37:182-186.
28. Vaisanen,T., M.R.Vaisanen, H.Autio-Harmainen, and T.Pihlajaniemi. 2005. Type XIII collagen expression is induced during malignant transformation in various epithelial and mesenchymal tumours. *J. Pathol.* 207:324-335.
29. Naviaux,R.K., E.Costanzi, M.Haas, and I.M.Verma. 1996. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J. Virol.* 70:5701-5705.
30. van de Peppel J., P.Kemmeren, H.van Bakel, M.Radonjic, D.van Leenen, and F.C.Holstege. 2003. Monitoring global messenger RNA changes in externally controlled microarray experiments. *EMBO Rep.* 4:387-393.
31. Tusher,V.G., R.Tibshirani, and G.Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. U. S. A* 98:5116-5121.
32. Kinter,M. and Sherman N.E. 2000. Protein sequencing identification using tandem mass spectrometry. Wiley and Sons, New York.
33. Sixma,J.J., P.G.de Groot, H.van Zanten, and M.IJsseldijk. 1998. A new perfusion chamber to detect platelet adhesion using a small volume of blood. *Thromb. Res.* 92:S43-S46.

5
CHAPTER

Identification of multiple potent binding sites for LAIR-1 on human collagens II and III

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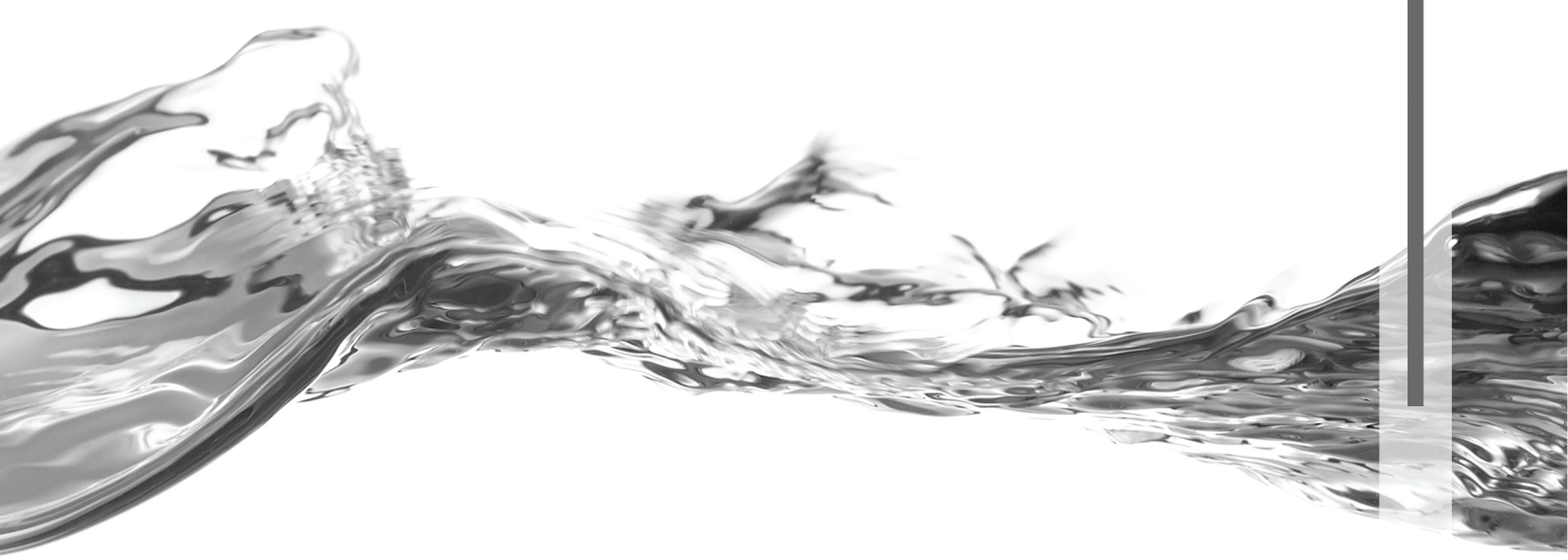

ABSTRACT

Immune responses are tightly controlled by the opposing actions of activating and inhibitory immune receptors. Recently we identified collagens as ligands for the inhibitory leukocyte-associated Ig-like receptor-1 (LAIR-1), revealing a novel mechanism of peripheral immune regulation by inhibitory immune receptors binding to extracellular matrix collagens. LAIR-1 specifically interacted with synthetic trimeric peptides containing 10 repeats of glycine-proline-hydroxyproline (GPO) residues which could directly inhibit immune cell activation *in vitro*. Here we studied the collagen/LAIR-1 interaction in more detail by using novel overlapping synthetic trimeric peptides encompassing the entire triple-helical domain of human collagens II and III. Human and mouse LAIR-1 bind many peptides of these collagen molecules with mouse LAIR-1 being more promiscuous than human LAIR-1. LAIR-1 binding to trimeric collagen peptides was influenced by GPO-content of the peptide, although additional non-GPO triplets contributed to the interaction. Furthermore, we identified several trimeric peptides that were potent LAIR-1 ligands and could efficiently induce inhibition of T cell activation and FcεRI-induced degranulation of RBL-2H3 cells through binding to human LAIR-1. A detailed understanding of the LAIR-1 recognition motifs within collagen may lead to the development of potent reagents that can be used in *in vitro*, *ex vivo*, and *in vivo* functional studies to dissect the biology and function of the collagen/LAIR-1 interaction.

INTRODUCTION

Collagens are the most abundant proteins in vertebrates and play crucial roles in the development, morphogenesis, and growth of many tissues (reviewed in [1]). Besides their mechanical properties, collagens serve as substrates for cell attachment, migration and coagulation. 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 $(Gly\text{-}X\text{-}Y)_n$, X being frequently proline (P) and, after post-translational modification, Y being hydroxyproline (O)[2]. The GPO triplet is almost exclusively present in collagenous molecules and allows the formation of the triple helix[1].

Specific molecules interact with collagens, such as integrins, von Willebrand Factor (VWF), discoidin domain receptors (DDRs), glycoprotein VI (GPVI), and proteoglycan receptors[3]. However, studies on the interaction of these receptors with intact collagen molecules are hampered by the large size and insoluble nature of extracellular matrix collagens. The development of reliable methods for synthesizing triple-helical peptide analogues of collagens however, allowed further advances in understanding the molecular basis of various receptor/collagen interactions[4]. Recently, a set of overlapping homotrimeric peptides covering the entire triple-helical domain of collagen III was generated (termed the Collagen III Toolkit[5]), which led to the identification of specific binding sites for VWF and α2β1 integrin on the human collagen III trimer[5;6]. Furthermore, trimeric collagen peptides containing ten glycine-proline-hydroxyproline (GPO) repeats in each strand of a triple helix (collagen-related peptide, CRP) proved potent activators of GPVI[7-9], a major player in platelet–collagen adhesive interactions leading to thrombus formation[10].

The inhibitory immune leukocyte-associated immunoglobulin-like receptor (LAIR)-1 (CD305) was recently identified as a high affinity receptor for various collagen molecules [11]. Like GPVI, LAIR-1 is a member of the immunoglobulin superfamily (IgSF) encoded on the leukocyte receptor complex (LRC) on human chromosome 19[12]. LAIR-1 is an inhibitory immune receptor which is expressed on the majority of peripheral blood mononuclear cells and thymocytes[12;13]. Antibody/ligand-induced cross-linking of the receptor *in vitro* delivers a potent inhibitory signal that is capable of inhibiting cellular functions of various immune cell types[11-19]. Inhibitory receptors are thought to play an important role in the prevention of autoimmunity[20]. To further understand the biological role of LAIR, characterization of the interaction between the LAIR molecules and collagen is essential.

Both human and mouse LAIR-1 specifically bind to CRP[11;19], whereas the receptors have no affinity for control peptides consisting of ten GPP triplet repeats[11;19]. Furthermore, BIAcore experiments indicated that both human and mouse LAIR-1 have similar, high affinity for human collagens I and III, and that a single collagen trimer contains ~10 binding sites for these receptors[11;19]. Although LAIR-1 interacts with both trans-membrane and extracellular matrix collagens such as human collagens I, II, and III[11;19], the actual LAIR-1 binding sites on collagens remain unknown.

We synthesized the entire triple-helical domain of human collagen II (termed the Collagen II Toolkit) as a set of overlapping homotrimeric peptides, similar to the Collagen III Toolkit[5], and used these to screen for LAIR-1 binding sites by using LAIR-1 expressing cells in functional and adhesion assays. With these peptides we identified multiple binding sites for human and mouse LAIR-1 on both human collagens II and III, which functioned as potent agonists of LAIR-1 mediated inhibition of immune cell function. These studies may lead to the development of potent therapeutics or reagents that can be used in functional studies to dissect the biology and function of the collagen/LAIR-1 interaction.

MATERIALS AND METHODS

Cells, transfectant, and cDNA

Cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured using standard techniques. Cell lines that were used in this study are: human embryonic kidney 293T cells and human erythroleukemia K562 cells. Mouse 2B4 NFAT-GFP reporter T cell hybridoma cells were kindly provided by H. Arase and L.L. Lanier (UCSF, USA).

hLAIR1a, mLAIR-1 and hLAIR-1-CD3ζ cloned in the pMX puro retroviral vector were described previously[11]. The mLAIR-1-CD3ζ chimeric reporter construct was cloned in the pMX retroviral vector by fusing the extracellular domain of mLAIR-1a to the trans-membrane and intracellular domain of human CD3ζ. Cell lines stably expressing hLAIR-1a, mLAIR-1, mLAIR-1- CD3ζ, or hLAIR-1- CD3ζ were generated as described previously [11].

Peptide synthesis

The set of 57 overlapping synthetic peptides encompassing the entire triple-helical domain of human collagen III (Collagen III Toolkit) was described previously[5]. The same approach was used to synthesize and verify 56 synthetic peptides encompassing the entire triple-helical domain of human collagen II (Toolkit collagen II). The primary sequences of the peptides of the Collagen II Toolkit and the Collagen III Toolkit are shown in Supplemental Table I and II respectively. Variant and truncated peptides of collagen III peptide 30 (III-30a to III-30f, see supplemental Table III) were synthesized similarly. Each peptide contains guest sequence[21]: 27 amino acids of the human collagen II or III primary protein sequence, in which the C-terminal 9 amino acids form the first 9 guest amino acids of the next peptide. Thus, a 9-amino acid overlap is included between adjacent peptides[21]. To ensure that the peptides are folded in a triple-helical conformation, each guest sequence was flanked between two $(GPP)_5$ host repeats, as described previously[5]. The triple-helical peptides composed of $GCO(GPO)_{10}GCOG\text{-}NH_2$ ($(GPO)_{10}$, also known as collagen-related peptide, CRP[8]) and $GCP(GPP)_{10}GCPG\text{-}NH_2$ ($(GPP)_{10}$) were described previously[8].

For GPO-requirement studies, we generated a set of CRPs in which the content of critical glycine-proline-hydroxyproline (GPO) triplets was varied in relation to the 'inert' scaffold sequence of GPP motifs (Toolbox peptides)[22]. The sequences of these Toolbox peptides are shown in Supplemental table III. Furthermore, mutants and deletion variants of collagen III peptide 30 (III-30) were generated as described above, their sequences are depicted in Supplemental Table III.

Cell adhesion assay

96-well MAXIsorp (Nunc) flat-bottom plates were coated overnight at 4°C with purified collagens I, III (Sigma), BSA, or synthetic trimeric peptides (100 μl/well, 10 μg/ml in PBS, 2 mM acetic acid). The cell adhesion assay was performed as described previously[11]. K562 cells stably transduced with mLAIR-1a or hLAIR-1a[11] were used in the assay. For collagen III peptide 30 variant-studies, cells were incubated with the peptides for 6 hours instead of 2 hours.

Reporter cell assay

For detection of LAIR-1 ligands: 2B4 T cell hybridoma cells stably transduced with an NFAT-GFP reporter and hLAIR-1-CD3ζ or mLAIR-1-CD3ζ were analyzed as described[11].

For detection of inhibition of mouse CD3 signaling: 2B4 T cell hybridoma cells stably transduced with an NFAT-GFP reporter and hLAIR-1a were generated. 1.25 μg/ml anti-mouse CD3 (PharMingen, San Diego, CA) was coated overnight at 4°C in 96-well MAXIsorp flat-bottom plates (Nunc) together with an indicated amount of synthetic trimeric peptides in a total volume of 100 μl/well. The next day, plates were washed and 200 μl of $2.5x10^5$ reporter cells/ml in medium were added to each well, and plates were incubated at 37°C for 22 hrs and then analyzed for GFP expression by flow cytometry on a FACSCalibur (BD Biosciences). Typically, ~90 percent of the reporters expressed GFP upon CD3 stimulation. The percentage of inhibition of CD3 stimulation was calculated as follows: percentage of inhibition = 100*[(%GFP^+ cells in BSA coated well – %GFP^+ cells in peptide coated well)/ (%GFP^+ cells in BSA coated well)].

Degranulation assay

96-well MAXIsorp flat-bottom plates (Nunc) were coated overnight at 4°C with BSA, or TNP conjugated to BSA (0.8 μg/ml) and indicated amounts of synthetic trimeric peptides (100 μl/well, 2 mM acetic acid in PBS). Meanwhile, untransfected or hLAIR-1a transfected RBL-2H3[23] cells were sensitized with IgE anti-TNP mAbs at 4°C for 30 minutes and subsequently washed for three times in medium (AIM-V; Gibco-BRL). After washings of the plates, $5.0x10^5$ cells in 70 μl medium were added to each well, and plates were incubated at 37°C for 1 hour. Subsequently, cultures were transferred to PCR tubes, centrifuged and the supernatants were assayed for β-glucuronidase activity as described[23].

RESULTS

Human LAIR-1 binds multiple sites on human collagens II and III

To identify sites in human collagens that act as functional binding sites for the LAIR-1 molecules we used overlapping synthetic trimeric peptides encompassing the entire triple-helical domain of human collagens II and III. Fluorescently labeled K562 cells stably expressing human (h) LAIR-1 were monitored for their capacity to bind the immobilized collagen peptides in a cell adhesion assay. Human collagen II peptides 17, 30 and 56 and collagen III peptides 1, 30, 38, 44, 45, and 51 specifically interacted with hLAIR-1 expressed on K562 cells (Figure 1A and 2A, >10% adherence), but not with the parental K562 cells (data not shown). Additionally, K562 hLAIR-1 cells slightly adhered to several other peptides of collagen II and III (Figure 1A and 2A). To analyze whether the collagen peptides were able to induce functional cross-linking of hLAIR-1, we used 2B4 NFAT-GFP reporter cells[24] expressing a chimeric protein consisting of the extracellular domain of hLAIR-1 fused to the transmembrane and intracellular domain of CD3ζ[11]. Receptor engagement of reporter cells expressing the hLAIR-1-CD3ζ chimera, via plate-bound collagen III, resulted in expression of GFP, indicating a functional triggering of the chimeric receptor (Fig. 1B and 2B). The parental reporter cells which do not express the chimeric LAIR-1 receptor did not respond to collagen III (data not shown). Human collagen II peptides 1, 30, 42, 49 and 56 (Fig. 1B) and collagen III peptides 1, 5, 30, 32, 38, 44, 45, and 51 (Fig. 2B) induced profound GFP expression (> 10% of cells turned GFP-positive) in hLAIR-1-CD3ζ expressing reporter cells. Furthermore, several peptides that induced a low percentage of GFP-expression (< 10%) were apparent. None of the peptides induced GFP-expression in the parental 2B4 NFAT-GFP reporter cells (data not shown). Although the NFAT-GFP reporter cell assay identified the same peptides as ligands for hLAIR-1 as compared to the K562 cell adhesion assay (Figure 1A and B, and 2A and B), the NFAT-GFP reporter cell assay identified additional peptides as ligands for hLAIR-1 and may therefore be a more sensitive ligand-sensing assay. The six most potent hLAIR-1 activators were in order of strength: III-30, II-56, II-30, III-38, II-42, and III-51. Intriguingly, collagen II peptide 17 (II-17) was a potent hLAIR-1 binder in the adhesion assay (Figure 1A), but was unable to induce significant signaling through the hLAIR-1-CD3ζ chimera.

Mouse LAIR-1 binds more collagen peptides as compared to hLAIR-1

To compare the binding requirements of human LAIR-1 with mouse LAIR-1, we performed adhesion and NFAT-GFP reporter cell assays with mLAIR-1 transfected K562 cells[11] and mLAIR-1-CD3ζ transfected 2B4 NFAT-GFP respectively. Intriguingly, mouse LAIR-1 bound and was functionally triggered by more collagen II (Fig. 1C and D) and III (Fig. 2C and D) peptides as compared to its human orthologue. In general, the same collagen sites that served as ligands for hLAIR-1 bound to mLAIR-1, although the order of strength in signaling was not preserved. Human collagen II peptide 30 for instance, was a potent inducer of hLAIR-1-CD3ζ signaling, whereas its signaling-inducing

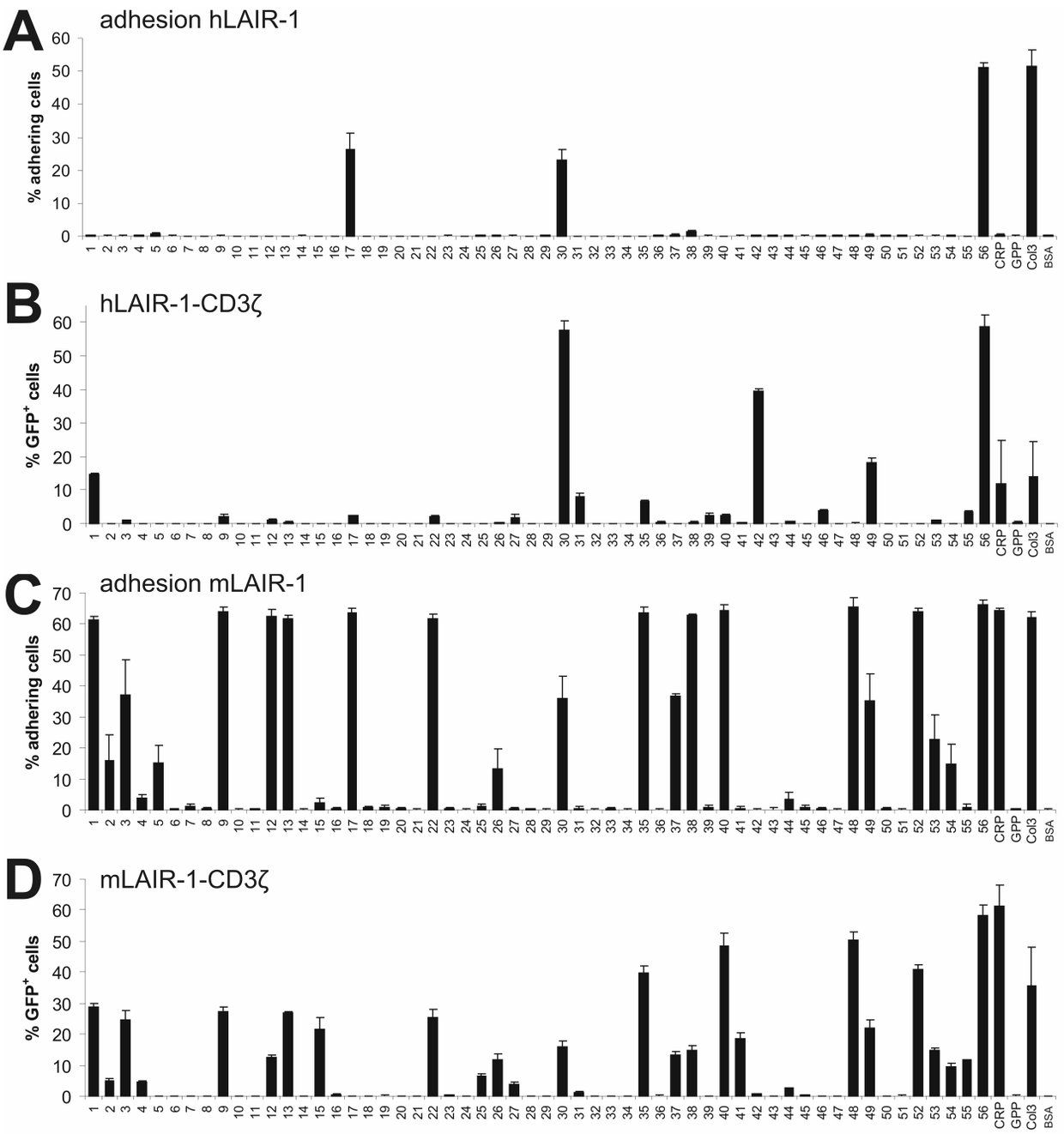

Figure 1. Identification of multiple LAIR-1 binding sites on human collagen II. A and C: K562 cell adhesion assay. Fluorescently labeled K562 cells stably expressing human (A) or mouse LAIR-1 (C) were monitored for their capacity to bind immobilized synthetic trimeric peptides encompassing the entire triple-helical domain of human collagen II (Collagen II Toolkit). Percentage of adhering cells relative to input is shown. B and D: 2B4 NFAT-GFP reporter cell assay. 2B4 NFAT-GFP reporter cells stably transfected with hLAIR-1-CD3ζ (B) or mLAIR-1-CD3ζ (D) chimeric molecules were incubated with the indicated immobilized collagens II synthetic trimeric peptides for 22 hours and GFP expression was analyzed by flow cytometry. Maximal stimulation in these reporter cell assays using trimeric collagen peptides typically resulted at most in ~70% GFP$^+$ cells. Parental K562 cells and 2B4 NFAT-GFP reporter cells did not adhere or respond to any of the peptides. The sequences of the indicated peptides are shown in supplemental table I. Data are expressed as mean values of three independent experiments plus standard deviation.

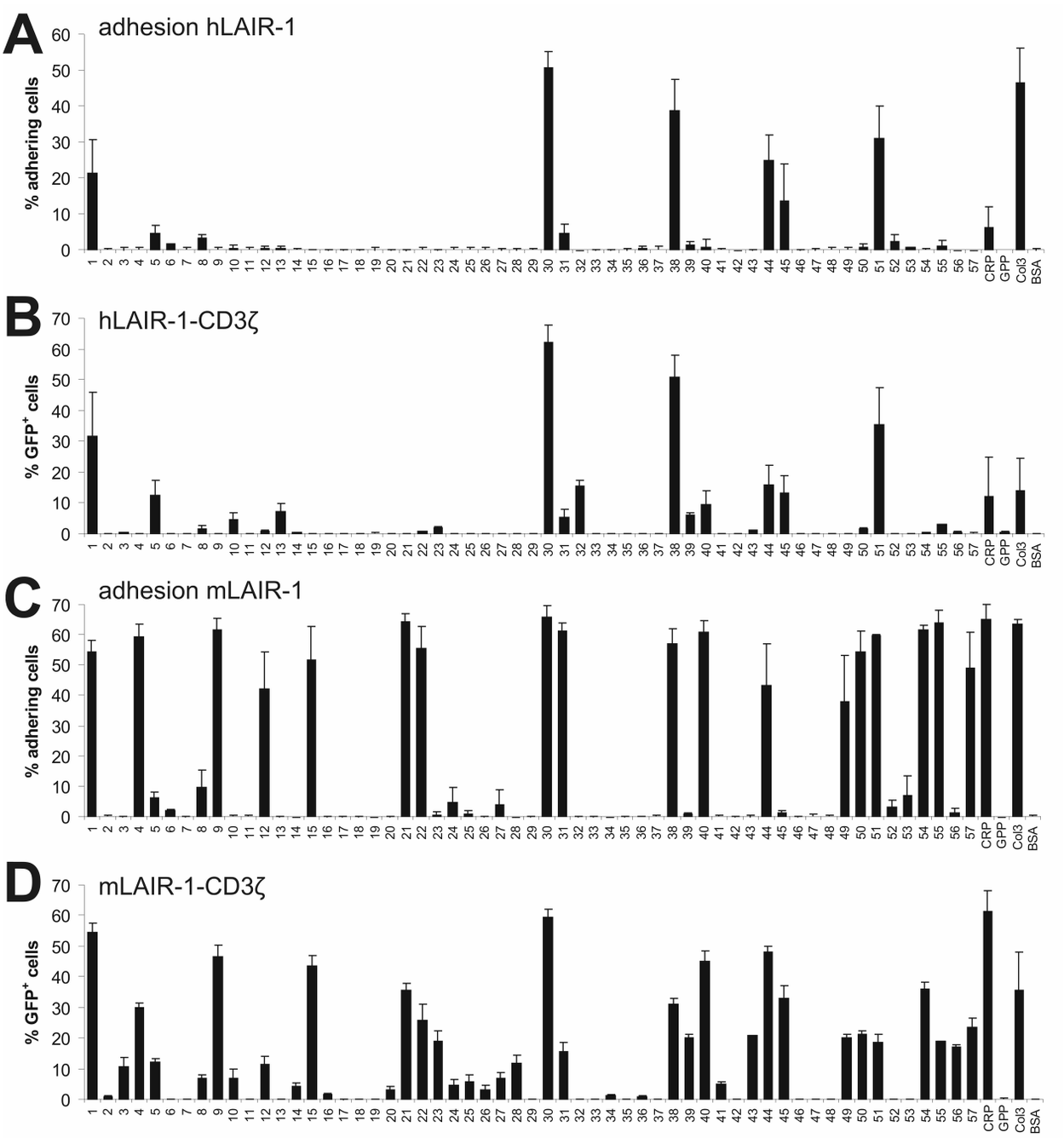

Figure 2. Identification of multiple LAIR-1 binding sites on human collagen III. A and C: K562 cell adhesion assay. Fluorescently labeled K562 cells stably expressing human (A) or mouse LAIR-1 (C) were monitored for their capacity to bind immobilized synthetic trimeric peptides encompassing the entire triple-helical domain of human collagen III (Collagen III Toolkit). Percentage of adhering cells relative to input is shown. B and D: 2B4 NFAT-GFP reporter cell assay. 2B4 NFAT-GFP reporter cells stably transfected with hLAIR-1-CD3ζ (B) or mLAIR-1-CD3ζ (D) chimeric molecules were incubated with the indicated immobilized collagens III synthetic trimeric peptides for 22 hours and GFP expression was analyzed by flow cytometry. Maximal stimulation in these reporter cell assays using trimeric collagen peptides typically resulted at most in ~70% GFP^+ cells. Parental K562 cells and 2B4 NFAT-GFP reporter cells did not adhere or respond to any of the peptides. The sequences of the indicated peptides are shown in supplemental table II. Data are expressed as mean values of three independent experiments plus standard deviation.

capacity on mLAIR-1-CD3ζ was more limited. The adhesion of mLAIR-1 expressing K562 cells to many collagen I and III peptides was maximal, whereas signaling via the mLAIR-1-CD3ζ chimera allowed a more quantitative assessment of the cross-linking capacity of the mLAIR-1 ligands. The peptides that induced the most profound functional cross-linking of mLAIR-1 (> 20% GFP-positive cells) were in order of strength: collagen II peptides 56, 48, 40, 52, 35, 1, 9, 13, 22, 3, 49, and 15, and collagen III peptides 30, 1, 44, 9, 40, 15, 54, 21, 45, 38, 4, 22, 57, 50, 43, 39, and 49. Additionally, collagen II peptide 42 did induce hLAIR-1 signaling, but no mLAIR-1 signaling. As observed for human LAIR-1, peptide II-17 did mediate adherence but not signaling via mLAIR-1. Hence, mLAIR-1 binds more collagen peptides as ligands and therefore may have less strict binding requirements as compared to its human orthologue. Although the collagen binding spectra of both receptors are similar, they are not identical.

Collagen II and III synthetic trimeric peptides identified by Toolkit are potent inhibitors of immune cell function

LAIR-1 is a potent inhibitory immune receptor that is broadly expressed on immune cells[12]. To assess whether the most effective peptides described above were also functional in cross-linking wild-type human LAIR-1 thereby inhibiting cellular immune responses, we performed two functional assays. First, we used 2B4 NFAT-GFP reporter cells[24] transfected with wild-type hLAIR-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 in approximately 90% of the cells (data not shown). Simultaneous cross-linking of hLAIR-1 via plate-bound CRP inhibited CD3 activation of hLAIR-1 transfected cells in a dose-dependent manner, but had little effect on the parental reporter cells (Fig 3, top right panel). In contrast, the control $(GPP)_{10}$ peptides did not inhibit either cell-type (Fig 3, top left panel). We next assessed the inhibitory potential of the four most potent peptides identified in figures 1 and 2 (II-30, II-56, III-38, and III-30). As expected, all four peptides were capable of efficiently inhibiting the CD3-induced activation of the hLAIR-1 transfected T cell hybridoma cells (Figure 3, middle panels). At low peptide concentrations (1.1 μg/ml), peptides II-56 and III-30 maintained their full inhibitory capacity, whereas the II-30 and III-38 peptides only inhibited ~60% of the T cells and the inhibitory potential of CRP was completely lost (Fig. 3). Two peptides from the collagen II peptide toolkit that did not respond in the previous assays also did not induce inhibition via hLAIR-1 (peptides II-20 and II-47, Figure 3, bottom panels). None of the peptides were efficient in inhibiting the parental reporter T cell hybridoma, although the most potent peptides (II-56 and III-30) did induce moderate inhibition (~30%) of these cells at high peptide concentrations (Fig. 3). This may be because the 2B4 NFAT-reporter cells express low amounts of endogenous mLAIR-1 which could account for the observed inhibition by the peptides (data not shown).

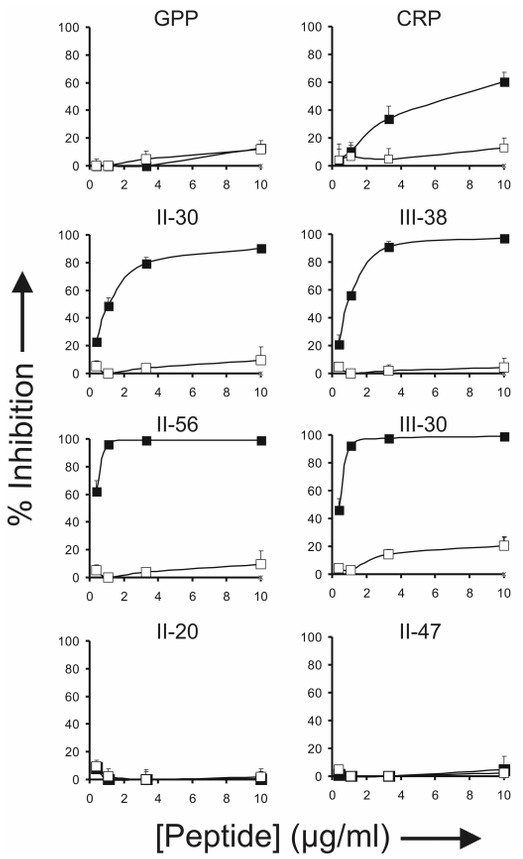

Figure 3. Peptides II-56 and III-30 are potent inhibitors of CD3-induced T cell activation via hLAIR-1. Human LAIR-1 transfected (closed squares) or untransfected (open squares) NFAT-GFP reporter T cells were incubated with immobilized anti-CD3 mAbs and indicated synthetic trimeric peptides for 22 hours and GFP expression was analyzed by flow cytometry. Percentage inhibition of anti-CD3-induced GFP expression is presented as a function of peptide concentration during coating (see materials and methods). Typically, ~90 percent of the reporters expressed GFP upon CD3 stimulation alone. The peptide concentrations used in the experiments were: 10, 3.3, 1.1, and 0.37 µg/ml. The percentage of inhibition of CD3 stimulation was calculated as follows: percentage of inhibition = 100*[(%GFP^+ cells in BSA coated well – %GFP^+ cells in peptide coated well)/ (%GFP^+ cells in BSA coated well)]. Data are expressed as mean values of three independent experiments plus standard deviation.

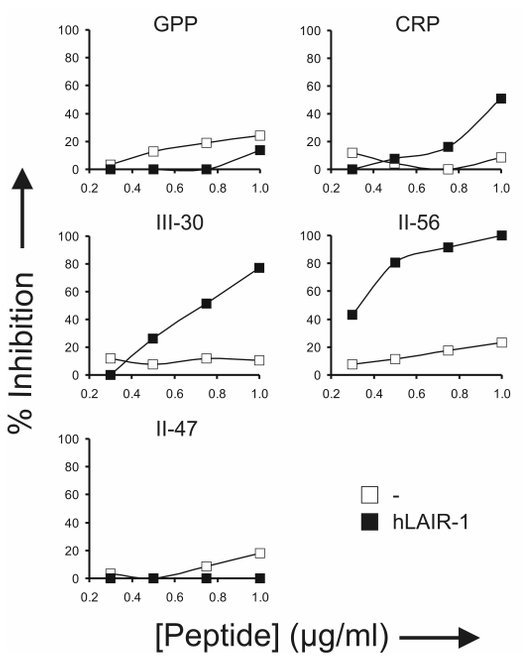

Figure 4. Peptides II-56 and III-30 are potent inhibitors of FcεRI-induced degranulation of RBL-2H3 cells. Human LAIR-1 transfected (closed squares) or untransfected (open squares) RBL-2H3 [23] cells were sensitized with IgE anti-TNP and subsequently incubated at 37°C in plates coated with TNP conjugated to BSA (0.8 µg/ml) and either 1, 0.75, 0.5, 0.3 µg/ml synthetic trimeric peptides. Degranulation was measured as described before [23]. Typical degranulation values ranged from 10 to 25% of the amount observed when all RBL cells were lysed by addition of 10% Triton. The relative percentage of degranulation was calculated as: 100*[(OD_{405} TNP+peptide - OD_{405} spontaneous release)/(OD_{405} TNP alone - OD_{405} spontaneous release)]. Measurements were performed using triplicate cultures. One representative experiment of two is shown.

In a second functional assay to test the inhibitory potential of the selected collagen peptides we used hLAIR-1 transfected RBL-2H3 cells, which express endogenous IgE receptor FcεRI[23]. We previously showed that collagens I, III, and CRP were capable of specifically inhibiting the FcεRI-induced degranulation of these cells[11]. As expected, peptides II-56 and III-30 were also potent inhibitors of the degranulation response (Fig. 4), whereas control peptides $(GPP)_{10}$ and II-47 were not. Again, peptides II-56 and III-30 proved more efficient functional ligands than CRP. Thus, the identified synthetic trimeric collagen peptides are potent inducers of LAIR-1-mediated inhibition of immune responses.

GPO-content in peptides correlates with binding potential to LAIR-1

Using BIAcore, we have shown that hLAIR-1 interacts with triple-helical peptides composed of 10 repeated GPO triplets (CRP), but not with corresponding peptides consisting of 10 repeated GPP triplets ($(GPP)_{10}$)[11]. To analyze whether the occurrence of GPO triplets in the collagen II and III Toolkit peptides correlates with LAIR-1 binding, we counted GPO triplets in peptides able to bind human or mouse LAIR-1. As expected, peptides with increased GPO-content were more likely to act as LAIR-1 ligands (Fig. 5A). Additionally, the 4 most potent hLAIR-1 activators II-30, II-56, III-30 and III-38 contained 2, 4, 3, and 3 GPO triplets respectively, whereas the average GPO content in the collagen II and III peptides is only ~0.91 per peptide. To investigate whether an increased content of GPO triplets in our synthetic peptides also resulted in more potent LAIR-1 ligands, we used peptides containing 0, 1, 2, 4, 6, or 10 adjacent GPOs triplets in a GPP backbone (Toolbox peptides)[22;25]. As expected, we observed a stepwise increase in both the inhibition of the degranulation response of RBL-2H3 cells (Fig. 5B) and the inhibition of CD3 stimulated 2B4 NFAT-GFP reporter cells (Fig. 5C) upon increased GPO content of the peptides. Nevertheless, although the Toolbox peptides could functionally interact with hLAIR-1, they were much less efficient in inhibiting immune responses compared with the newly identified collagen peptides described above (II-30, II-56, III-30 and III-38). For example, whereas peptide II-56 retained full inhibitory potential via LAIR-1 at even low peptide concentrations (1.1 μg/ml), the inhibitory potential of the most potent Toolbox peptide GPO_{10} (CRP) was completely absent (Fig. 3). As observed before, mLAIR-1 was a much more efficient Toolbox-peptide binder; a single GPO triplet was sufficient to induce profound adherence of mLAIR-1 expressing K562 cells, whereas 10 GPO triplets gave hardly detectable adherence of hLAIR-1 expressing K562 cells (data not shown).

Intriguingly, although the $(GPP)_{10}$ control peptides showed no/hardly binding to LAIR-1, several GPO-deficient peptides from the collagen II and III Toolkit were potent LAIR-1 binders and activators. These were (in order of effectiveness): collagen II toolkit peptides 15, 37, 12, and 44 and collagen III peptides 15, 51, 28, 8, and 20 for mouse LAIR-1 and collagen III peptides 51 and 32 for human LAIR-1. Although these peptides did contain hydroxyproline residues, the content of these residues was not elevated as compared to GPO-lacking peptides that did not bind human or mouse LAIR-1 (data not shown).

Additionally, many peptides that did contain one or more GPO triplets, did not serve as ligands for LAIR-1. (Fig. 1 and 2).

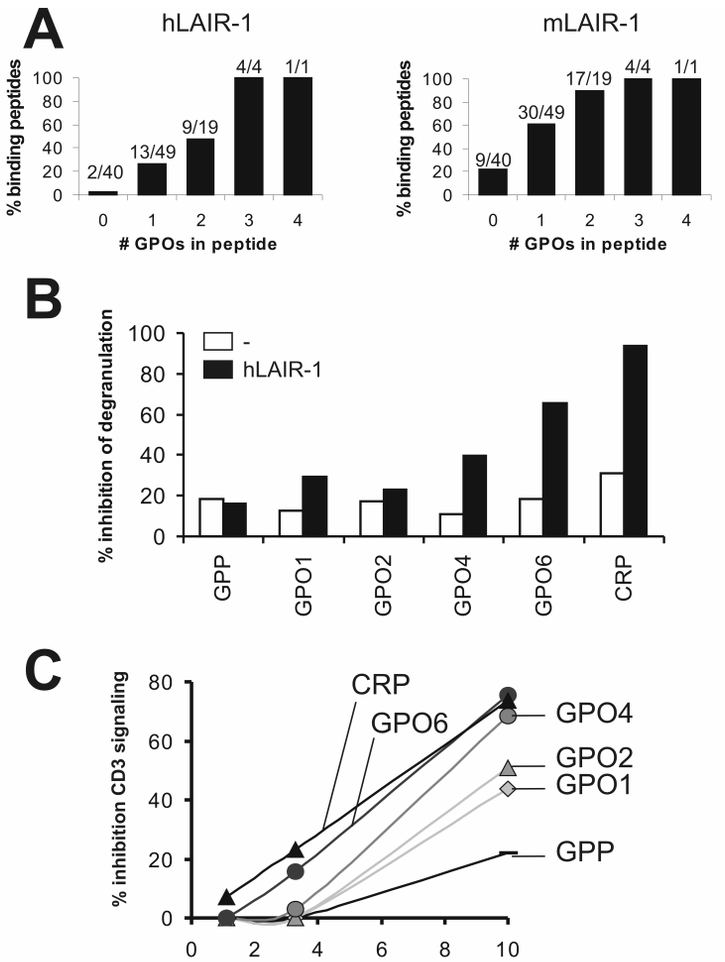

Figure 5. Increased GPO-content in peptides correlates with increased binding potential to LAIR-1. (A) The number of GPO triplets in the collagen II and III Toolkit peptides was correlated with the ability of the peptides to induce functional cross-linking of hLAIR-1-CD3ζ (left panel) or mLAIR-1-CD3ζ (right panel) chimeric molecules on the surface of 2B4 NFAT-GFP reporter cells. Peptides that induced GFP-expression in >3% of the reporter cells were scored as positive. The percentage of responding peptides of the total amount of peptides containing a given number of GPO triplets is indicated. From the total 113 Toolkit peptides, 40 peptides contained 0 GPO triplets, 49 contained 1 GPO triplet, 19 contained 2 GPO triplets, 4 contained 3 GPO triplets, and 1 peptide contained 4 GPO triplets. The number of peptides that induced GFP expression out of the total number of peptides with a given number of GPO triplets is indicated (B) An increased GPO-content in Toolbox peptides results in increased inhibition of the degranulation response via hLAIR-1. Human LAIR-1 transfected (closed bars) or untransfected (open bars) RBL-2H3 cells were sensitized with IgE anti-TNP and subsequently incubated at 37°C in plates coated with TNP conjugated to BSA (0.8 μg/ml) and synthetic trimeric peptides (1 μg/ml). The assay was identical as described in Fig. 4. The sequences of the indicated peptides are shown in supplemental table III. One representative experiment of two is shown. (C) An increased GPO-content in Toolbox peptides results in increased inhibition of CD3-induced T cell activation via hLAIR-1. Human LAIR-1 transfected NFAT-GFP reporter T cells were incubated with immobilized anti-CD3 mAbs and 10, 3.3, or 1.1 μg/ml immobilized synthetic trimeric peptides for 22 hours and GFP expression was analyzed by flow cytometry. The assay was identical as described in Fig. 3. One representative experiment of three is shown.

The C-terminal GPO triplets in III-30 are crucial for hLAIR-1 interaction

To study the effect of GPO to GPP substitution in potent LAIR-1 binding peptides derived from the collagen III toolkit, we generated novel peptides of III-30 in which we substituted and omitted various regions (Fig. 6). By changing the first or second GPO triplet into non-hydroxylated GPP (III-30a and III-30b respectively), we observed a small decrease in adherence of K562 cells expressing hLAIR-1 to these peptides as compared to the intact III-30 peptide (Fig. 6, right panel). Furthermore, III-30a and III-30b were slightly less potent in inducing hLAIR-1-mediated inhibition of CD3 stimulation in the NFAT-GFP reporter T cells (Fig. 6, left panel). Mutating the third GPO to GPP however (III-30c), resulted in a more profound decrease in LAIR-1 induced adherence and inhibition (Fig. 6), the latter especially at low coating concentration. We next generated truncated variants of III-30 to further map the LAIR-1 binding site. By removing the first two N-terminal triplets, GAOGLR, adherence of K562 cells through hLAIR-1 was further reduced, whereas the peptide retained comparable signaling capacity as the III-30c peptide (Fig. 6). Removal of the two C-terminal GPO triplets (III-30e) however, completely abrogated hLAIR-1-induced adherence and inhibition. Correspondingly, removal of both the N- and C-terminal two triplets gave similar results (III-30f). Peptide III-30e and III-30f showed moderate inhibitory potential at high peptide concentrations (~ 30 and 15% respectively at 10 μg/ml, data not shown), indicating that hLAIR-1 retained some affinity for these peptides. To conclude, presence of both C-terminal GPO triplets in III-30 are crucial for functional cross-linking of hLAIR-1. Furthermore, the most C-terminal GPO triplet is the dominant GPO triplet for hLAIR-1 binding.

Figure 6. Both C-terminal GPO triplets in III-30 are crucial for LAIR-1 binding and functioning. Indicated substitution and deletion variants of the III-30 peptide were monitored for their capacity to induce adherence of hLAIR-1 expressing K562 cells (right panel, similar as in Fig. 1,) and cause inhibition of CD3-induced T cell activation via hLAIR-1 (left panel, similar as in Fig. 3). Mutated GPO triplets are underlined (III-30a, III-30b, and III-30c). GPO triplets are indicated in bold. The inhibition of CD3-signaling is expressed as mean values of three independent experiments plus standard deviation. Data are expressed as mean values of three independent experiments plus standard deviation.

DISCUSSION

In this report we set out to characterize the collagen binding requirements of LAIR-1. By using overlapping synthetic trimeric peptides encompassing the entire triple-helical domain of human collagens II and III, we identified several sites on collagen molecules that serve as functional binding sites for human and mouse LAIR-1. We show that immobilized collagen-derived peptides can signal through LAIR-1, and inhibit both CD3-mediated T cell activation and FcεRI-induced degranulation of RBL-2H3 cells. Human collagens II and III have at least 5 and 8 hLAIR-1 binding sites per collagen trimer respectively and both contain more than 14 sites for mLAIR-1, determined using GFP expression in LAIR-1-CD3ζ-transfected cells. The detected amount of LAIR-1 binding sites on collagen III is in good agreement with the estimated 9.7 ± 0.6 binding sites, as determined by BIAcore experiments[11]. However, mLAIR-1 was estimated to interact with 8.9 ± 0.2 sites on human collagen III[19]. This discrepancy may be caused by the use of trimeric collagen peptides instead of intact collagen molecules. LAIR-1 may bind sites on synthetic trimeric peptides which are not recognized as such in an intact collagen molecule. Potentially, residues in the vicinity of a binding site that negatively influence LAIR-1 binding may be excluded in the synthetic peptides. Additionally, binding of LAIR-1 to a specific collagen motif in intact collagens may mask adjacent binding sites due to steric hindrance or conformational change of the collagen helix. Furthermore, the triple-helical structure forced on the trimeric peptides by the flanking GPP residues may create binding sites that are not present in the same sequence within an intact collagen molecule. Alternatively, the discrepancy could be caused by a difference between the membrane-bound LAIR-1 molecules and purified LAIR-1-IgG fusion proteins used in the BIAcore experiments[11]. In the latter case, the recombinant LAIR proteins are forced into a specific dimeric state which may affect the binding characteristics of the protein, as was previously described for the collagen receptor, glycoprotein VI (GPVI)[26]. Due to the presence of a cysteine in the CD3ζ chain, the LAIR-1-CD3ζ chimeric molecules are also expressed as dimers on the cell surface, whereas the stoichiometry of wild-type membrane-expressed LAIR-1 expressed is unknown and could be monomeric. This may explain the profound difference in the amount of detected binding peptides between the adhesion assay and the LAIR-1-CD3ζ NFAT-GFP reporter cell assay.

Human and mouse LAIR-1 were more likely to bind to synthetic peptides that contained a high GPO triplet content and the most potent peptides from the collagen II and III Toolkits were high in GPO content. These findings are in agreement with our previous studies in which we showed that hLAIR-1 specifically binds CRP (containing ten GPO triplets) but not $(GPP)_{10}$ peptides[11] . However, CRP was much less efficient in functionally cross-linking hLAIR-1 as compared to peptides II-30, II-56, III-30, and III-38 which contain only two to four GPO triplets (see Fig. 3 and 4). Hence, although GPO content is an important factor for hLAIR-1 binding, it is not the only determinant. In line with this, several GPO-deficient peptides from the collagen II and III toolkit were potent LAIR-1 binders and activators (e.g. III-51 which completely lacks GPO triplets), again

suggesting that other non-GPO residues in the collagen trimer contribute to LAIR-1 binding. Also our studies with collagen III Toolkit peptide 30 indicate that the C-terminal GPO triplets are crucial for LAIR-1 induced inhibition and adherence. However, also removal of the two N-terminal triplets (GAOGLR) reduced LAIR-1 adherence, whereas LAIR-1 induced inhibition was less affected. Since a single Ig-fold is too small to accommodate more than eight triplets of a synthetic collagen peptide[27], the LAIR-1 binding motif on collagens is likely less than 8 triplets in size. III-30, containing 9 triplets, thus may harbor two recognition motifs for LAIR-1, which could explain the complex binding characteristics observed in the III-30 variants. The first motif (including residues from the N-terminal GAOGLR) has profound impact on binding to LAIR-1, but less on the functional triggering of the receptor, whereas the second motif (including residues from the C-terminal two GPO triplets) affects both adhesion and signaling. Whether this can also be observed in other Toolkit peptides needs to be determined.

Intriguingly, mLAIR-1 interacted with more collagen II and III toolkit peptides than its human orthologue. Furthermore, mLAIR-1 responded much better than hLAIR-1 to the Toolbox peptides. Hence, mLAIR-1 may have a higher affinity for GPO-bearing peptides as compared to its human orthologue, resulting in a wider range of peptides as ligands. Alternatively, mLAIR-1 may be more promiscuous in requirements for collagen binding sites as human LAIR-1. Given the moderate protein sequence identity (~40%) between human and mouse LAIR-1[28], it is conceivable that specific amino acid differences between both receptors may affect their potential to bind synthetic collagen peptides, without affecting affinity for intact collagen molecules. Such species-specific variation is known for GPVI, where a charge reversal, E replacing K, in the collagen-binding site reduces mGPVI affinity for CRP compared with hGPVI, yet both are good receptors for collagen fibres[29].

The platelet activating collagen receptor GPVI and LAIR-1 are both encoded in the LRC on human chromosome 19[11], suggesting they evolved from a common collagen-binding ancestral gene. Both receptors bind specifically to CRP, but not to GPP_{10} (reviewed in [10]). Furthermore the receptors both bind peptides 1 and 30 from the collagen III Toolkit (personal communication, Farndale R.W.). However, LAIR-1 appears to have less strict collagen binding requirements, since LAIR-1 interacts with more peptides from the collagen III Toolkit (personal communication, Farndale R.W.). Furthermore, whereas a single GPO triplet in the Toolbox peptide-set is sufficient to induce hLAIR-1 inhibition of immune cells, GPVI requires chemically cross-linked peptides containing at least two GPOs in tandem (GPO_2) to induce platelet aggregation[30]. Hence, although both LAIR-1 and GPVI bind collagen molecules and interact with similar synthetic collagen peptides, their collagen binding requirements are not identical.

Recently, the crystal structure of the two Ig-like domains of human GPVI was solved which allowed computational determination of CRP binding sites on the GPVI protein[27]. As expected, human GPVI formed a dimer in the crystal and mainly residues in the N-terminal Ig-domain (D1) of the protein were predicted to be involved in CRP-binding[27]. Unlike GPVI, hLAIR-1 contains a single Ig-like domain[12] and the stoichiometry of

membrane-expressed LAIR-1 remains unknown. Mutational studies on the GPVI/collagen interaction have revealed several residues in the human GPVI D1 domain that are important in GPVI binding to collagens and CRP. Of these, GPVI residues G30 and V34 are conserved in human LAIR-1 (G51 and V55 respectively), and the lysine at position 41 is replaced by the similar arginine (R62). This is suggestive for these residues to be involved in LAIR-1 binding to collagen and synthetic trimeric collagen peptides.
To conclude, by using synthetic trimeric peptides encompassing the entire triple-helical domain of human collagens II and III, we identified multiple functional binding sites for the LAIR-1 molecules which proved potent agonists for these inhibitory immune receptors *in vitro*. Furthermore, we identified a single peptide (II-17) that bound to LAIR-1 but was unable to induce LAIR-1 signaling. This peptide may represent a potent blocker of LAIR-1 binding to collagens. In general, the GPO content of the synthetic peptides appeared the dominant determinant for LAIR-1 binding, although other residues in the collagen sequence had considerable impact on the binding characteristics. Co-crystallization studies of LAIR-1 in complex with synthetic trimeric collagen peptides are required to further elucidate the requirements for interaction between LAIR and collagens. A detailed understanding of the LAIR-1 recognition motifs within collagen may lead to the development of more potent reagents that can be used *in vitro*, *ex vivo*, and *in vivo* functional studies on the biology of the LAIR-1 receptors.

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REFERENCES

1. Myllyharju,J. and K.I.Kivirikko. 2004. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet.* 20:33-43.
2. Ricard-Blum,S. and F.Ruggiero. 2005. The collagen superfamily: from the extracellular matrix to the cell membrane. *Pathol. Biol. (Paris)* 53:430-442.
3. Gelse,K., E.Poschl, and T.Aigner. 2003. Collagens--structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* 55:1531-1546.
4. Farndale,R.W., J.J.Sixma, M.J.Barnes, and P.G.de Groot. 2004. The role of collagen in thrombosis and hemostasis. *J. Thromb. Haemost.* 2:561-573.
5. Raynal,N., S.W.Hamaia, P.R.Siljander, B.Maddox, A.R.Peachey, R.Fernandez, L.J.Foley, D.A.Slatter, G.E.Jarvis, and R.W.Farndale. 2006. Use of synthetic peptides to locate novel integrin alpha2beta1-binding motifs in human collagen III. *J. Biol. Chem.* 281:3821-3831.
6. Lisman,T., N.Raynal, D.Groeneveld, B.Maddox, A.R.Peachey, E.G.Huizinga, P.G.de Groot, and R.W.Farndale. 2006. A single high-affinity binding site for von Willebrand Factor in collagen III, identified using synthetic triple-helical peptides. *Blood. 2006. Aug. 15.*
7. Kehrel,B., S.Wierwille, K.J.Clemetson, O.Anders, M.Steiner, C.G.Knight, R.W.Farndale, M.Okuma, and M.J.Barnes. 1998. Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa, and von Willebrand factor do not. *Blood* 91:491-499.
8. Knight,C.G., L.F.Morton, D.J.Onley, A.R.Peachey, T.Ichinohe, M.Okuma, R.W.Farndale, and M.J.Barnes. 1999. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. *Cardiovasc. Res.* 41:450-457.
9. Morton,L.F., P.G.Hargreaves, R.W.Farndale, R.D.Young, and M.J.Barnes. 1995. Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. *Biochem. J.* 306 (Pt 2):337-344.
10. Moroi,M. and S.M.Jung. 2004. Platelet glycoprotein VI: its structure and function. *Thromb. Res.* 114:221-233.
11. Lebbink,R.J., T.de Ruiter, J.Adelmeijer, A.B.Brenkman, J.M.van Helvoort, M.Koch, R.W.Farndale, T.Lisman, A.Sonnenberg, P.J.Lenting, and L.Meyaard. 2006. Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. *J. Exp. Med.* 203:1419-1425.
12. Meyaard,L., G.J.Adema, C.Chang, E.Woollatt, G.R.Sutherland, L.L.Lanier, and J.H.Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
13. Poggi,A., N.Pella, L.Morelli, F.Spada, V.Revello, S.Sivori, R.Augugliaro, L.Moretta, and A.Moretta. 1995. p40, a novel surface molecule involved in the regulation of the non-major histocompatibility complex-restricted cytolytic activity in humans. *Eur. J. Immunol.* 25:369-376.
14. Meyaard,L., J.Hurenkamp, H.Clevers, L.L.Lanier, and J.H.Phillips. 1999. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. *J. Immunol.* 162:5800-5804.
15. Saverino,D., M.Fabbi, A.Merlo, G.Ravera, C.E.Grossi, and E.Ciccone. 2002. Surface density expression of the leukocyte-associated Ig-like receptor-1 is directly related to inhibition of human T-cell functions. *Hum. Immunol.* 63:534-546.
16. Maasho,K., M.Masilamani, R.Valas, S.Basu, J.E.Coligan, and F.Borrego. 2005. The inhibitory leukocyte-associated Ig-like receptor-1 (LAIR-1) is expressed at high levels by human naive T cells and inhibits TCR mediated activation. *Mol. Immunol.* 42:1521-1530.

17. van der Vuurst de Vries AR, H.Clevers, T.Logtenberg, and L.Meyaard. 1999. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur. J. Immunol.* 29:3160-3167.
18. Poggi,A., E.Tomasello, E.Ferrero, M.R.Zocchi, and L.Moretta. 1998. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. *Eur. J. Immunol.* 28:2086-2091.
19. Lebbink, R. J., Kaptijn, G. J., de Ruiter, T., Jansen, C. A., Lenting, P. J., and Meyaard, L. Mouse Leukocyte-associated Ig-like receptor-1 (LAIR-1) functions as an inhibitory collagen-binding receptor on immune cells . 2006. Submitted for publication.
20. Ravetch,J.V. and L.L.Lanier. 2000. Immune inhibitory receptors. *Science* 290:84-89.
21. Shah,N.K., J.A.Ramshaw, A.Kirkpatrick, C.Shah, and B.Brodsky. 1996. A host-guest set of triple-helical peptides: stability of Gly-X-Y triplets containing common nonpolar residues. *Biochemistry.* 35:10262-10268.
22. O'Connor,M.N., P.A.Smethurst, L.W.Davies, L.Joutsi-Korhonen, D.J.Onley, A.B.Herr, R.W.Farndale, and W.H.Ouwehand. 2006. Selective blockade of glycoprotein VI clustering on collagen helices. *J. Biol. Chem. 2006. Sep. 6.*
23. Verbrugge,A., T.de Ruiter, H.Clevers, and L.Meyaard. 2003. Differential contribution of the Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) of human Leukocyte Associated Ig-like Receptor (LAIR)-1 to inhibitory function and phosphatase recruitment. *Int. Immunol.* 15:1349-1358.
24. Voehringer,D., D.B.Rosen, L.L.Lanier, and R.M.Locksley. 2004. CD200 receptor family members represent novel DAP12-associated activating receptors on basophils and mast cells. *J. Biol. Chem.* 279:54117-54123.
25. Farndale,R.W., P.R.Siljander, D.J.Onley, P.Sundaresan, C.G.Knight, and M.J.Barnes. 2003. Collagen-platelet interactions: recognition and signalling. *Biochem. Soc. Symp. 94.*81-94.
26. Miura,Y., T.Takahashi, S.M.Jung, and M.Moroi. 2002. Analysis of the interaction of platelet collagen receptor glycoprotein VI (GPVI) with collagen. A dimeric form of GPVI, but not the monomeric form, shows affinity to fibrous collagen. *J. Biol. Chem.* 277:46197-46204.
27. Horii,K., M.L.Kahn, and A.B.Herr. 2006. Structural basis for platelet collagen responses by the immune-type receptor glycoprotein VI. *Blood.*
28. Lebbink,R.J., T.de Ruiter, A.Verbrugge, W.S.Bril, and L.Meyaard. 2004. The mouse homologue of the leukocyte-associated Ig-like receptor-1 is an inhibitory receptor that recruits Src homology region 2-containing protein tyrosine phosphatase (SHP)-2, but not SHP-1. *J. Immunol.* 172:5535-5543.
29. Smethurst,P.A., L.Joutsi-Korhonen, M.N.O'Connor, E.Wilson, N.S.Jennings, S.F.Garner, Y.Zhang, C.G.Knight, T.R.Dafforn, A.Buckle, M.J.IJsseldijk, P.G.de Groot, N.A.Watkins, R.W.Farndale, and W.H.Ouwehand. 2004. Identification of the primary collagen-binding surface on human glycoprotein VI by site-directed mutagenesis and by a blocking phage antibody. *Blood* 103:903-911.
30. Smethurst, P. A., Onley, D. J., Jarvis, G. E., O'Connor, M. N., Knight, C. G., Herr, A. B., Ouwehand, W., and Farndale, R. W. The platelet-collagen interaction: the smallest effective motif for Glycoprotein VI-mediated platelet adhesion and activation contains two Glycine-Proline-Hydroxyproline triplets. 2006. Submitted for publication.

6
CHAPTER

Review: Non-MHC ligands for inhibitory immune receptors: novel insights and implications for immune regulation

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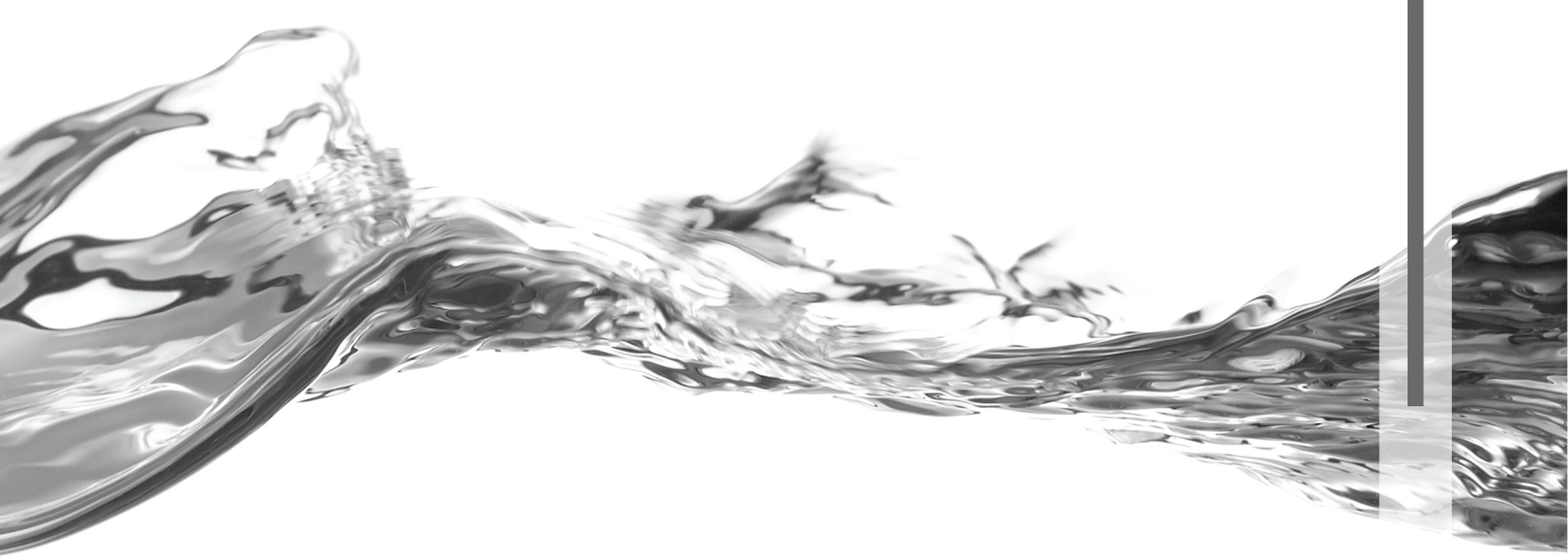

ABSTRACT

Regulation of cellular responses by inhibitory receptors is crucial for proper function of the immune system. The prototype inhibitory immune receptors are major histocompatibility complex (MHC) class I binding Killer-Ig like Receptors (KIRs) present on effector cells such as natural killer (NK) cells and effector T cells. However, the recent identification of non-MHC class I ligands for inhibitory immune receptors, such as KLRG1, KLRB1 and LAIR-1, indicates that also MHC class I independent inhibitory immune receptors play crucial roles in inducing peripheral tolerance. The presence of these receptors on many other immune cell types besides effector cells suggests that tight regulation of cell activation is necessary in all facets of the immune response in both normal and diseased tissue.

Here, we review novel insights and implications of non-MHC class I ligand binding to inhibitory immune receptors. We give an overview of the known ligand-receptor pairs by grouping the ligands according to their properties and discuss implications of these interactions for the maintenance of immune balance and for the defense against tumors and pathogens.

INTRODUCTION

The immune system is in a constant balance by opposing signals that on the one side activate immune cells allowing them to eradicate diseased cells and pathogens, and on the other side inhibit these same cells to limit and ultimately terminate an immune response[1;2]. Loss of inhibitory signaling is often associated with unchecked inflammatory responses resulting in a state of autoimmunity[1;3]. One means of inhibition is provided by inhibitory immune receptors that attenuate activation signals initiated by activating receptors. The cellular response depends on the net outcome of activating and inhibitory signals, which is determined by the expression of activating and inhibiting receptors on the cells and the availability of their ligands in the vicinity.

Almost all inhibitory immune receptors contain one or several Immuno receptor Tyrosine-based Inhibitory Motifs (ITIMs) in their cytoplasmic tail, relaying the inhibitory signal. These motifs serve as docking sites for downstream effectors that mediate the inhibition of cellular action, such as proliferation, differentiation and cytotoxicity. These effectors include the phosphatases SHP-1,2 and SHIP[2] as well as the tyrosine kinase Csk[4;5].

The most frequently studied inhibitory immune receptors bind major histocompatibility complex (MHC) class I. These receptors include killer cell immunoglobulin-like receptor (KIR)s and leukocyte Ig-like receptors (LILR) in humans, Ly49 in mice and CD94 /NKG2A in both (reviewed in [1]). They are predominantly expressed on natural killer (NK) cells and effector T cells, and their interaction with MHC class I ligands results in the abrogation of the signaling pathways in the immune cell thus preventing cytotoxic activity and ultimately killing of the target cells[6]. As predicted by the "missing self hypothesis"[7], NK cells can sense and eradicate virally infected cells that have down regulated their MHC class I as a viral strategy to evade killing by $CD8^+$ T cells[1].

Recently, non-MHC related ligands have been identified for several orphan inhibitory immune receptors. Intriguingly, these ligands have a broad expression pattern and the inhibitory receptors are expressed on not only effector immune cells, but on almost all immune cell types. Hence, non-MHC/inhibitory immune receptor interactions may play important roles in self-tolerance in many different stages of immune cell function. Here, we review novel insights and implications of various classes of non-MHC class I ligand binding to inhibitory immune receptors (see Fig. 1). Of note, many of the identified ligands are involved in the regulation of cell adhesion. We discuss implications of these interactions for maintaining immune balance and describe immune evasion strategies of tumors and pathogens that target inhibitory receptor/ligand pairs. From this overview it can be concluded that immune cells express inhibitory receptors and encounter ligands for these receptors throughout their life-span, which function as thresholds to safely guide them through all phases of an immune response.

Adhesion molecules as ligands for inhibitory immune receptors

Regulation of adhesive contacts between cells underlies many morphogenetic processes during the development of new tissues and the controlled growth and turnover of adult tissues[8]. These functions are mediated by adhesion receptor recognition of extracellular matrix molecules or counter-receptors on other cells, and are achieved through receptor control of cytoskeletal architecture and signalling fluxes[8]. Recently several inhibitory immune receptors have been reported to interact with molecules that have previously been looked upon solely as adhesion molecules. These interactions are reviewed below.

Cadherins

Cadherins mediate Ca^{2+}-dependent cell-cell adhesion important in the formation and maintenance of adherens junctions and are involved in the organized turnover of rapidly growing tissues such as the lining of the gut and the epidermis[9]. Killer cell lectin-like receptor G1 (KLRG1), a C-type lectin-like receptor containing one ITIM, recently has been shown to interact with at least three members of the cadherin family: epithelial (E)-, neuronal (N)-, and retinal (R)-cadherin[10;11]. E-cadherin is expressed on many types of epithelial cells whereas N- and R-cadherin are widely expressed in the nervous system[9]. The inhibitory KLRG1 is expressed by subsets of NK and cytotoxic T cells and its interaction with cadherins inhibits immune responses of these cells[10;11]. This inhibition may have important implications for tumor immunosurveillance by NK and T cells, as will be discussed later in this review.

CAMs

Two transmembrane receptors belonging to the family of Cellular Adhesion Molecules (CAMs), PECAM-1 (CD31) and CEACAM1 (CD66a), are homotypic adhesion molecules with dual functions. Besides functioning as adhesion molecules, both molecules contain ITIMs and as such inhibit immune responses.

CEACAM1 is expressed on epithelial and endothelial cells, but also on activated NK cells, T cells, and other lymphoid and myeloid cells[12]. CEACAM1 expressed on NK cells has been shown to inhibit NK cell cytotoxicity against several melanoma cell lines[13]. Furthermore, inhibitory functions of this receptor have been reported to inhibit T cell[14;15] and B cell[16] function. Surprisingly, CEACAM1 knock-out mice do not show major vascular abnormalities or dramatic phenotypic alterations that one would expect from deficient adhesion molecule expression[17;18], but lymphocytes isolated from these mice display altered proliferation and cytokine secretion[17;18].

PECAM-1-deficient mice were first evaluated in the context of cell adhesion[19] but later demonstrated a much more prominent phenotype related to immune activation, having enhanced sensitivity for autoimmune diseases[20;21]. PECAM-1 is a versatile cell adhesion molecule abundantly expressed on endothelial cells, platelets, monocytes, neutrophils and

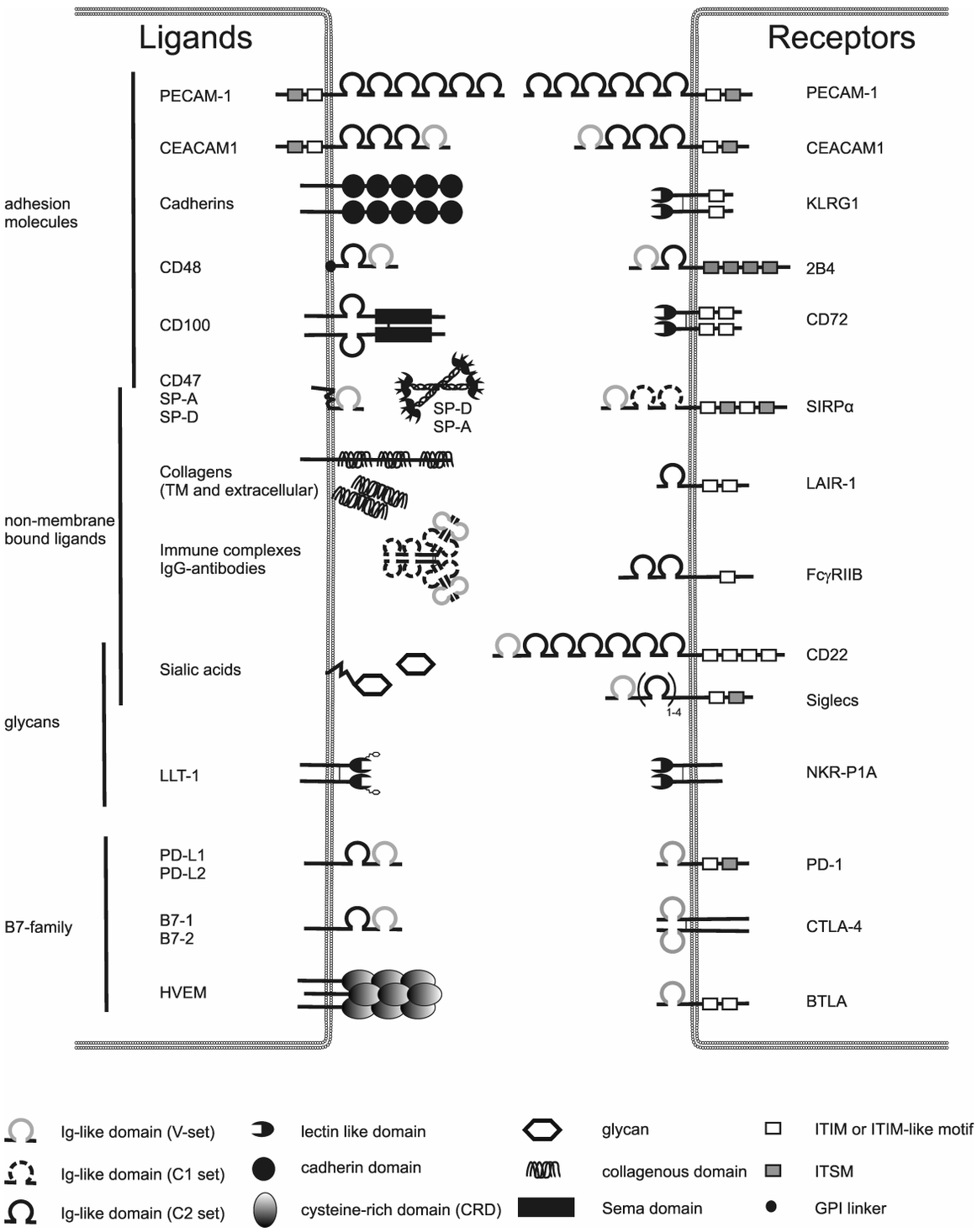

Figure 1. Non-MHC ligands for inhibitory immune receptors. Schematic overview of the various ligand-receptor pairs, grouped according to ligand properties as discussed in the text. Structurally diverse domains are indicated. The relative size of the domains are not to scale. The ligand for CD32 is not depicted as immune complex. Members of the Siglec family (except CD22) are represented by a single receptor. The presence of ITSM motifs in the inhibitory immune receptors was based on a review by Sidorenko and Clark28.

a subset of T cells. On these cells, PECAM-1 is thought to play a role in establishing homophilic interactions and in mediating transmigration of immune cells through the endothelium (reviewed in[22]). Only recently the receptor was recognized to contain ITIM-like structures in its cytoplasmic tail[23]. Indeed, PECAM-1 has been demonstrated to abrogate T cell[24] and B cell[25] receptor-mediated signal transduction and to act as an inhibitory receptor in the context of platelet activation[25-27]. The importance of the inhibitory function of PECAM-1 is supported by the phenotype of the PECAM-1-deficient mice.

CD48

CD48 is a glycophosphatidylinositol (GPI) anchored adhesion molecule, and together with CD58, CD84, Ly9 (CD229), SLAM (CD150) and 2B4 (CD244) belongs to the immunoglobulin superfamily (IgSF) CD2-subset leukocyte antigens that are expressed on all peripheral blood lymphocytes[28]. Many of these receptors have been shown to bind homophilically or heterophilically to other molecules within the family, and thus mediate inter-lymphocyte communication[29]. CD48 is the natural ligand for 2B4[30;31], a receptor expressed on mouse $CD8^+$ T cells, γδ-T cells and monocytes and in humans also on NK cells and granulocytes[32]. Interestingly, mouse 2B4 is considered an inhibitory receptor, while in humans it has a dual function; peripheral blood NK cells express activating 2B4 receptors, but this receptor is inhibitory in decidual NK cells[33]. This dual function is dependent on the association with signaling lymphocyte activation molecule (SLAM)-associated protein (SAP, a.k.a. SH2D1A), a small cytoplasmic protein interacting with tyrosine phosphorylated 2B4 that prevents 2B4 association with SHP-1 and as such determines whether 2B4 acts inhibitory or activating[34;35]. Indeed, inhibitory signaling of 2B4 was related to deficient expression of SAP[33]. SAP binds to cytoplasmic immuno-receptor tyrosine-based switch motifs (ITSM) in the cytoplasmic tail of 2B4. These domains share similarities with ITIMs, although they bind distinct but overlapping sets of molecular components of signal transduction pathways thereby relaying either activating or inhibitory signals (reviewed in [28]). ITSM motifs have been found in several other inhibitory immune receptors, such as CEACAM1, PECAM-1, PD-1, SIRPα, and several members of the Siglec family[28].

Semaphorins

Semaphorins are a large family of secreted and transmembrane signaling proteins that were initially identified through their ability to regulate axonal guidance in the developing nervous system[36]. CD100/SEMA4D is a transmembrane molecule containing both a semaphorin and an Ig-like domain in its extracellular region[37], and the protein has been described to be involved in many cell functions such as cell aggregation and adhesion, cell migration, and cell proliferation (reviewed in [38]). The protein is expressed on immune cells and several non-immune cell types and it can be released from the surface through a metalloprotease-dependent proteolytic process[39]. CD100 is a ligand for inhibitory C-type lectin transmembrane receptor CD72 (LYB-2) and is exceptional since ligation results in

activation rather than inhibition of the cellular function. The interaction of CD100 with CD72 enhances B-cell responses because it induces dephosphorylation of the ITIM and release of SHP-1 from CD72[40]. Indeed, in CD100-deficient B cells, SHP-1 is constitutively associated with CD72, resulting in impaired B-cell responses[40;41]. Consequently, CD100-deficient mice exhibit several immunological defects including reduced in vitro proliferative response and impaired humoral immunity[41]. As expected, this phenotype is nearly the opposite of that seen in CD72-deficient mice which have hyper proliferative B cells in response to various stimuli and show enhanced kinetics in their intracellular Ca^{2+} response following IgM cross-linking[42].

CD47

CD47, is an tetra-spanning Ig-like receptor that regulates integrin function and cellular responses to RGD-containing extracellular matrix proteins, such as vitronectin, fibrinogen, and collagens[43]. Furthermore, CD47 is important in regulating trans-endothelial and trans-epithelial migration of neutrophils, monocytes, and lymphocytes[43]. Besides these functions, CD47 was also found to function as a ligand for the ITIM-bearing Ig-superfamily member signal regulatory protein alpha (SIRPα) [44]. SIRPα is expressed on neurons and myeloid cells with phagocytic capacity, such as granulocytes, monocytes, macrophages and dendritic cells[45], in which the inhibitory potential of the receptor has been documented[43]. There are several reports describing CD47 as a marker for self, e.g. murine red blood cells lacking CD47 expression are rapidly cleared from the bloodstream by splenic red pulp macrophages, whereas CD47 on normal red blood cells prevents this elimination by binding to the inhibitory receptor SIRPα[46]. In terms of structure, tissue distribution and biological function on myeloid cells, SIRPα shows most similarity to another inhibitory Ig-like receptor CD200 receptor (CD200R). This receptor is expressed on myeloid, T and B cells[47], and interacts with CD200[48], a membrane-associated glycoprotein expressed by numerous cell types, including neurons, ovarian cells, tropoblasts, thymocytes, T cells, B cells, and dendritic cells[49]. Although CD200R does not contain ITIMs in its cytoplasmic tail, it does function as an inhibitory immune receptor[50;51]. Indeed, deletion of the ligand CD200 in mice results in myeloid cell dysregulation and enhanced susceptibility to autoimmune inflammation such as experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA)[52].

Adhesion or inhibitory immune signaling?

The interaction of adhesion molecules with inhibitory immune receptors is an interesting phenomenon that may represent an important mechanism to protect these cells from immune cell attack. For some of the adhesion molecules the question now arises whether their primary function lies in immune regulation rather than in their function as adhesion molecule. Recently, it was postulated that loss of cell polarity and tissue architecture due to growth of tumor cells or injury, may favor lymphocyte-endothelial cell interactions over epithelial-epithelial interactions[53]. In a normal healthy situation, adhesion molecules expressed on endothelial and epithelial cells form tight complexes that tether the cells

together, leaving no room for immune cells to interact with the molecules present in the contact area. However, disruption of cell-cell contacts through e.g. injury, may cause exposure of various ligands for activating immune receptors, such as members of the nectin and nectin-like proteins family (necls)[54], thereby triggering immune cell cytotoxicity. Simultaneous exposure of ligands for inhibitory immune receptors may counteract the activation of immune cells, thereby dampening the local immune response and preventing unwanted killing of intact cells present in the exposed tissue. The net outcome, as determined by the relative strength of the opposing activating and inhibiting signals, determines whether the exposed cells become eradicated or remain untouched by the immune system. This discrimination may be crucial to allow removal of damaged or malignant cells that escaped the cell-cell contact dependent state, whereas 'healthy' cells that become exposed to the immune system are not deleted and may promote healing of the damaged tissue.

Glycans as ligands for inhibitory immune receptors

Glycosylation produces different types of glycans or glycoconjugates that are attached to proteins and lipids[55]. Proteins that specifically interact with glycan molecules are termed lectins. One class of these receptors are the Immunoglobulin (I)-type lectin family, with sialic acid binding immunoglobulin-like lectins (Siglecs) constituting its major sub-group[56;57]. Siglecs bind the terminal sialic acids on glycoconjugates, where each Siglec binds a specific oligosaccharide structure. This is determined by the nature of sialic acid, its linkage to substituted sugars, and the underlying neutral oligosaccharides[57]. The majority of Siglecs (CD22, CD33 and CD33-related siglecs) contain ITIMs and ITSMs in their cytoplasmic tails and are expressed in a cell type-specific manner on all cells of the immune system[57]. In line with this, several Siglecs have been shown to inhibit immune cell functions upon receptor cross-linking using monoclonal antibodies[56;57].

The best studied Siglec-member is CD22, a B cell-specific Siglec that regulates BCR and CD19 signaling[58]. CD22 binds 2,6-linked sialic acid-bearing (sialoglycoconjugate) ligands expressed by hematopoietic and nonhematopoietic cells and serum proteins, such as CD22 itself, CD45RO, IgM, members of the Ly-6 family of glycoproteins, and other structurally diverse proteins and lipids[59-63]. In agreement with a inhibitory signaling role for CD22, B cells from CD22–/– mice show augmented calcium responses following BCR cross-linking and exhibit an a phenotype that is characteristic of stimulated B cells[64-66].

Another glycan-recognizing receptor is human CD161 (NKR-P1A), a C-type lectin receptor expressed by most human NK cells, CD1d-restricted NKT cells and subsets of circulating and tissue-infiltrating T cells[67;68]. Recently, human NKR-P1A has been shown to interact with terminal Galα(1,3)Gal epitopes[69], although functional triggering of the receptor by these structures remains to be established. Additionally, two research groups independently identified Lectin-Like Transcript 1 (LLT1) as a functional ligand for human

NKR-P1A[70;71], suggesting that a terminal Galα(1,3)Gal epitope present on LLT1 may be responsible for NKR-P1A binding[69]. Engagement of NKR-P1A on NK cells with LLT1 expressed on target cells inhibits NK cell-mediated cytotoxicity[70;71] and IFN-γ secretion[70]. Intriguingly, the LLT1/NKR-P1A interaction in the presence of a TCR signal was shown to increase IFN-γ production in T cells, suggesting that NKR-P1A may also act as an activating receptor[70].

Given the broad expression of glycan-ligands in the body on cell-bound and soluble molecules and the widespread expression of glycan-binding receptors on immune cells, signaling via glycan-recognizing inhibitory immune receptors likely has a profound impact on all aspects of immunobiology. Detailed analysis on the exact glycan binding residues serving as ligands for the inhibitory glycan-receptors and mapping of the proteins that complex with these structures is needed to appreciate the exact biological function of these versatile immune receptors.

B7-family members as ligands for inhibitory immune receptors

The B7 family of co-regulatory ligands plays a central role in the positive and negative regulation of antigen-specific T cell-mediated immune responses. The ligands, B7-1, B7-2, ICOS ligand, PD-L1 (B7-H1), PD-L2 (B7-DC), B7-H3, and B7-H4 (B7x/B7-S1) are closely related members of the IgSF and are often inducibly expressed on professional antigen-presenting cells as well as on cells within non-lymphoid organs, regulating T cell activation and tolerance in peripheral tissues[72]. The B7-family members serve as ligands for both inhibitory and activating immune receptors. B7.1 and B7.2 binding to CD28 promotes T-cell activation, whereas binding to the inhibitory cytotoxic T lymphocyte antigen-4 (CTLA-4) attenuates responses[73]. Additionally, programmed death-1 ligand-1 and -2 (PD-L1, PD-L2) binding to the inhibitory immune receptor PD-1 inhibits immune responses[74]. The inhibitory immune receptor B and T lymphocyte attenuator (BTLA) expressed on activated NK, T and B cells has been suggested to interact with B7-H4 (B7S1, B7x) as a ligand[75], although a direct interaction between BTLA and B7-H4 could not be verified in subsequent studies76. Recently however, the TNF-family member herpesvirusentry mediator (HVEM) was identified as a functional ligand for BTLA[76;77]. HVEM is expressed on almost all cells of the immune system and its interaction with BTLA has been reported to induce inhibition of immune responses[78].

Non-membrane bound ligands for inhibitory immune receptors

Most ligands for activating and inhibitory immune receptors are membrane-bound antigens, implying a regulatory role in cell-cell interaction. However, several soluble and insoluble secreted ligands that serve as binding partners for inhibitory immune receptors exist.

Immune complexes

The inhibitory Fc-receptor FcγRIIb (CD32) specifically interacts with IgG molecules in complex with antigen[79]. These immune complexes can be either soluble or insoluble, depending on the size of the complex. CD32 is expressed on all cells of the immune system, with the exception of T and NK cells, and it is the only classical Fc receptor on B cells where it regulates activating signals delivered by immune complexes to the B cell receptor[80]. Additionally, the receptor modulates the activation threshold for other activating Fc-receptors on monocytes, macrophages, and dendritic cell subsets[81-83]. The importance of this receptor is illustrated by CD32 deficient animals, which spontaneously develop lupus-like autoimmunity and progress to fulminate glomerulonephritis and premature mortality[84].

Surfactant proteins A and D

Besides CD47, SIRPα has also been shown to interact with soluble ligands, surfactant proteins A and D (SP-A and SP-D)[85]. These multimeric proteins are present in high amounts in the lung and belong to the collectin family of proteins, which contain globular C-type carbohydrate-recognition (lectin) domains coupled to collagen-like tails[86]. In healthy lungs, SP-A and SP-D keep the activity of alveolar macrophages in check by binding with their lectin domains to SIRPα, which can down regulate local immune responses and prevent damage to the airways caused by activated macrophages[86]. However, if a lung becomes damaged or infected, the globular heads of SPs interact with pathogen associated molecular patterns (PAMPs) on foreign organisms, apoptotic cells, or cell debris. The collagenous domains of SPs are subsequently presented in an aggregated state to calreticulin/CD91, which can initiate phagocytosis and/or proinflammatory responses needed to eradicate the diseased state[85]. Furthermore, SP-A and SP-D bind to the same regions of SIRPα as CD47, and as such can block subsequent binding of CD47[85].

Collagens

Collagens represent the most abundant type of proteins in vertebrates and play crucial roles in the development, morphogenesis, and growth of many tissues[87]. Besides their mechanical properties, collagens serve as substrates for cell attachment, migration, and coagulation[88]. Recently, we identified collagens as functional, high affinity ligands for the inhibitory immune receptor leukocyte-associated immunoglobulin-like receptor (LAIR)-1[89]. LAIR-1 is an ITIM-bearing receptor expressed on most cells of the immune system[90], which in the blood are not exposed to collagens[91]. Their extravasation however, will result in interaction with collagen-rich sub-endothelial structures, which may trigger LAIR-1 thereby increasing the threshold for activation needed to control potentially dangerous immune cells. When these cells reach an inflammatory locus, the presence of specific and strong activating stimuli given by antigen-presenting cells, cytokines or pathogens, will override the threshold and allow cells to become activated and mediate their function[89]. Since collagens are present at many sites in the body, the collagen-LAIR-1 interaction

could play an important role at various stages of immune responses. For example, presence of collagens in stroma of lymphoid organs could, via LAIR-1, influence differentiation of immune cells towards certain cell lineages. In line with this hypothesis, collagen X null mice, show defects in hematopoiesis[92].
Intriguingly, also the SIRPα ligands SP-A and SP-D contain collagenous domains[86] which may interact with the inhibitor LAIR-1. Hence, LAIR-1 could play a similar role as SIRPα in down regulating immune responses in healthy lungs. Additionally, LAIR-1 may modulate the activating potential of the calreticulin/CD91complex.

Soluble variants of membrane-bound ligands
Several inhibitory immune receptors can interact with soluble versions of their membrane-bound ligands, which may regulate the interaction between receptor and membrane-bound ligand. These soluble ligands are either generated via proteolytic cleavage of ligand ectodomains or alternative splicing of mRNA transcripts[93]. For example, soluble variants of the ITIM-bearing receptor PECAM-1 have been shown to interfere with the function of the membrane-bound receptor (reviewed in [94]). In addition, soluble versions of rat CEACAM1 inhibit CEACAM1-dependent adhesion and induce CEACAM1-mediated inhibitory signaling, consistent with its homophilic binding[12;95]. Furthermore, soluble versions have been identified for other inhibitory receptor ligands such as cadherins[96], B7-family members[97;98], CD100[99], and CD48[100]. Besides presence of soluble ligands, ectodomains of inhibitory receptors themselves may also be released from the cell surface and either attenuate or promote signalling by competing for ligand(s) with the membrane-bound receptor to decrease receptor-mediated signal generation or by stabilizing ligands in the extracellular milieu[93;101]. Although soluble variants of inhibitory immune receptors are frequently found: sLAIR-1[102], sKLRG1[103], sSIGLEC1[104], sSIGLEC5[105], sPD-1[106], sCTLA-4[107], s2B4[108], and sCD200R[109], their role in immunobiology remains largely unadressed.

Modulation of inhibitory immune receptor ligands by tumors and pathogens

Cytotoxic lymphocytes, such as NK and CD8+ T cells, provide essential defence mechanisms against intracellular pathogens and tumors. These effector immune cells recognize structures on malignant cells that evoke anti-tumor responses resulting in clearance of the diseased cells[1]. However, tumors and virally infected cells have found delicate systems to specifically inhibit immune cells that may harm their existence. Amongst other strategies, malignant cells and pathogens evolved various immune-evasion strategies by targeting inhibitory receptor signalling.

Ligands of inhibitory immune receptors in tumor escape
Several inhibitory receptors have been implicated in the immune escape by tumor cells. Introduction of the ligand for the ITIM-bearing receptor PD-1 into tumor cells for example, renders these cells less susceptible for CTL-mediated kill *in vitro* and enhances

their tumorigenesis and invasiveness *in vivo*[110]. Since PD-L1 expression is low on normal tissues but upregulated in many cancers, the receptor has been proposed to play an important role in tumor immune evasion[111]. Compared to normal B cells, also the ligand for the inhibitory receptor CD200R was shown to be expressed at increased levels on primary chronic lymphocytic leukemia cells, which downregulated T cell responses[112]. There are many other examples of tumors that manifest increased expression of ligands for inhibitory immune receptors (see Table I). Several aggressive breast tumors initially down-regulate E-cadherin to gain motility and metastatic potential, but then re-express the protein which may promote the attachment of metastases and avoid immune attack through the action of the inhibitory KLRG1[53;113]. Additionally, CEACAM1 is over-expressed in some epithelial tumors. Increased CEACAM1 expression has a significant association with poor prognosis, a feature that may be caused by an increased inhibition of immune cell function through homotypic interactions with CEACAM1[12].
Another interesting receptor in this respect is LAIR-1. Multiple studies have been published in which increased collagen expression by tumors was related to malignancy, metastic capacity, and/or tumor progression[114-119]. By over expressing collagens, tumor cells may prevent immune cell activation by ligation of LAIR-1, thereby escaping anti-tumor responses[89].

Table 1: Ligands of inhibitory immune receptors upregulated in cancer cells

Ligand	*Type of tumor*	*Receptor*	*Ref.*
CD47	ovarian carcinoma cells	SIRPα	143
CD48	acute myeloid leukaemia (AML) and chronic lymphocytic leukaemia (B-CLL)	2B4	144;145
CD100	head and neck squamous cell carcinomas	CD72	146
gangliosides	renal-cell carcinomas and melanomas	SIGLEC7	147;148
collagens	various types of tumors	LAIR-1	114-118
PD-L1	various types of tumors	PD-1	111
CD200	B cell lymphocytic leukemia cells	CD200R	112
E-cadherin*	breast tumors	KLRG1	53;113
CEACAM1*	epithelial tumors	CEACAM1	12

*These ligands are reported to be upregulated or downregulated on tumor cells, see text for details. Note: Although these examples suggest that tumor cells exploit a wide range of inhibitory immune receptor to downmodulate immune responses, the actual contribution of many of these actions in tumor protection awaits clarification.

In contrast to increased expression of inhibitory ligands to protect tumors against immune cell cytolysis, reduced expression of such ligands may evoke a 'danger' signal thereby promoting tumor killing. In line with this, E-cadherin expression is frequently down-regulated in breast cancers[53;113], which may decrease the activation threshold of cytotoxic immune cells via KLRG1, rendering the tumor cells more susceptible for immune cell attack. Counteractively, reduced E-cadherin expression triggers a more invasive tumor phenotype caused by a loss of contact inhibition and a higher proliferative capacity[53;113]. Also CEACAM1 has been reported to be downregulated in various cancers[12], and forced expression of CEACAM1 in cancer cells suppresses their

tumorigenic phenotype in an ITIM-dependent manner in both cell culture and *in vivo* mouse studies[12;120;121].

Pathogens exploiting inhibitory immune receptor/ligand pairs

In response to the selective pressure that is exerted by immune cells, many viruses and pathogens have evolved strategies to evade detection by the immune system or to modulate the activity of immune cells[122]. Whereas many immune evading strategies by pathogens are aimed at interfering with MHC class I function[122], several non-MHC inhibitory immune receptors are also targeted by these pathogens resulting in inhibition of immune responses through inhibitory signaling (see Fig. 2).

One strategy viruses use to target inhibitory immune receptors is by encoding viral ligands that are expressed on the membrane of infected cells and functionally interact with endogenous inhibitory receptor on immune cells (Fig. 2, part A). Human herpesvirus 8, rat cytomegalovirus (RCMV), myxoma virus M141R, and rhesus rhadinovirus R15 encode viral homologues of CD200[123-126], which directly down-regulate macrophage activation through the inhibitory action of the CD200R receptor[124;126]. Additionally, some poxvirus isolates encode CD47 homologues [127-129], which may interact with the inhibitory SIRPα once they are expressed on the surface of infected cells[129]. Indeed, rabbits infected with a M128L myxoma virus in which the CD47 homologue was deleted showed greater activation of monocytes and macrophages in infected and lymphoid tissues when compared to wild-type myxoma-infected rabbits[129], suggesting that the virus uses SIRPα to evade an immune response. Furthermore, UL144, a TNF receptor homologue encoded by human CMV, has been shown to compete with BTLA for HVEM binding and potently inhibits T-cell activation[130].

Another strategy that pathogens may use is the upregulation of host-encoded ligands for inhibitory immune receptors. PD-L1 and PD-L2, the ligands for the inhibitory immune receptor PD-1 can become upregulated upon viral infection[131;132]. Furthermore, *Schistosoma mansoni* worms have been shown to induce T cell anergy by upregulating PD-L1 expression on macrophages[133]. Additionally, CD48, the ligand for the receptor 2B4, is induced on B cells upon Epstein–Barr virus (EBV) infection[134]. Since 2B4 can both activate and inhibit immune cells, CD48 expression may either trigger or inhibit anti-viral activity.

Viral pathogens can use inhibitory immune receptors or ligands as host-cell entry molecules. Binding of the HSV viral envelope glycoprotein D (gD) to HVEM for example, is part of the HSV viral entry mechanism[78]. Recent studies also have identified B7-1 and B7-2 as cellular attachment receptors for adenovirus serotype 3[135]. Furthermore, CEACAM1-specific adhesions allow various *Neisseria* and *Haemophilus* bacterial strains to attach to, invade or transcytose polarized epithelia and endothelia, providing them with a system to colonize host tissues[12]. Besides employing CEACAM1 as entry molecule, opacity-associated (Opa) proteins of *N. gonorrhoeae* can directly ligate the receptor and as such repress the activation and proliferation of human $CD4^+$ T cells[136]. Furthermore, CEACAM1 is directly targeted by the viral murine hepatitis

virus (MHV) spike glycoprotein, which inhibits the differentiation of naive cells into Th1 but not Th2 cells and activation of Th1 but not Th2 cytokine production[137].

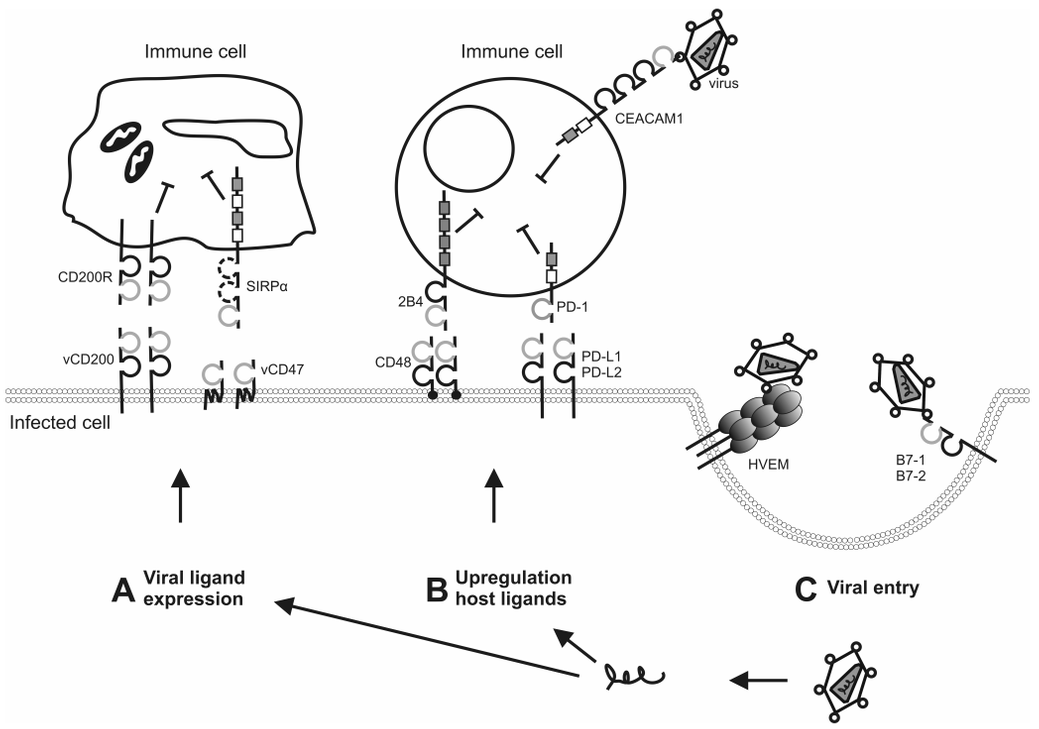

Figure 2. Viral immune evasion strategies that target inhibitory immune receptor/ligand pairs. Three immune evasion strategies are depicted: A) Expression of virus-encoded ligands (vCD200 and vCD47) that interact with inhibitory immune receptors on immune cells. B) Virus-induced upregulation of host-encoded ligands (CD48, PD-L1, and PD-L2) for inhibitory immune receptors. C) Ligands (HVEM, B7-1, and B7-2) of inhibitory immune receptors act as viral entry molecules. Furthermore, viral particles may directly inhibit immune cells through direct binding to inhibitory immune receptors (e.g. CEACAM1). A more detailed description of the figure is given in the text. The schematic representation of the domains of receptors and ligands is described in figure 1.

CONCLUDING REMARKS

With the recent identification of ligands for orphan inhibitory immune receptors, it becomes clear that an inhibitory signal provided by self-MHC is not sufficient to prevent autoimmunity. Indeed, several lines of experimental evidence point to a relation between disturbed function of inhibitory receptors recognizing non-MHC ligands and autoimmune disease. Mice that carry natural or targeted deletions in inhibitory receptors have manifestations resembling human autoimmune disease[138]. For example, mice deficient in FcγRIIB or PD-1 develop lupus-like pathology[84;139] and CD72 knock out mice have hyper responsive B cells[42]. Other evidence suggests a role of genetic variation of these receptors in human autoimmune disease. Genetic variation in the gene cluster that includes FcγRIIB has been reported to be associated to SLE and RA[140;141] and a locus close to PD-1 was identified as a SLE disease susceptibility locus[142].

Most cells in the immune system express at least one and often many inhibitory receptors which often play crucial and non-redundant roles in the regulation of the immune system. Furthermore, it is becoming increasingly clear that their ligands are broadly expressed molecules, allowing receptor/ligand interactions to occur in many facets of the immune response in both normal and diseased tissue. Apparently, every phase of the immune response requires a tight regulation by inhibitory receptor-ligand interaction, and defects in these systems have profound consequences for immune surveillance. The challenge now is to unravel when and where, which receptor-ligand interactions occur, and how the interplay between the various activating and inhibiting immune receptor on a single cell dictates what the outcome will be on immune function.

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REFERENCES

1. Lanier,L.L. 2005. NK Cell Recognition. *Annu. Rev. Immunol.*225-274.
2. Ravetch,J.V. and L.L.Lanier. 2000. Immune inhibitory receptors. *Science* 290:84-89.
3. Lanier,L.L. 1998. NK cell receptors. *Annu. Rev. Immunol.* 16:359-393.
4. Verbrugge,A., E.S.Rijkers, T.de Ruiter, and L.Meyaard. 2006. Leukocyte-associated Ig-like receptor-1 has SH2 domain-containing phosphatase-independent function and recruits C-terminal Src kinase. *Eur. J. Immunol.* 36:190-198.
5. Sayos,J., A.Martinez-Barriocanal, F.Kitzig, T.Bellon, and M.Lopez-Botet. 2004. Recruitment of C-terminal Src kinase by the leukocyte inhibitory receptor CD85j. *Biochem. Biophys. Res. Commun.* 324:640-647.
6. Blery,M., L.Olcese, and E.Vivier. 2000. Early signaling via inhibitory and activating NK receptors. *Hum. Immunol.* 61:51-64.
7. Karre,K., H.G.Ljunggren, G.Piontek, and R.Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature.* 319:675-678.
8. Gumbiner,B.M. 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell.* 84:345-357.
9. Gumbiner,B.M. 2005. Regulation of cadherin-mediated adhesion in morphogenesis. *Nat. Rev. Mol. Cell Biol.* 6:622-634.
10. Ito,M., T.Maruyama, N.Saito, S.Koganei, K.Yamamoto, and N.Matsumoto. 2006. Killer cell lectin-like receptor G1 binds three members of the classical cadherin family to inhibit NK cell cytotoxicity. *J Exp. Med.* 203:289-295.
11. Grundemann,C., M.Bauer, O.Schweier, N.von Oppen, U.Lassing, P.Saudan, K.F.Becker, K.Karp, T.Hanke, M.F.Bachmann, and H.Pircher. 2006. Cutting edge: identification of E-cadherin as a ligand for the murine killer cell lectin-like receptor G1. *J Immunol.* 176:1311-1315.
12. Gray-Owen,S.D. and R.S.Blumberg. 2006. CEACAM1: contact-dependent control of immunity. *Nat. Rev. Immunol.* 6:433-446.
13. Markel,G., N.Lieberman, G.Katz, T.I.Arnon, M.Lotem, O.Drize, R.S.Blumberg, E.Bar-Haim, R.Mader, L.Eisenbach, and O.Mandelboim. 2002. CD66a interactions between human melanoma and NK cells: a novel class I MHC-independent inhibitory mechanism of cytotoxicity. *J. Immunol.* 168:2803-2810.
14. Chen,D., H.Iijima, T.Nagaishi, A.Nakajima, S.Russell, R.Raychowdhury, V.Morales, C.E.Rudd, N.Utku, and R.S.Blumberg. 2004. Carcinoembryonic antigen-related cellular adhesion molecule 1 isoforms alternatively inhibit and costimulate human T cell function. *J. Immunol. 2004. Mar. 15. ;172. (6.):3535. -43.* 172:3535-3543.
15. Markel,G., D.Wolf, J.Hanna, R.Gazit, D.Goldman-Wohl, Y.Lavy, S.Yagel, and O.Mandelboim. 2002. Pivotal role of CEACAM1 protein in the inhibition of activated decidual lymphocyte functions. *J. Clin. Invest.* 110:943-953.
16. Chen,T., W.Zimmermann, J.Parker, I.Chen, A.Maeda, and S.Bolland. 2001. Biliary glycoprotein (BGPa, CD66a, CEACAM1) mediates inhibitory signals. *J. Leukoc. Biol.* 70:335-340.
17. Hemmila,E., C.Turbide, M.Olson, S.Jothy, K.V.Holmes, and N.Beauchemin. 2004. Ceacam1a-/- mice are completely resistant to infection by murine coronavirus mouse hepatitis virus A59. *J. Virol.* 78:10156-10165.
18. Kuespert,K., S.Pils, and C.R.Hauck. 2006. CEACAMs: their role in physiology and pathophysiology. *Curr. Opin. Cell Biol.* 18:565-571.
19. Duncan,G.S., D.P.Andrew, H.Takimoto, S.A.Kaufman, H.Yoshida, J.Spellberg, d.l.P.Luis, A.Elia, A.Wakeham, B.Karan-Tamir, W.A.Muller, G.Senaldi, M.M.Zukowski, and T.W.Mak. 1999. Genetic evidence for functional redundancy of Platelet/Endothelial cell adhesion molecule-1 (PECAM-1):

CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. *J. Immunol.* 162:3022-3030.

20. Graesser,D., A.Solowiej, M.Bruckner, E.Osterweil, A.Juedes, S.Davis, N.H.Ruddle, B.Engelhardt, and J.A.Madri. 2002. Altered vascular permeability and early onset of experimental autoimmune encephalomyelitis in PECAM-1-deficient mice. *J. Clin. Invest.* 109:383-392.
21. Wilkinson,R., A.B.Lyons, D.Roberts, M.X.Wong, P.A.Bartley, and D.E.Jackson. 2002. Platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) acts as a regulator of B-cell development, B-cell antigen receptor (BCR)-mediated activation, and autoimmune disease. *Blood.* 100:184-193.
22. Fujiwara,K. 2006. Platelet endothelial cell adhesion molecule-1 and mechanotransduction in vascular endothelial cells. *J. Intern. Med.* 259:373-380.
23. Newman,P.J. 1999. Switched at birth: a new family for PECAM-1. *J. Clin. Invest.* 103:5-9.
24. Newton-Nash,D.K. and P.J.Newman. 1999. A new role for platelet-endothelial cell adhesion molecule-1 (CD31): inhibition of TCR-mediated signal transduction. *J. Immunol.* 163:682-688.
25. Henshall,T.L., K.L.Jones, R.Wilkinson, and D.E.Jackson. 2001. Src homology 2 domain-containing protein-tyrosine phosphatases, SHP-1 and SHP-2, are required for platelet endothelial cell adhesion molecule-1/CD31-mediated inhibitory signaling. *J. Immunol.* 166:3098-3106.
26. Cicmil,M., J.M.Thomas, M.Leduc, C.Bon, and J.M.Gibbins. 2002. Platelet endothelial cell adhesion molecule-1 signaling inhibits the activation of human platelets. *Blood.* 99:137-144.
27. Falati,S., S.Patil, P.L.Gross, M.Stapleton, G.Merrill-Skoloff, N.E.Barrett, K.L.Pixton, H.Weiler, B.Cooley, D.K.Newman, P.J.Newman, B.C.Furie, B.Furie, and J.M.Gibbins. 2006. Platelet PECAM-1 inhibits thrombus formation in vivo. *Blood.* 107:535-541.
28. Sidorenko,S.P. and E.A.Clark. 2003. The dual-function CD150 receptor subfamily: the viral attraction. *Nat. Immunol.* 4:19-24.
29. McNerney,M.E. and V.Kumar. 2006. The CD2 family of natural killer cell receptors. *Curr. Top. Microbiol. Immunol.* 298:91-120.
30. Brown,M.H., K.Boles, P.A.van der Merwe, V.Kumar, P.A.Mathew, and A.N.Barclay. 1998. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J. Exp. Med.* 188:2083-2090.
31. Latchman,Y., P.F.McKay, and H.Reiser. 1998. Identification of the 2B4 molecule as a counter-receptor for CD48. *J. Immunol.* 161:5809-5812.
32. Vaidya,S.V. and P.A.Mathew. 2006. Of mice and men: different functions of the murine and human 2B4 (CD244) receptor on NK cells. *Immunol. Lett.* 105:180-184.
33. Vacca,P., G.Pietra, M.Falco, E.Romeo, C.Bottino, F.Bellora, F.Prefumo, E.Fulcheri, P.L.Venturini, M.Costa, A.Moretta, L.Moretta, and M.C.Mingari. 2006. Analysis of Natural Killer cells isolated from human decidua: evidence that 2B4 (CD244) functions as an inhibitory receptor and blocks NK cell function. *Blood. 2006. Aug. 24.*
34. Eissmann,P., L.Beauchamp, J.Wooters, J.C.Tilton, E.O.Long, and C.Watzl. 2005. Molecular basis for positive and negative signaling by the natural killer cell receptor 2B4 (CD244). *Blood.* 105:4722-4729.
35. Chen,R., F.Relouzat, R.Roncagalli, A.Aoukaty, R.Tan, S.Latour, and A.Veillette. 2004. Molecular dissection of 2B4 signaling: implications for signal transduction by SLAM-related receptors. *Mol. Cell Biol.* 24:5144-5156.
36. Kolodkin,A.L. 1996. Semaphorins: mediators of repulsive growth cone guidance. *Trends Cell Biol.* 6:15-22.
37. Hall,K.T., L.Boumsell, J.L.Schultze, V.A.Boussiotis, D.M.Dorfman, A.A.Cardoso, A.Bensussan, L.M.Nadler, and G.J.Freeman. 1996. Human CD100, a novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. *Proc. Natl. Acad. Sci. U. S. A.* 93:11780-11785.
38. Yazdani,U. and J.R.Terman. 2006. The semaphorins. *Genome Biol.* 7:211.

39. Elhabazi,A., S.Delaire, A.Bensussan, L.Boumsell, and G.Bismuth. 2001. Biological activity of soluble CD100. I. The extracellular region of CD100 is released from the surface of T lymphocytes by regulated proteolysis. *J. Immunol.* 166:4341-4347.
40. Kumanogoh,A., C.Watanabe, I.Lee, X.Wang, W.Shi, H.Araki, H.Hirata, K.Iwahori, J.Uchida, T.Yasui, M.Matsumoto, K.Yoshida, H.Yakura, C.Pan, J.R.Parnes, and H.Kikutani. 2000. Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. *Immunity.* 13:621-631.
41. Shi,W., A.Kumanogoh, C.Watanabe, J.Uchida, X.Wang, T.Yasui, K.Yukawa, M.Ikawa, M.Okabe, J.R.Parnes, K.Yoshida, and H.Kikutani. 2000. The class IV semaphorin CD100 plays nonredundant roles in the immune system: defective B and T cell activation in CD100-deficient mice. *Immunity.* 13:633-642.
42. Pan,C., N.Baumgarth, and J.R.Parnes. 1999. CD72-deficient mice reveal nonredundant roles of CD72 in B cell development and activation. *Immunity.* 11:495-506.
43. Olsson,M., A.Nilsson, and P.A.Oldenborg. 2006. Target cell CD47 regulates macrophage activation and erythrophagocytosis. *Transfus. Clin. Biol.* 13:39-43.
44. Jiang,P., C.F.Lagenaur, and V.Narayanan. 1999. Integrin-associated protein is a ligand for the P84 neural adhesion molecule. *J. Biol. Chem.* 274:559-562.
45. Seiffert,M., C.Cant, Z.Chen, I.Rappold, W.Brugger, L.Kanz, E.J.Brown, A.Ullrich, and H.J.Buhring. 1999. Human signal-regulatory protein is expressed on normal, but not on subsets of leukemic myeloid cells and mediates cellular adhesion involving its counterreceptor CD47. *Blood.* 94:3633-3643.
46. Oldenborg,P.A., A.Zheleznyak, Y.F.Fang, C.F.Lagenaur, H.D.Gresham, and F.P.Lindberg. 2000. Role of CD47 as a marker of self on red blood cells. *Science.* 288:2051-2054.
47. Wright,G.J., H.Cherwinski, M.Foster-Cuevas, G.Brooke, M.J.Puklavec, M.Bigler, Y.Song, M.Jenmalm, D.Gorman, T.McClanahan, M.R.Liu, M.H.Brown, J.D.Sedgwick, J.H.Phillips, and A.N.Barclay. 2003. Characterization of the CD200 receptor family in mice and humans and their interactions with CD200. *J Immunol.* 171:3034-3046.
48. Wright,G.J., M.J.Puklavec, A.C.Willis, R.M.Hoek, J.D.Sedgwick, M.H.Brown, and A.N.Barclay. 2000. Lymphoid/neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. *Immunity.* 13:233-242.
49. Barclay,A.N., G.J.Wright, G.Brooke, and M.H.Brown. 2002. CD200 and membrane protein interactions in the control of myeloid cells. *Trends Immunol.* 23:285-290.
50. Zhang,S., H.Cherwinski, J.D.Sedgwick, and J.H.Phillips. 2004. Molecular mechanisms of CD200 inhibition of mast cell activation. *J. Immunol.* 173:6786-6793.
51. Jenmalm,M.C., H.Cherwinski, E.P.Bowman, J.H.Phillips, and J.D.Sedgwick. 2006. Regulation of myeloid cell function through the CD200 receptor. *J. Immunol.* 176:191-199.
52. Hoek,R.M., S.R.Ruuls, C.A.Murphy, G.J.Wright, R.Goddard, S.M.Zurawski, B.Blom, M.E.Homola, W.J.Streit, M.H.Brown, A.N.Barclay, and J.D.Sedgwick. 2000. Down-regulation of the macrophage lineage through interaction with OX2 (CD200). *Science* 290:1768-1771.
53. Colonna,M. 2006. Cytolytic responses: cadherins put out the fire. *J Exp. Med.* 203:261-264.
54. Fuchs,A. and M.Colonna. 2006. The role of NK cell recognition of nectin and nectin-like proteins in tumor immunosurveillance. *Semin. Cancer Biol. 2006. Jul. 7. ;. ..*
55. Ohtsubo,K. and J.D.Marth. 2006. Glycosylation in cellular mechanisms of health and disease. *Cell.* 126:855-867.
56. Crocker,P.R. 2005. Siglecs in innate immunity. *Curr. Opin. Pharmacol.* 5:431-437.
57. Varki,A. and T.Angata. 2006. Siglecs--the major subfamily of I-type lectins. *Glycobiology* 16:1R-27R.
58. Nitschke,L. 2005. The role of CD22 and other inhibitory co-receptors in B-cell activation. *Curr. Opin. Immunol.* 17:290-297.

59. Peaker,C.J. and M.S.Neuberger. 1993. Association of CD22 with the B cell antigen receptor. *Eur. J. Immunol.* 23:1358-1363.
60. Leprince,C., K.E.Draves, R.L.Geahlen, J.A.Ledbetter, and E.A.Clark. 1993. CD22 associates with the human surface IgM-B-cell antigen receptor complex. *Proc. Natl. Acad. Sci. U. S. A.* 90:3236-3240.
61. Pflugh,D.L., S.E.Maher, and A.L.Bothwell. 2002. Ly-6 superfamily members Ly-6A/E, Ly-6C, and Ly-6I recognize two potential ligands expressed by B lymphocytes. *J. Immunol.* 169:5130-5136.
62. Stamenkovic,I., D.Sgroi, A.Aruffo, M.S.Sy, and T.Anderson. 1991. The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and alpha 2-6 sialyltransferase, CD75, on B cells. *Cell.* 66:1133-1144.
63. Han,S., B.E.Collins, P.Bengtson, and J.C.Paulson. 2005. Homomultimeric complexes of CD22 in B cells revealed by protein-glycan cross-linking. *Nat. Chem. Biol.* 1:93-97.
64. Sato,S., A.S.Miller, M.Inaoki, C.B.Bock, P.J.Jansen, M.L.Tang, and T.F.Tedder. 1996. CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity.* 5:551-562.
65. O'Keefe,T.L., G.T.Williams, S.L.Davies, and M.S.Neuberger. 1996. Hyperresponsive B cells in CD22-deficient mice. *Science.* 274:798-801.
66. Otipoby,K.L., K.B.Andersson, K.E.Draves, S.J.Klaus, A.G.Farr, J.D.Kerner, R.M.Perlmutter, C.L.Law, and E.A.Clark. 1996. CD22 regulates thymus-independent responses and the lifespan of B cells. *Nature.* 384:634-637.
67. Lanier,L.L., C.Chang, and J.H.Phillips. 1994. Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *J. Immunol.* 153:2417-2428.
68. Exley,M., J.Garcia, S.P.Balk, and S.Porcelli. 1997. Requirements for CD1d recognition by human invariant Valpha24+ CD4-CD8- T cells. *J. Exp. Med.* 186:109-120.
69. Christiansen,D., E.Mouhtouris, J.Milland, A.Zingoni, A.Santoni, and M.S.Sandrin. 2006. Recognition of a carbohydrate xenoepitope by human NKRP1A (CD161). *Xenotransplantation.* 13:440-446.
70. Aldemir,H., V.Prod'homme, M.J.Dumaurier, C.Retiere, G.Poupon, J.Cazareth, F.Bihl, and V.M.Braud. 2005. Cutting edge: lectin-like transcript 1 is a ligand for the CD161 receptor. *J. Immunol.* 175:7791-7795.
71. Rosen,D.B., J.Bettadapura, M.Alsharifi, P.A.Mathew, H.S.Warren, and L.L.Lanier. 2005. Cutting edge: lectin-like transcript-1 is a ligand for the inhibitory human NKR-P1A receptor. *J. Immunol. 2005. Dec. 15. ;175. (12.):7796. -9.* 175:7796-7799.
72. Carreno,B.M. and M.Collins. 2003. BTLA: a new inhibitory receptor with a B7-like ligand. *Trends Immunol.* 24:524-527.
73. Sansom,D.M., C.N.Manzotti, and Y.Zheng. 2003. What's the difference between CD80 and CD86? *Trends Immunol. 2003. Jun. ;24. (6.):314. -9.* 24:314-319.
74. Okazaki,T. and T.Honjo. 2006. The PD-1-PD-L pathway in immunological tolerance. *Trends Immunol.* 27:195-201.
75. Watanabe,N., M.Gavrieli, J.R.Sedy, J.Yang, F.Fallarino, S.K.Loftin, M.A.Hurchla, N.Zimmerman, J.Sim, X.Zang, T.L.Murphy, J.H.Russell, J.P.Allison, and K.M.Murphy. 2003. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat. Immunol.* 4:670-679.
76. Sedy,J.R., M.Gavrieli, K.G.Potter, M.A.Hurchla, R.C.Lindsley, K.Hildner, S.Scheu, K.Pfeffer, C.F.Ware, T.L.Murphy, and K.M.Murphy. 2005. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat. Immunol.* 6:90-98.
77. Gonzalez,L.C., K.M.Loyet, J.Calemine-Fenaux, V.Chauhan, B.Wranik, W.Ouyang, and D.L.Eaton. 2005. A coreceptor interaction between the CD28 and TNF receptor family members B and T lymphocyte attenuator and herpesvirus entry mediator. *Proc. Natl. Acad. Sci. U. S. A.* 102:1116-1121.
78. Murphy,K.M., C.A.Nelson, and J.R.Sedy. 2006. Balancing co-stimulation and inhibition with BTLA and HVEM. *Nat. Rev. Immunol.* 6:671-681.

79. Ravetch,J.V. and S.Bolland. 2001. IgG Fc receptors. *Annu. Rev. Immunol.* 19:275-290.
80. Nimmerjahn,F. and J.V.Ravetch. 2006. Fcgamma receptors: old friends and new family members. *Immunity.* 24:19-28.
81. Cassel,D.L., M.A.Keller, S.Surrey, E.Schwartz, A.D.Schreiber, E.F.Rappaport, and S.E.McKenzie. 1993. Differential expression of Fc gamma RIIA, Fc gamma RIIB and Fc gamma RIIC in hematopoietic cells: analysis of transcripts. *Mol. Immunol.* 30:451-460.
82. Yada,A., S.Ebihara, K.Matsumura, S.Endo, T.Maeda, A.Nakamura, K.Akiyama, S.Aiba, and T.Takai. 2003. Accelerated antigen presentation and elicitation of humoral response in vivo by FcgammaRIIB- and FcgammaRI/III-mediated immune complex uptake. *Cell Immunol.* 225:21-32.
83. Tridandapani,S., K.Siefker, J.L.Teillaud, J.E.Carter, M.D.Wewers, and C.L.Anderson. 2002. Regulated expression and inhibitory function of Fcgamma RIIb in human monocytic cells. *J. Biol. Chem.* 277:5082-5089.
84. Bolland,S. and J.V.Ravetch. 2000. Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis. *Immunity.* 13:277-285.
85. Gardai,S.J., Y.Q.Xiao, M.Dickinson, J.A.Nick, D.R.Voelker, K.E.Greene, and P.M.Henson. 2003. By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell.* 115:13-23.
86. Barclay,A.N. and M.H.Brown. 2006. The SIRP family of receptors and immune regulation. *Nat. Rev. Immunol.* 6:457-464.
87. Myllyharju,J. and K.I.Kivirikko. 2004. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet.* 20:33-43.
88. Gelse,K., E.Poschl, and T.Aigner. 2003. Collagens--structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* 55:1531-1546.
89. Lebbink,R.J., T.de Ruiter, J.Adelmeijer, A.B.Brenkman, J.M.van Helvoort, M.Koch, R.W.Farndale, T.Lisman, A.Sonnenberg, P.J.Lenting, and L.Meyaard. 2006. Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. *J. Exp. Med.* 203:1419-1425.
90. Meyaard,L., G.J.Adema, C.Chang, E.Woollatt, G.R.Sutherland, L.L.Lanier, and J.H.Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
91. Farndale,R.W., J.J.Sixma, M.J.Barnes, and P.G.de Groot. 2004. The role of collagen in thrombosis and hemostasis. *J. Thromb. Haemost.* 2:561-573.
92. Gress,C.J. and O.Jacenko. 2000. Growth plate compressions and altered hematopoiesis in collagen X null mice. *J. Cell Biol.* 149:983-993.
93. Levine,S.J. 2004. Mechanisms of soluble cytokine receptor generation. *J Immunol.* 173:5343-5348.
94. Ilan,N. and J.A.Madri. 2003. PECAM-1: old friend, new partners. *Curr. Opin. Cell Biol.* 15:515-524.
95. Budt,M., B.Michely, M.M.Muller, W.Reutter, and L.Lucka. 2002. Secreted CEACAM1 splice variants in rat cell lines and in vivo in rat serum. *Biochem. Biophys. Res. Commun.* 292:749-755.
96. Charalabopoulos,K., A.Gogali, O.K.Kostoula, and S.H.Constantopoulos. 2004. Cadherin superfamily of adhesion molecules in primary lung cancer. *Exp. Oncol.* 26:256-260.
97. Hock,B.D., J.L.O'Donnell, K.Taylor, A.Steinkasserer, J.L.McKenzie, A.G.Rothwell, and K.L.Summers. 2006. Levels of the soluble forms of CD80, CD86, and CD83 are elevated in the synovial fluid of rheumatoid arthritis patients. *Tissue Antigens.* 67:57-60.
98. Simon,I., S.Zhuo, L.Corral, E.P.Diamandis, M.J.Sarno, R.L.Wolfert, and N.W.Kim. 2006. B7-h4 is a novel membrane-bound protein and a candidate serum and tissue biomarker for ovarian cancer. *Cancer Res.* 66:1570-1575.
99. Kumanogoh,A. and H.Kikutani. 2004. Biological functions and signaling of a transmembrane semaphorin, CD100/Sema4D. *Cell Mol. Life Sci.* 61:292-300.

100. Smith,G.M., J.Biggs, B.Norris, P.Anderson-Stewart, and R.Ward. 1997. Detection of a soluble form of the leukocyte surface antigen CD48 in plasma and its elevation in patients with lymphoid leukemias and arthritis. *J. Clin. Immunol.* 17:502-509.
101. Heaney,M.L. and D.W.Golde. 1996. Soluble cytokine receptors. *Blood* 87:847-857.
102. Ouyang,W., J.Xue, J.Liu, W.Jia, Z.Li, X.Xie, X.Liu, J.Jian, Q.Li, Y.Zhu, A.Yang, and B.Jin. 2004. Establishment of an ELISA system for determining soluble LAIR-1 levels in sera of patients with HFRS and kidney transplant. *J Immunol. Methods* 292:109-117.
103. Bocek,P., Jr., M.D.Guthmann, and I.Pecht. 1997. Analysis of the genes encoding the mast cell function-associated antigen and its alternatively spliced transcripts. *J. Immunol.* 158:3235-3243.
104. Crocker,P.R., S.Mucklow, V.Bouckson, A.McWilliam, A.C.Willis, S.Gordon, G.Milon, S.Kelm, and P.Bradfield. 1994. Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains. *EMBO J.* 13:4490-4503.
105. Connolly,N.P., M.Jones, and S.M.Watt. 2002. Human Siglec-5: tissue distribution, novel isoforms and domain specificities for sialic acid-dependent ligand interactions. *Br. J. Haematol.* 119:221-238.
106. Nielsen,C., L.Ohm-Laursen, T.Barington, S.Husby, and S.T.Lillevang. 2005. Alternative splice variants of the human PD-1 gene. *Cell Immunol.* 235:109-116.
107. Oaks,M.K., K.M.Hallett, R.T.Penwell, E.C.Stauber, S.J.Warren, and A.J.Tector. 2000. A native soluble form of CTLA-4. *Cell Immunol.* 201:144-153.
108. Kumaresan,P.R., S.E.Stepp, M.Bennett, V.Kumar, and P.A.Mathew. 2000. Molecular cloning of transmembrane and soluble forms of a novel rat natural killer cell receptor related to 2B4. *Immunogenetics. 2000. Apr;51. (4-5):306. -13.* 51:306-313.
109. Vieites,J.M., T.R.de la, M.A.Ortega, T.Montero, J.M.Peco, A.Sanchez-Pozo, A.Gil, and A.Suarez. 2003. Characterization of human cd200 glycoprotein receptor gene located on chromosome 3q12-13. *Gene.* 311:99-104.
110. Iwai,Y., M.Ishida, Y.Tanaka, T.Okazaki, T.Honjo, and N.Minato. 2002. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc. Natl. Acad. Sci. U. S. A.* 99:12293-12297.
111. Blank,C., T.F.Gajewski, and A.Mackensen. 2005. Interaction of PD-L1 on tumor cells with PD-1 on tumor-specific T cells as a mechanism of immune evasion: implications for tumor immunotherapy. *Cancer Immunol. Immunother.* 54:307-314.
112. McWhirter,J.R., A.Kretz-Rommel, A.Saven, T.Maruyama, K.N.Potter, C.I.Mockridge, E.P.Ravey, F.Qin, and K.S.Bowdish. 2006. Antibodies selected from combinatorial libraries block a tumor antigen that plays a key role in immunomodulation. *Proc. Natl. Acad. Sci. U. S. A.* 103:1041-1046.
113. Cowin,P., T.M.Rowlands, and S.J.Hatsell. 2005. Cadherins and catenins in breast cancer. *Curr. Opin. Cell Biol.* 17:499-508.
114. Parikka,M., T.Kainulainen, K.Tasanen, A.Vaananen, L.Bruckner-Tuderman, and T.Salo. 2003. Alterations of collagen XVII expression during transformation of oral epithelium to dysplasia and carcinoma. *J. Histochem. Cytochem.* 51:921-929.
115. Roepman,P., L.F.Wessels, N.Kettelarij, P.Kemmeren, A.J.Miles, P.Lijnzaad, M.G.Tilanus, R.Koole, G.J.Hordijk, P.C.van der Vliet, M.J.Reinders, P.J.Slootweg, and F.C.Holstege. 2005. An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nat. Genet.* 37:182-186.
116. Vaisanen,T., M.R.Vaisanen, H.Autio-Harmainen, and T.Pihlajaniemi. 2005. Type XIII collagen expression is induced during malignant transformation in various epithelial and mesenchymal tumours. *J. Pathol.* 207:324-335.
117. Iizasa,T., H.Chang, M.Suzuki, M.Otsuji, S.Yokoi, M.Chiyo, S.Motohashi, K.Yasufuku, Y.Sekine, A.Iyoda, K.Shibuya, K.Hiroshima, and T.Fujisawa. 2004. Overexpression of collagen XVIII is

associated with poor outcome and elevated levels of circulating serum endostatin in non-small cell lung cancer. *Clin. Cancer Res.* 10:5361-5366.

118. Nielsen,G.P., J.X.O'Connell, B.M.Wehrli, and A.E.Rosenberg. 2003. Collagen-rich tumors of soft tissues: an overview. *Adv. Anat. Pathol.* 10:179-199.
119. Banyard,J., L.Bao, and B.R.Zetter. 2003. Type XXIII collagen, a new transmembrane collagen identified in metastatic tumor cells. *J. Biol. Chem.* 278:20989-20994.
120. Kunath,T., C.Ordonez-Garcia, C.Turbide, and N.Beauchemin. 1995. Inhibition of colonic tumor cell growth by biliary glycoprotein. *Oncogene.* 11:2375-2382.
121. Hsieh,J.T., W.Luo, W.Song, Y.Wang, D.I.Kleinerman, N.T.Van, and S.H.Lin. 1995. Tumor suppressive role of an androgen-regulated epithelial cell adhesion molecule (C-CAM) in prostate carcinoma cell revealed by sense and antisense approaches. *Cancer Res.* 55:190-197.
122. Lodoen,M.B. and L.L.Lanier. 2005. Viral modulation of NK cell immunity. *Nat. Rev. Microbiol.* 3:59-69.
123. Langlais,C.L., J.M.Jones, R.D.Estep, and S.W.Wong. 2006. Rhesus rhadinovirus R15 encodes a functional homologue of human CD200. *J. Virol.* 80:3098-3103.
124. Cameron,C.M., J.W.Barrett, L.Liu, A.R.Lucas, and G.McFadden. 2005. Myxoma virus M141R expresses a viral CD200 (vOX-2) that is responsible for down-regulation of macrophage and T-cell activation in vivo. *J. Virol.* 79:6052-6067.
125. Voigt,S., G.R.Sandford, G.S.Hayward, and W.H.Burns. 2005. The English strain of rat cytomegalovirus (CMV) contains a novel captured CD200 (vOX2) gene and a spliced CC chemokine upstream from the major immediate-early region: further evidence for a separate evolutionary lineage from that of rat CMV Maastricht. *J. Gen. Virol.* 86:263-274.
126. Foster-Cuevas,M., G.J.Wright, M.J.Puklavec, M.H.Brown, and A.N.Barclay. 2004. Human herpesvirus 8 K14 protein mimics CD200 in down-regulating macrophage activation through CD200 receptor. *J. Virol.* 78:7667-7676.
127. Cameron,C., S.Hota-Mitchell, L.Chen, J.Barrett, J.X.Cao, C.Macaulay, D.Willer, D.Evans, and G.McFadden. 1999. The complete DNA sequence of myxoma virus. *Virology.* 264:298-318.
128. Campbell,I.G., P.S.Freemont, W.Foulkes, and J.Trowsdale. 1992. An ovarian tumor marker with homology to vaccinia virus contains an IgV-like region and multiple transmembrane domains. *Cancer Res.* 52:5416-5420.
129. Cameron,C.M., J.W.Barrett, M.Mann, A.Lucas, and G.McFadden. 2005. Myxoma virus M128L is expressed as a cell surface CD47-like virulence factor that contributes to the downregulation of macrophage activation in vivo. *Virology.* 337:55-67.
130. Cheung,T.C., I.R.Humphreys, K.G.Potter, P.S.Norris, H.M.Shumway, B.R.Tran, G.Patterson, R.Jean-Jacques, M.Yoon, P.G.Spear, K.M.Murphy, N.S.Lurain, C.A.Benedict, and C.F.Ware. 2005. Evolutionarily divergent herpesviruses modulate T cell activation by targeting the herpesvirus entry mediator cosignaling pathway. *Proc. Natl. Acad. Sci. U. S. A.* 102:13218-13223.
131. Muhlbauer,M., M.Fleck, C.Schutz, T.Weiss, M.Froh, C.Blank, J.Scholmerich, and C.Hellerbrand. 2006. PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. *J. Hepatol.* 45:520-528.
132. Stanciu,L.A., C.M.Bellettato, V.Laza-Stanca, A.J.Coyle, A.Papi, and S.L.Johnston. 2006. Expression of programmed death-1 ligand (PD-L) 1, PD-L2, B7-H3, and inducible costimulator ligand on human respiratory tract epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines. *J. Infect. Dis.* 193:404-412.
133. Smith,P., C.M.Walsh, N.E.Mangan, R.E.Fallon, J.R.Sayers, A.N.McKenzie, and P.G.Fallon. 2004. Schistosoma mansoni worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages. *J. Immunol.* 173:1240-1248.

134. Thorley-Lawson,D.A., R.T.Schooley, A.K.Bhan, and L.M.Nadler. 1982. Epstein-Barr virus superinduces a new human B cell differentiation antigen (B-LAST 1) expressed on transformed lymphoblasts. *Cell.* 30:415-425.
135. Short,J.J., A.V.Pereboev, Y.Kawakami, C.Vasu, M.J.Holterman, and D.T.Curiel. 2004. Adenovirus serotype 3 utilizes CD80 (B7.1) and CD86 (B7.2) as cellular attachment receptors. *Virology.* 322:349-359.
136. Boulton,I.C. and S.D.Gray-Owen. 2002. Neisserial binding to CEACAM1 arrests the activation and proliferation of CD4+ T lymphocytes. *Nat. Immunol. Feb.* 3:229-236.
137. Iijima,H., M.F.Neurath, T.Nagaishi, J.N.Glickman, E.E.Nieuwenhuis, A.Nakajima, D.Chen, I.J.Fuss, N.Utku, D.N.Lewicki, C.Becker, T.M.Gallagher, K.V.Holmes, and R.S.Blumberg. 2004. Specific regulation of T helper cell 1-mediated murine colitis by CEACAM1. *J. Exp. Med. 2004.* 199:471-482.
138. Pritchard,N.R. and K.G.Smith. 2003. B cell inhibitory receptors and autoimmunity. *Immunology* 108:263-273.
139. Nishimura,H., M.Nose, H.Hiai, N.Minato, and T.Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity.* 11:141-151.
140. Kyogoku,C., H.M.Dijstelbloem, N.Tsuchiya, Y.Hatta, H.Kato, A.Yamaguchi, T.Fukazawa, M.D.Jansen, H.Hashimoto, J.G.Van De Winkel, C.G.Kallenberg, and K.Tokunaga. 2002. Fcgamma receptor gene polymorphisms in Japanese patients with systemic lupus erythematosus: contribution of FCGR2B to genetic susceptibility. *Arthritis Rheum.* 46:1242-1254.
141. Morgan,A.W., J.H.Barrett, B.Griffiths, D.Subramanian, J.I.Robinson, V.H.Keyte, M.Ali, E.A.Jones, R.W.Old, F.Ponchel, A.W.Boylston, R.D.Situnayake, A.F.Markham, P.Emery, and J.D.Isaacs. 2005. Analysis of Fcgamma receptor haplotypes in rheumatoid arthritis: FCGR3A remains a major susceptibility gene at this locus, with an additional contribution from FCGR3B. *Arthritis Res. Ther.* 8:R5.
142. Moser,K.L., B.R.Neas, J.E.Salmon, H.Yu, C.Gray-McGuire, N.Asundi, G.R.Bruner, J.Fox, J.Kelly, S.Henshall, D.Bacino, M.Dietz, R.Hogue, G.Koelsch, L.Nightingale, T.Shaver, N.I.Abdou, D.A.Albert, C.Carson, M.Petri, E.L.Treadwell, J.A.James, and J.B.Harley. 1998. Genome scan of human systemic lupus erythematosus: evidence for linkage on chromosome 1q in African-American pedigrees. *Proc. Natl. Acad. Sci. U. S. A.* 95:14869-14874.
143. Lindberg,F.P., H.D.Gresham, E.Schwarz, and E.J.Brown. 1993. Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane-spanning domains implicated in alpha v beta 3-dependent ligand binding. *J. Cell Biol.* 123:485-496.
144. Costello,R.T., S.Sivori, F.Mallet, D.Sainty, C.Arnoulet, D.Reviron, J.A.Gastaut, A.Moretta, and D.Olive. 2002. A novel mechanism of antitumor response involving the expansion of CD3+/CD56+ large granular lymphocytes triggered by a tumor-expressed activating ligand. *Leukemia.* 16:855-860.
145. Sun,H., B.J.Norris, K.Atkinson, J.C.Biggs, and G.M.Smith. 1998. Preclinical antitumor activity of an antibody against the leukocyte antigen CD48. *Clin. Cancer Res.* 4:895-900.
146. Basile,J.R., R.M.Castilho, V.P.Williams, and J.S.Gutkind. 2006. Semaphorin 4D provides a link between axon guidance processes and tumor-induced angiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 103:9017-9022.
147. Ito,A., K.Handa, D.A.Withers, M.Satoh, and S.Hakomori. 2001. Binding specificity of siglec7 to disialogangliosides of renal cell carcinoma: possible role of disialogangliosides in tumor progression. *FEBS Lett.* 504:82-86.
148. Urmacher,C., C.Cordon-Cardo, and A.N.Houghton. 1989. Tissue distribution of GD3 ganglioside detected by mouse monoclonal antibody R24. *Am. J. Dermatopathol.* 11:577-581.

CHAPTER 7

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

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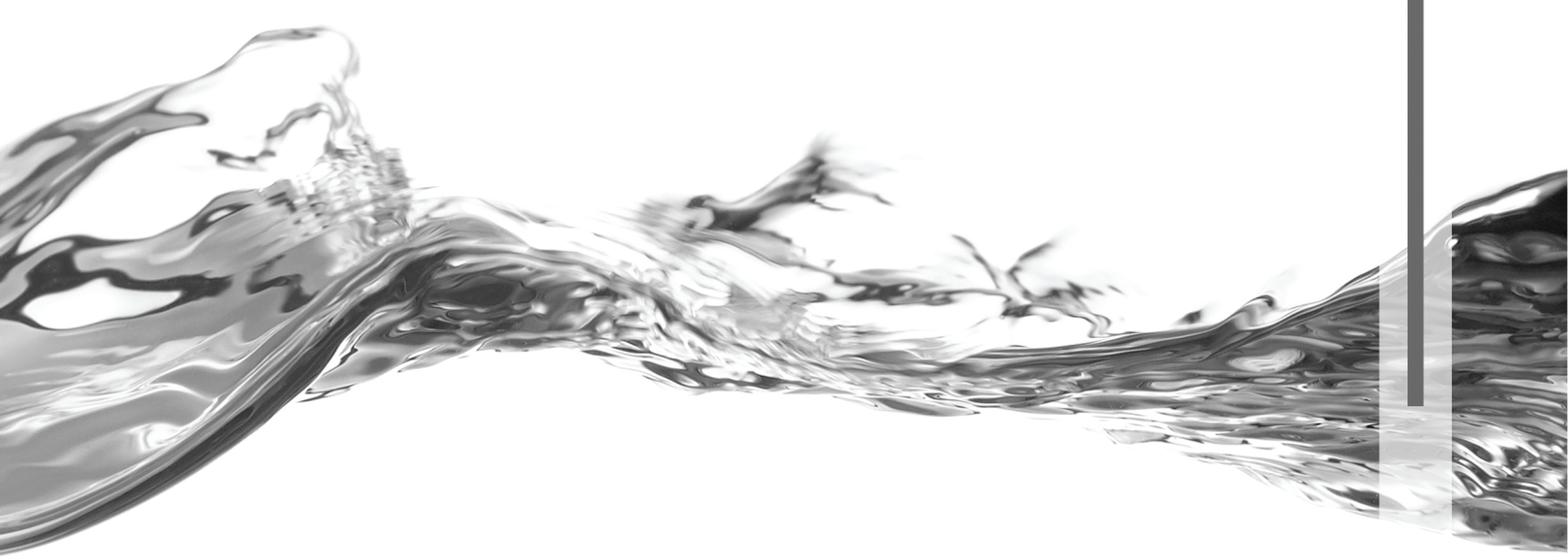

ABSTRACT

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 LAIR-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, mouse LAIR-1 is inducibly expressed on blood granulocytes *in vivo* and is downregulated upon T cell activation *in vitro*. Unexpectedly, mouse LAIR-1 was not expressed on splenic and blood $B220^+$ B cells. Similar to its human homolog, mouse LAIR-1 interacted with high affinity with a wide range of collagen molecules. Furthermore, mouse LAIR-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*. Given the similarities between the mouse and human receptors, mouse LAIR-1 may serve as a good model to assess the role of the LAIR-1 receptors in regulation of immune responses.

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 knock-out 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 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 of 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 immunoglobulin-like receptor (LAIR)-1 (CD305) is a member of the immunoglobulin superfamily (IgSF), which is expressed on the majority of peripheral blood mononuclear cells and thymocytes[5;6]. Cross-linking of human (h) LAIR-1 by monoclonal antibodies (mAb) *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 precursors[11]. Recently we identified orthologs of 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 RT-PCR analysis indicates that both proteins have a similar expression profile[12]. Furthermore, the mouse LAIR-1 cytoplasmic tail can be phosphorylated thereby recruiting SH2-containing tyrosine phosphatase-2 (SHP-2)[12] and C-terminal Src kinase (Csk)[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 $(Gly\text{-}X\text{-}Y)_n$, 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 human LAIR-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 human LAIR-1 and collagens 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 monoclonal antibodies and study its interaction with its natural ligand collagen.

MATERIALS AND METHODS

Cells, transfectant, and cDNA

Cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured using standard techniques. Cell lines used in this study: D011.10 and B097.10 mouse T cell lines, human embryonic kidney 293T cells, mouse SP2/0 B cells, and human erythroleukemia 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 dendritic cells (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-eco 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, 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 phycoerythrin (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 (Cricetulus migratorius; Cytogen, West Roxbury, MA) was injected i.p. with an ARHO12 clone stably expressing mLAIR-1 fused to a C-terminal MYC-tag[12]. Five injections with 10^7 irradiated cells (50 Gray) in PBS were given at weekly intervals. Two weeks after the fifth injection, the hamster was boosted i.p. with 10^7 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 subcloned 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 and peripheral blood of wild-type BALB/c mice. To increase circulating numbers of granulocytes, when indicated, BALB/c mice were injected s.c. with saline or 20 μg/mouse (150 μl/mouse in PBS) PEG-modified recombinant human 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), 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 25μg/ml 2.4G2 mAb (anti-CD16/CD32; PharMingen). Allophycocyanine (APC)-conjugated streptavidin (BD Biosciences) and R-phycoerythrin (RPE)-conjugated goat anti-Armenian hamster Ig (BD Biosciences) were used as secondary detecting reagents.

Surface plasmon resonance experiments

Surface plasmon resonance (BIAcore) binding studies were performed by using a BIAcore2000 system (BIAcore AB, Uppsala, Sweden). Approximately 2000-3000 response units (RU) 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$(GPO)_{10}$GCOG-NH2 ($(GPO)_{10}$, also known as collagen-related peptide (CRP) and GCP$(GPP)_{10}$GCPG-NH2 ($(GPP)_{10}$) were described previously[20] and were kindly provided by Prof. 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 for collagen I and III interaction-studies and 5 μl/min for the immobilized peptides. Binding of mLAIR-1-IgG to collagen I and III was specific, because nonspecific binding to an uncoated control channel was less than 1% compared to 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).

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 Biaevaluation 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. 1.25 μg/ml anti-mouse CD3 (PharMingen, San Diego, CA) was coated overnight at 4°C in 96-well MAXIsorp flat-bottom plates (Nunc) together with 10 μg/ml purified collagens I, III, BSA, $(GPO)_{10}$, $(GPP)_{10}$ or anti-mLAIR-1 Abs (113) in a total volume of 100 μl/well. The next day, 200 μl of $2.5x10^5$ reporter cells/ml in medium were added to each well, and plates were incubated at 37°C for 22 hrs and then analyzed for GFP expression 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 ARHO12 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 ARHO12 cells stably expressing mLAIR-1 (Figure 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 a ~46 kDa monomer when analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) under both non-reducing and reducing conditions (Figure 1E), whereas no specific mLAIR-1 molecule was precipitated from mouse Ba/F3, B16.F10, or IIA1.6 cell lines (Figure 1E and data not shown). Mouse LAIR-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, a proteolytic cleavage-product, or non-glycosylated protein is unknown.

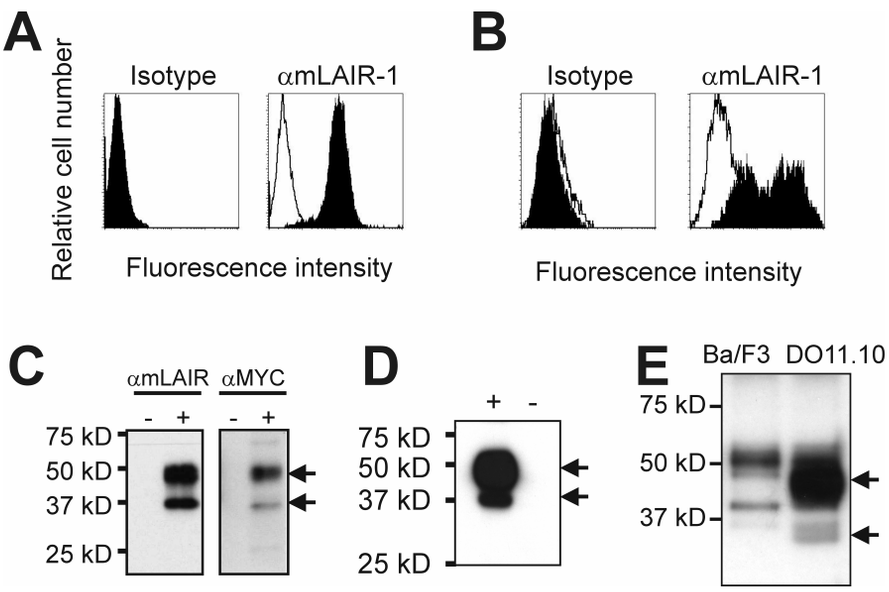

Figure 1: Generation of anti-mLAIR-1 mAbs. (A) Flow cytometric analysis of hybridoma supernatants. Untransfected ARHO12 cells (open histograms) and ARHO12 cells stably expressing MYC-tagged mLAIR-1 (closed histograms) were stained with the supernatant of hybridoma 113 (right panel) or Armenian hamster isotype control IgG (left panel) and visualized by phycoerythrin (PE)–conjugated goat anti-Armenian hamster IgG. (B) 293T cells transiently transfected with control cDNA (open histogram) 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 phycoerythrin (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) Mouse LAIR-1 is endogenously expressed as a ~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.

Expression of mLAIR-1

Human LAIR-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 dendritic cells express hLAIR-1, where it plays a role in the 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 both splenocytes and peripheral blood cells, mLAIR-1 was expressed on $CD3^+CD4^+$, $CD3^+CD8^+$ 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 dendritic cells (Fig. 2B).
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-1^{dim} granulocytes expressed no or low amounts of mLAIR-1, and mature GR-1^{bright} 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 upregulation of mLAIR-1 in both immature GR-1^{dim} and mature GR-1^{bright} granulocytes (Fig. 2C), indicating that mLAIR-1 expression is regulated on this cell type.

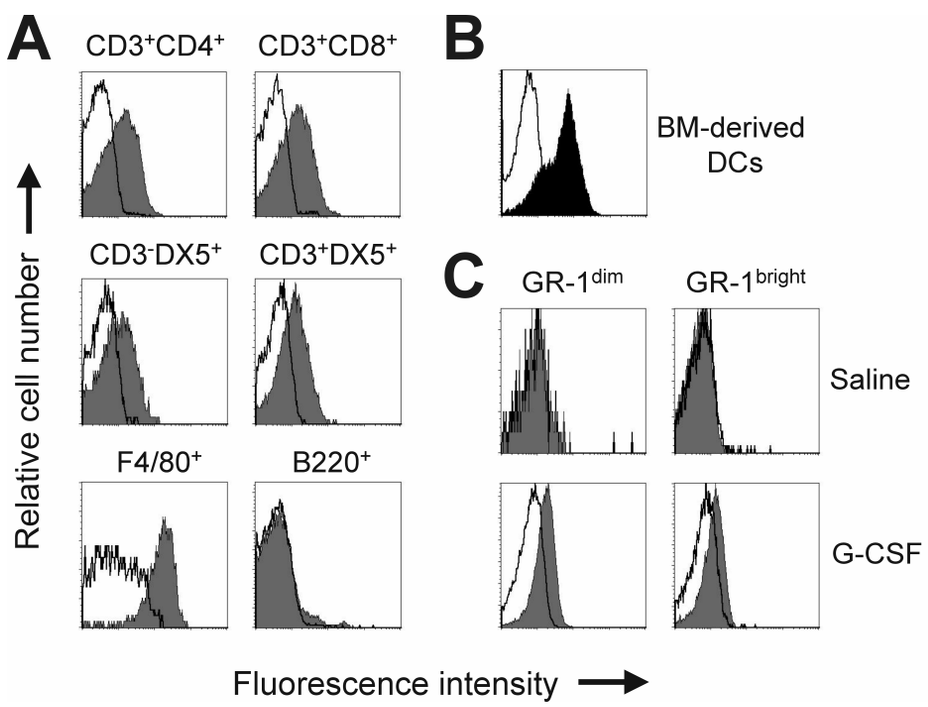

Figure 2: Mouse LAIR-1 is expressed on various immune cell types. (A) Staining of BALB/c splenocytes using the 113 mAb (closed histograms) or Armenian hamster isotype control IgG (open histograms) followed by phycoerythrin (PE)–conjugated goat anti-Armenian hamster IgG as detecting antibody. T cells (CD3, CD4, CD8), B cells (B220), NK cells (DX5), and monocytes (F4/80) are shown based on gating using the indicated cell-type specific antibodies and gating on forward and side light scatter. (B) Mouse bone-marrow derived dendritic cells (BALB/c) were stained with biotinylated anti-mLAIR-1 mAb (113, closed histogram) or biotinylated Armenian hamster isotype control IgG (open histogram) and visualized by PE-conjugated streptavidin. The data shown are representative of three independent experiments. (C) Peripheral blood cells from BALB/c mice were gated on granulocytes based on forward and side light scatter and GR-1 expression and analyzed for mLAIR-1 expression by using biotinylated anti-mLAIR-1 mAbs (closed histograms) or biotinylated isotype control IgG (open histograms). Mature GR-1^{bright} (right panels) and immature GR-1^{dim} (left panels) granulocytes of peripheral blood of mice that were injected with 20 μg PEG-modified G-CSF (bottom panels) or saline (top panels) are shown.

To study the expression of mLAIR-1 upon activation of T cells, we stimulated B097.10 and DO11.10 T cell lines with phorbol 12-myristate 13-acetate (PMA) and ionomycin and studied mLAIR-1 expression. CD69 upregulation was used as a positive control for activation. Mouse LAIR-1 expression was downregulated upon T cell activation (Fig 3). 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 regulated on various immune cell types.

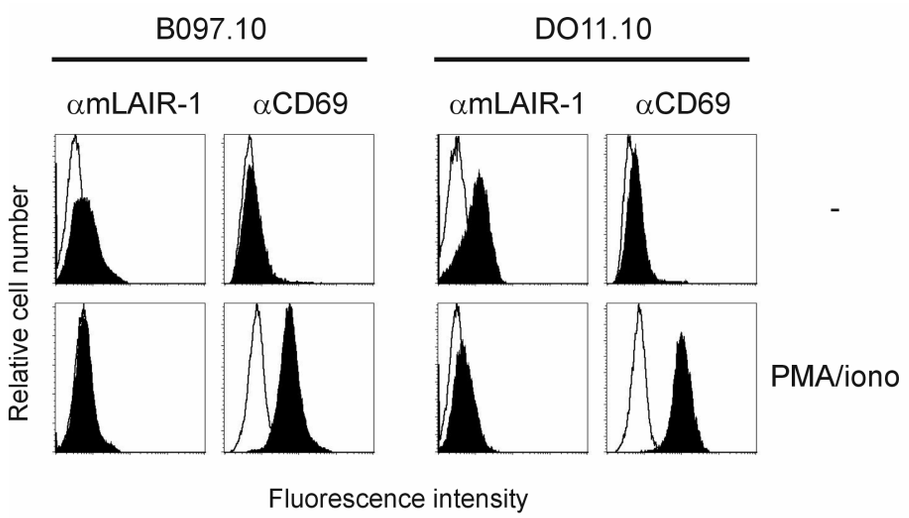

Figure 3: Mouse LAIR-1 expression is downregulated on T-cell lines upon stimulation. B097.10 and DO11.10 T-cell lines were cultured for 4 days in the absence or presence of PMA and ionomycin and subsequently stained using Armenian hamster isotype control IgG (open histograms) or anti-mLAIR-1 mAbs (113, closed histograms). Staining was visualized by phycoerythrin (PE)–conjugated goat anti-Armenian hamster IgG. The activation status of the cells was assessed by using anti-mouse CD69 mAbs (closed histograms) and isotype matched control IgG (open histograms). Results are representative of two experiments.

Mouse LAIR-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 XIII, 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-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. Mouse LAIR-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[15] and is relatively slow with a rapid initial phase and a slower secondary phase. A single triple-helical collagen I or III molecule interacted with approximately 9.7 and 8.9 mLAIR-1 proteins respectively (Fig. 4B), a value that is comparable to hLAIR-1 interacting with human collagens I and III[15].

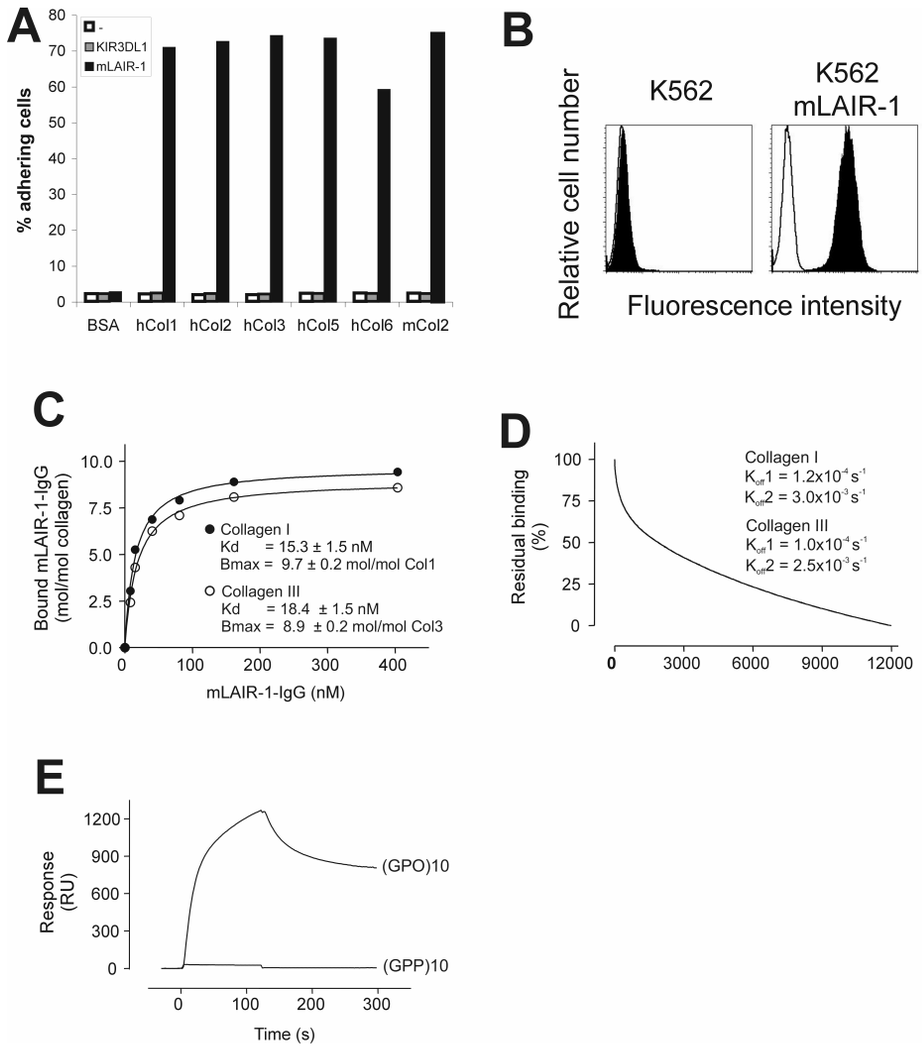

Figure 4. Mouse LAIR-1 binds with high affinity to collagen molecules. (A) Fluorescently labeled wt K562 cells (open bars) and K562 cells expressing mLAIR-1 (black bars) or KIR3DL1 (gray bars) were monitored for their capacity to bind immobilized human collagens I, II, III, IV, and VI and mouse collagen II. Percentage of adhering cells relative to input is shown. One of three independent experiments is shown. (B) Parental K562 cells (left panel) and K562 cells stably expressing mLAIR-1 (right panel) were either left untreated (open histogram) or incubated with 50 μg/ml Oregon Green 488-labeled human collagen IV (closed histograms) and analyzed by flow cytometry. (C) The indicated concentrations mLAIR-1-IgG were injected at 20 μl/min sequentially through a BIAcore flow cell containing ~2000-3000 RU of directly immobilized collagen I (○), III (□) or nothing. Each symbol represents the resonance unit at equilibrium and the corresponding concentration of the fusion protein; these data were used to determine the indicated Kd-values. Isotype-matched control fusion-proteins did not associate with collagen. (D) Rate of dissociation of mLAIR-1-IgG from collagen I and III as monitored by surface plasmon resonance. (E) Mouse LAIR-1 binds immobilized $(GPO)_{10}$, but not $(GPP)_{10}$. mLAIR-1-IgG (480 nM) was injected at 5 μl/min through a BIAcore flow cell containing ~250 RU of immobilized $(GPO)_{10}$ or $(GPP)_{10}$.

Like hLAIR-1, mLAIR-1 binds common collagen motifs in a hydroxyproline-dependent manner, since it interacts with trimeric peptides containing 10 repeated GPO triplets ($(GPO)_{10}$[20]), but not with the control $(GPP)_{10}$ trimeric peptide (Fig. 4E). Interestingly, whereas the affinity of human and mLAIR-1 for collagens I and III is similar, mLAIR-1 binds better to $(GPO)_{10}$ than does hLAIR-1[15].

Collagen inhibits CD3-induced T cell stimulation 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 mLAIR-1 fused to the transmembrane and intracellular domain of CD3ζ. 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, $(GPO)_{10}$, but not $(GPP)_{10}$ trimeric peptides, resulted in triggering of the receptor (Fig. 5B). Collagens I, III and $(GPO)_{10}$ trimeric peptides are thus capable of cross-linking mLAIR-1.

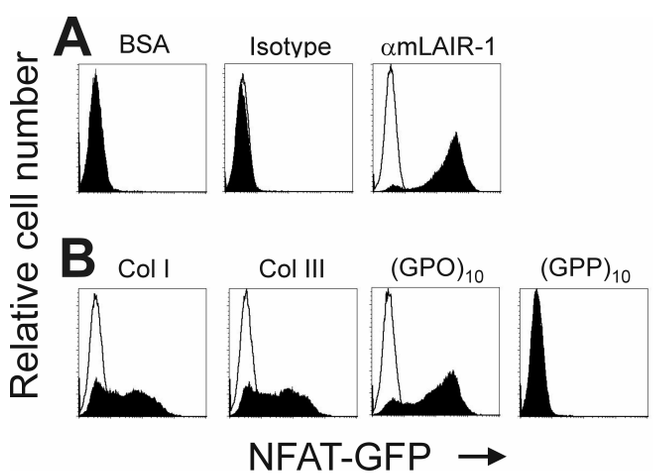

Figure 5: Collagens directly cross-link mouse LAIR-1. (A) Untransfected (open histograms) or mLAIR-1-CD3ζ chimera transfected (closed histograms) NFAT-GFP reporter cells were incubated with immobilized BSA, anti-mLAIR-1 mAbs (113) or isotype control IgG mAbs for 22 hours and GFP expression was analyzed by flow cytometry. (B) Untransfected (open histograms) or mLAIR-1-CD3ζ chimera transfected (closed histograms) NFAT-GFP reporter cells were incubated with immobilized collagens I, III, $(GPO)_{10}$, or $(GPP)_{10}$ for 22 hours and GFP expression was analyzed by flow cytometry. Maximal stimulation in these reporter cell assays using trimeric collagen peptides typically resulted at most in ~70% GFP^+ cells.

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. 6). 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 6). 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 6). Hence, collagens are able to down modulate mouse CD3 signaling via mLAIR-1 and thus are potential inhibitors of T-cell responses *in vivo*.

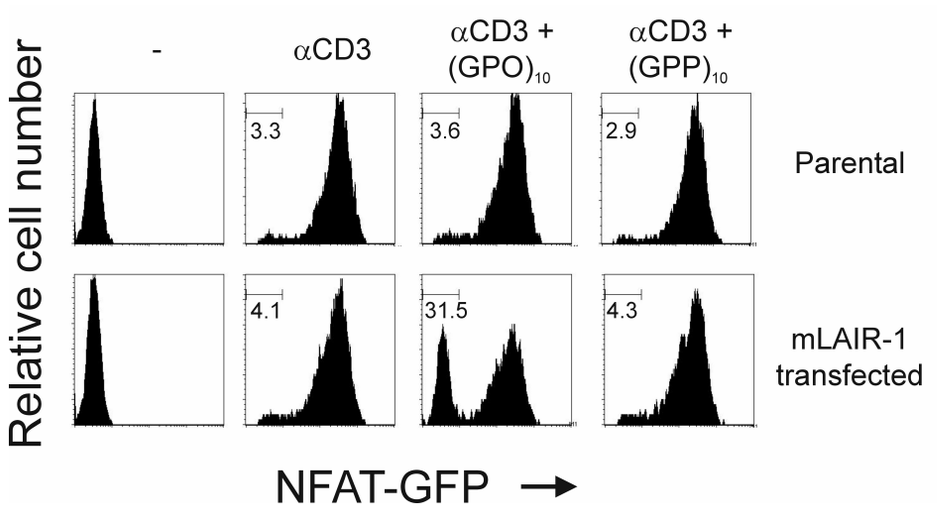

Figure 6: Mouse LAIR-1/collagen interaction directly inhibits CD3-signaling in mouse T cell hybridomas. Untransfected (top panels) or wild-type mLAIR-1 transfected (bottom panels) NFAT-GFP reporter cells were incubated with immobilized anti-CD3 mAbs in the presence or absence of immobilized trimeric peptides $(GPO)_{10}$ or $(GPP)_{10}$ for 22 hours 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.

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, 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 monoclonal antibodies.

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 dendritic cells, 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, may establish a threshold for DC activation.

In vivo administration of G-CSF in wild-type C57BL/6J and BALB/c mice resulted in upregulation of mLAIR-1 expression on $GR1^{bright}$ and $GR1^{dimm}$ 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.

We found that mLAIR-1 was more abundantly expressed on immune cells from the BALB/c mouse strain as compared to C57/BL6J strain (data not shown). This inter strain variability might be caused by an allelic difference among the used BALB/c and C57/BL6J strains resulting in decreased reactivity of the used antibody, a phenomenon previously described for the NK1.1 antigen[26].

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;27;28] 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 human LAIR-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 mouse LAIR-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 Immunoglobulin Superfamily (IgSF) and is encoded in the Leukocyte Receptor Complex (LRC) on human chromosome 19[29]. 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 FcεRI expressed on 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, indicating that this cell type may be subject to regulation via LAIR-1 *in vivo* as well. Of note, although all reporter cells stably expressed 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.

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 mLAIR-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.

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REFERENCES

1. Lanier,L.L. 2005. NK Cell Recognition. *Annu. Rev. Immunol.*225-274.
2. Ravetch,J.V. and L.L.Lanier. 2000. Immune inhibitory receptors. *Science* 290:84-89.
3. Pritchard,N.R. and K.G.Smith. 2003. B cell inhibitory receptors and autoimmunity. *Immunology* 108:263-273.
4. Vilches,C. and P.Parham. 2002. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu. Rev. Immunol.* 20:217-251.
5. Meyaard,L., G.J.Adema, C.Chang, E.Woollatt, G.R.Sutherland, L.L.Lanier, and J.H.Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
6. Poggi,A., N.Pella, L.Morelli, F.Spada, V.Revello, S.Sivori, R.Augugliaro, L.Moretta, and A.Moretta. 1995. p40, a novel surface molecule involved in the regulation of the non-major histocompatibility complex-restricted cytolytic activity in humans. *Eur. J. Immunol.* 25:369-376.
7. Meyaard,L., J.Hurenkamp, H.Clevers, L.L.Lanier, and J.H.Phillips. 1999. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. *J. Immunol.* 162:5800-5804.
8. Saverino,D., M.Fabbi, A.Merlo, G.Ravera, C.E.Grossi, and E.Ciccone. 2002. Surface density expression of the leukocyte-associated Ig-like receptor-1 is directly related to inhibition of human T-cell functions. *Hum. Immunol.* 63:534-546.
9. Maasho,K., M.Masilamani, R.Valas, S.Basu, J.E.Coligan, and F.Borrego. 2005. The inhibitory leukocyte-associated Ig-like receptor-1 (LAIR-1) is expressed at high levels by human naive T cells and inhibits TCR mediated activation. *Mol. Immunol.* 42:1521-1530.
10. van der Vuurst de Vries AR, H.Clevers, T.Logtenberg, and L.Meyaard. 1999. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur. J. Immunol.* 29:3160-3167.
11. Poggi,A., E.Tomasello, E.Ferrero, M.R.Zocchi, and L.Moretta. 1998. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. *Eur. J. Immunol.* 28:2086-2091.
12. Lebbink,R.J., T.de Ruiter, A.Verbrugge, W.S.Bril, and L.Meyaard. 2004. The mouse homologue of the leukocyte-associated Ig-like receptor-1 is an inhibitory receptor that recruits Src homology region 2-containing protein tyrosine phosphatase (SHP)-2, but not SHP-1. *J. Immunol.* 172:5535-5543.
13. Lebbink,R.J., T.de Ruiter, G.J.Kaptijn, and L.Meyaard. 2005. Identification and characterization of the rat homologue of LAIR-1. *Immunogenetics* 57:344-351.
14. Verbrugge,A., E.S.Rijkers, T.de Ruiter, and L.Meyaard. 2006. Leukocyte-associated Ig-like receptor-1 has SH2 domain-containing phosphatase-independent function and recruits C-terminal Src kinase. *Eur. J. Immunol.* 36:190-198.
15. Lebbink,R.J., T.de Ruiter, J.Adelmeijer, A.B.Brenkman, J.M.van Helvoort, M.Koch, R.W.Farndale, T.Lisman, A.Sonnenberg, P.J.Lenting, and L.Meyaard. 2006. Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. *J. Exp. Med.* 203:1419-1425.
16. Myllyharju,J. and K.I.Kivirikko. 2004. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet.* 20:33-43.
17. Ricard-Blum,S. and F.Ruggiero. 2005. The collagen superfamily: from the extracellular matrix to the cell membrane. *Pathol. Biol. (Paris)* 53:430-442.
18. Naviaux,R.K., E.Costanzi, M.Haas, and I.M.Verma. 1996. The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *J. Virol.* 70:5701-5705.
19. Gravestein,L.A., J.D.Nieland, A.M.Kruisbeek, and J.Borst. 1995. Novel mAbs reveal potent co-stimulatory activity of murine CD27. *Int. Immunol.* 7:551-557.

20. Knight,C.G., L.F.Morton, D.J.Onley, A.R.Peachey, T.Ichinohe, M.Okuma, R.W.Farndale, and M.J.Barnes. 1999. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. *Cardiovasc. Res.* 41:450-457.
21. Verbrugge,A., T.de Ruiter, C.Geest, P.J.Coffer, and L.Meyaard. 2006. Differential expression of leukocyte-associated Ig-like receptor-1 during neutrophil differentiation and activation. *J. Leukoc. Biol.* 79:828-836.
22. Pojda,Z., G.Molineux, and T.M.Dexter. 1990. Hemopoietic effects of short-term in vivo treatment of mice with various doses of rhG-CSF. *Exp. Hematol.* 18:27-31.
23. Voehringer,D., D.B.Rosen, L.L.Lanier, and R.M.Locksley. 2004. CD200 receptor family members represent novel DAP12-associated activating receptors on basophils and mast cells. *J. Biol. Chem.* 279:54117-54123.
24. Liu,Y.J., H.Kanzler, V.Soumelis, and M.Gilliet. 2001. Dendritic cell lineage, plasticity and cross-regulation. *Nat. Immunol.* 2:585-589.
25. Shi,L., K.Luo, D.Xia, T.Chen, G.Chen, Y.Jiang, N.Li, and X.Cao. 2006. DIgR2, dendritic cell-derived immunoglobulin receptor 2, is one representative of a family of IgSF inhibitory receptors and mediates negative regulation of dendritic cell-initiated antigen-specific T cell responses. *Blood.*
26. Carlyle,J.R., A.Mesci, B.Ljutic, S.Belanger, L.H.Tai, E.Rousselle, A.D.Troke, M.F.Proteau, and A.P.Makrigiannis. 2006. Molecular and genetic basis for strain-dependent NK1.1 alloreactivity of mouse NK cells. *J. Immunol.* 176:7511-7524.
27. Canavez,F., N.T.Young, L.A.Guethlein, R.Rajalingam, S.I.Khakoo, B.P.Shum, and P.Parham. 2001. Comparison of chimpanzee and human leukocyte Ig-like receptor genes reveals framework and rapidly evolving genes. *J. Immunol.* 167:5786-5794.
28. Wende,H., A.Volz, and A.Ziegler. 2000. Extensive gene duplications and a large inversion characterize the human leukocyte receptor cluster. *Immunogenetics* 51:703-713.
29. Clemetson,J.M., J.Polgar, E.Magnenat, T.N.Wells, and K.J.Clemetson. 1999. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors. *J. Biol. Chem.* 274:29019-29024.

8
CHAPTER

Leukocyte-associated immunoglobulin-like receptor-2 (LAIR-2) is a soluble competitor of the collagen/LAIR-1 inhibitory immune interaction

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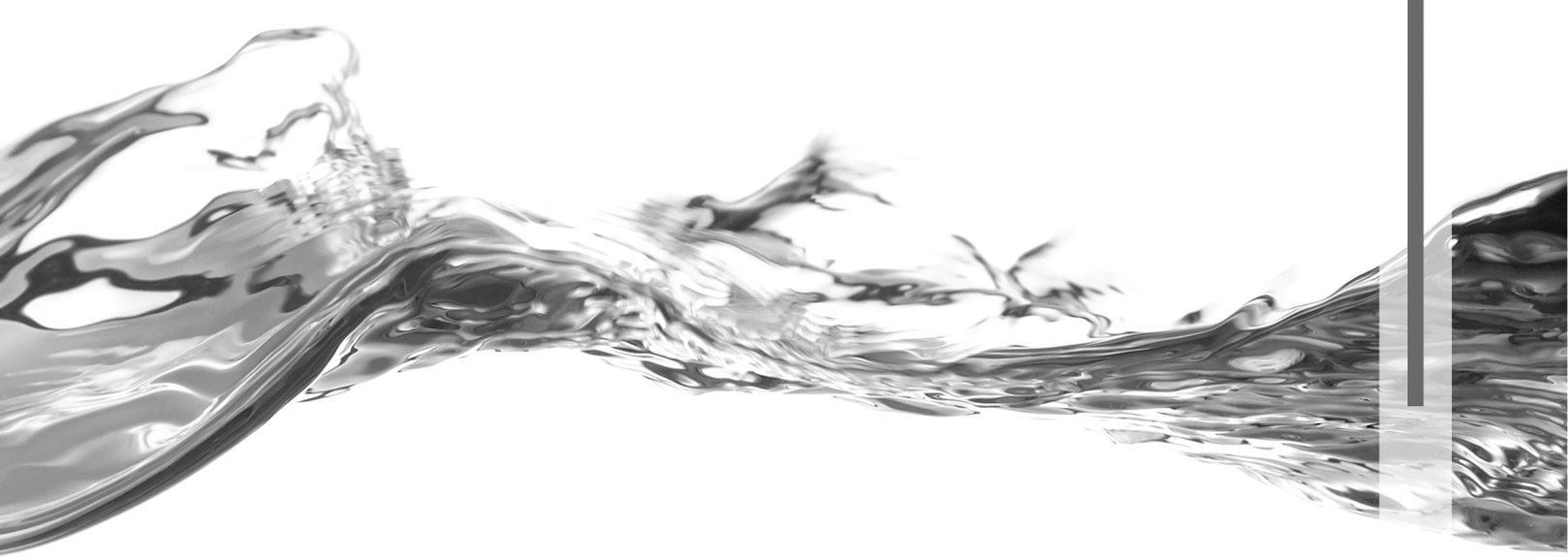

ABSTRACT

Leukocyte-associated Ig-like receptor-1 is a collagen-receptor that inhibits immune cell function upon collagen binding. LAIR-2, a putative soluble homologue, may function as a regulator of this interaction. For the first time we show that the *LAIR-2* gene was broadly transcribed in human peripheral blood mononuclear cells, mirroring the expression profile of LAIR-1. LAIR-2 is expressed as a soluble receptor exhibiting high affinity for various collagen molecules to which it binds in a hydroxyproline-dependent manner. We detected 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 were increased in synovial fluid (SF) of patients with rheumatoid arthritis (RA) as compared to osteoarthritis (OA) patients. We hypothesized that soluble LAIR-2 may function as a natural competitor for LAIR-1, thereby regulating its inhibitory potential. Indeed, LAIR-2 prevented binding of human LAIR-1 to collagens *in vitro*, suggesting that the protein has an immuno-regulatory 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.

INTRODUCTION

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 immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails, essential for the inhibitory signal[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[3-6]. 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 generated via proteolytic cleavage of the receptors' ectodomains or alternative splicing of mRNA transcripts[7]. 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 [8])

The leukocyte-associated Ig-like receptor-1 (LAIR-1, CD305) is an ITIM-containing inhibitory immune receptor, belonging to the Ig superfamily (IgSF)[9]. Human (h) LAIR-1 is expressed on the majority of peripheral blood mononuclear cells and thymocytes[4;9-11]. Cross-linking of hLAIR-1 by monoclonal antibodies (mAb) *in vitro* delivers a potent inhibitory signal that is capable of inhibiting immune cell function[4;9-14]. Recently, we identified collagens as natural, high affinity ligands for the LAIR molecules[15]. 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[15].

Given the broad expression profile of LAIR-1 on immune cells[9] and the high abundance of collagen molecules in the human body[16], a fine-tuned regulation of the collagen-LAIR interaction is needed to allow proper inhibitory signaling of the LAIR-1 receptor. One level of regulation occurs by modulating LAIR-1 expression at different stages of differentiation/activation of immune cells, as was demonstrated for B cells[11], T cells[4;17], neutrophils[18], and dendritic cells (our own unpublished observations). Furthermore, LAIR-1 can be shed from immune cells upon cellular activation[19]. Another system to regulate the interaction between collagens and LAIR-1 may involve the putative secreted homologue LAIR-2 (CD306). LAIR-2 is encoded by a gene located in the vicinity of the *LAIR-1* gene in the leukocyte receptor complex (LCR) on human chromosome 19q13.4[9;20]. The predicted LAIR-2 protein has a single Ig-like domain sharing 84% sequence homology with hLAIR-1[9], and the molecule lacks a transmembrane and cytoplasmic region suggesting it is a secreted protein[9]. In contrast to hLAIR-1, LAIR-2

has no orthologues in mice or rats[20;21]. 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 LAIR-1. Our data reveal a novel mechanism of immune regulation by the soluble receptor LAIR-2 regulating the inhibitory potential of the membrane-bound LAIR-1 via competing 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. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood from healthy donors by Ficoll-Hypaque centrifugation. K562 cells and 2B4 NFAT-GFP T cell reporter cells (kindly provided by L.L. Lanier and H. Arase (USCF, USA)) were stably transfected with hLAIR-1 or hLAIR-1a-CD3ζ respectively as described previously[15].

Subjects

For LAIR-2 measurements in urine: 16 healthy individuals (10 males and 6 females) and 6 healthy pregnant women (16-37 weeks 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 out of 16 RA patients were positive for rheumatoid factor. Samples were subjected to centrifugation to remove cells prior storage at -80°C. Approval to use synovial fluid obtained upon joint aspiration was given by the medical ethical committee of the UMC Utrecht.

Antibodies

The 1A7 and 2H12 IgG1 hLAIR-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, Freiburg, Germany). Biotinylated anti-human LAIR2 antibody was obtained from R&D systems (BAF2665).

RT PCR

Total RNA was isolated from several human cell lines using the TRIzol method according to the manufacturer's instructions (Invitrogen). Total RNA was converted to first-strand DNA with oligo(dT)$_{20}$ primers and M-MLV reverse transcriptase using the SuperScript™ III First- Strand Synthesis System for RT-PCR (Invitrogen). The cDNA mixtures were amplified by PCR using hLAIR-2 specific forward (5'-GTTGGGGTTCAAACATTCCG-3') and reverse (5'-TCATGGTGCATCAAATCCGG-3') primers and the AmpliTaq Gold DNA polymerase system (PE 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'-GCATCCTGGGCTACACTGAGC-3') primers.

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

Western blot analysis

293T cells were transiently transfected with a vector encoding hLAIR-2b, 48 hours after transfection the supernatant was collected and separated by SDS-polyacrylamide gel electrophoresis (12% gel) under reducing conditions and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blot analysis was performed with 1A7 anti-LAIR-2 mAbs, followed by peroxidase-conjugated rabbit anti mouse mAb (DAKO) as secondary antibody. Proteins were detected by enhanced chemiluminescence (GE, UK).

Surface plasmon resonance

The surface plasmon resonance binding studies were performed as described before[15]. Briefly; approximately 2000-3000 response units of acid-soluble human collagen type I or III (Sigma) were immobilized on a CM5 biosensor chip using a amine coupling kit and approximately 250 response units of (GPP)$_{10}$ or (GPO)$_{10}$ peptide trimers (described previously (11)) were immobilized using a cysteine coupling kit, both according to the manufacturer's instructions. Analysis was performed at 25°C at flow rate of 20 µl/min for collagen I and III and at 5 µl/min for the peptide trimers. hLAIR-2-IgG dimer concentration was calculated based on a theoretical mass of 82.5 kDa. Increased concentrations of hLAIR-2-IgG were injected and allowed to reach equilibrium for 10 minutes. In the peptide binding studies, the biosensor chips were regenerated by injection of 0.1 M H3PO4 (5 µl/min, 2 min). Dissociation constants (Kd) and Bmax were calculated as described using 82.5 kDa as the theoretical mass of hLAIR-2-IgG and for collagen I and III, 416.7 and 415.7 kDa respectively.

Flow cytometry

For intracellular hLAIR-2 staining, the Zenon Mouse IgG Labeling Kit (Molecular Probes) was used according to the manufacturer's instructions. Cells were analyzed by flow cytometry (FACSCaliber™, BD).

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

For fusion protein blocking studies, chimeric proteins of the extracellular domain of hLAIR-1 or hLAIR-2 fused to the Fc region of human IgG1 were prepared as described previously[20]. HT29 cells were pre-treated with 10% normal mouse serum to block aspecific interactions. Subsequently, HT29 cells were incubated with unlabeled hLAIR-1-IgG or hLAIR-2-IgG for 10 minutes at room temperature and subsequently incubated with biotinylated hLAIR-1-IgG or hLAIR-2-IgG for 30 minutes at room temperature. Binding of biotinylated proteins was detected with allophycocyanin (APC) conjungated streptavidin (BD Biosciences, San Diego, CA) and cells were analyzed by flow cytometry (FACSCaliber™, BD).

Binding of K562 transfectants to plate-bound collagen

As described before[15], 96-well MAXIsorp flat-bottom plates (Nunc) were coated overnight at 4°C with purified collagens I or III (Sigma, 2 and 5 μg/ml respectively) or BSA (5 μg/ml) in 100μl PBS, supplemented with 2mM acetic acid. $5x10^6$ cells/ml wild-type K562 or K562 stably transfected with hLAIR-1 were fluorescently labeled with 5 μM calceine AM (Molecular Probes) in PBS for 30 minutes at 37°C. Meanwhile, after washings, the wells were incubated for 30 min with different concentrations hLAIR-2-IgG or wt LAIR-2b from supernatant from transiently transfected 293T cells (in 50 μl). After washing the cells twice with RPMI-1640 containing 1% FCS, the cells were added to each well ($1.5*10^5$ in 50 μl) and incubated for 3 hours at 37°C. Input fluorescence was measured by using a fluorescent plate reader (Fluoreskan Ascent, Thermo Labsystems). The plates were firmly flicked and washed in culture medium. The retained fluorescence was determined for each well as a percentage of the input fluorescence.

Reporter cell assay

2B4 T cell hybridoma cells were stably transduced with an NFAT-GFP reporter construct[22] and hLAIR-1-CD3ζ[15] and analyzed as described[15;22]. Briefly, 96-well MAXIsorp flat-bottom plates (Nunc) were coated overnight at 4°C with purified BSA and collagens I and III (100 μl/well 10 μg/ml in PBS supplemented with 2 mM acetic acid). After washings, the plates were incubated with hLAIR-2-IgG (30 μg/ml in RPMI 1640, 100 μl/well) for 30 min, subsequently $3.5x10^5$ cells/ml in 100 μl medium were

added to each well. Plates were incubated at 37°C for 20 hours and analyzed for GFP expression by flow cytometry (FACSCaliber™, BD).

Sandwich ELISA

96-well flat bottom MAXIsorp plates (Nunc) were coated overnight at 4°C with 1A7 anti-LAIR-2 mAbs (6 μg/ml in 50 μl/well PBS). After washings, the plates were incubated with 3% BSA in PBS to block aspecific interactions. Meanwhile, synovial fluid (SF) were treated with hyaluronidase type IV, 20 units/ml (Sigma, Munich, Germany), for 20 minutes 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 hLAIR-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 two hours at room temperature. After three washes, the wells were incubated with a biotinylated LAIR-2 mAb (R&D systems) for two hours at room temperature. After washings, the wells were incubated with StreptABComplex/HRP (Dako) for one hour and color development was performed by adding 100 μl/well ABTS reagent (Roche Diagnostics). A Mann-Whitney 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). Since 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 antibody with an isotype-matched control antibody (BD Biosciences, San Diego, CA). 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 hematopoietic origin (Fig. 1A), corresponding with the expression pattern of hLAIR-1[4;9-11]. LAIR-2 was detected in primary cells (PBMCs), monocytes and T cells, but not in non-hematopoietic cells. Besides the previously described hLAIR-2a and hLAIR-2b[13] isoforms, an additional product was evident after RT-PCR (Fig. 1A). 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 amino acid deletion as compared to LAIR-2a. The 31 aa deletion encompasses a region in the LAIR-1 protein where one of the conserved cysteines involved in intradomain disulfide bond formation is located[13]. Thus this splice variant does not encode an intact Ig-like domain.

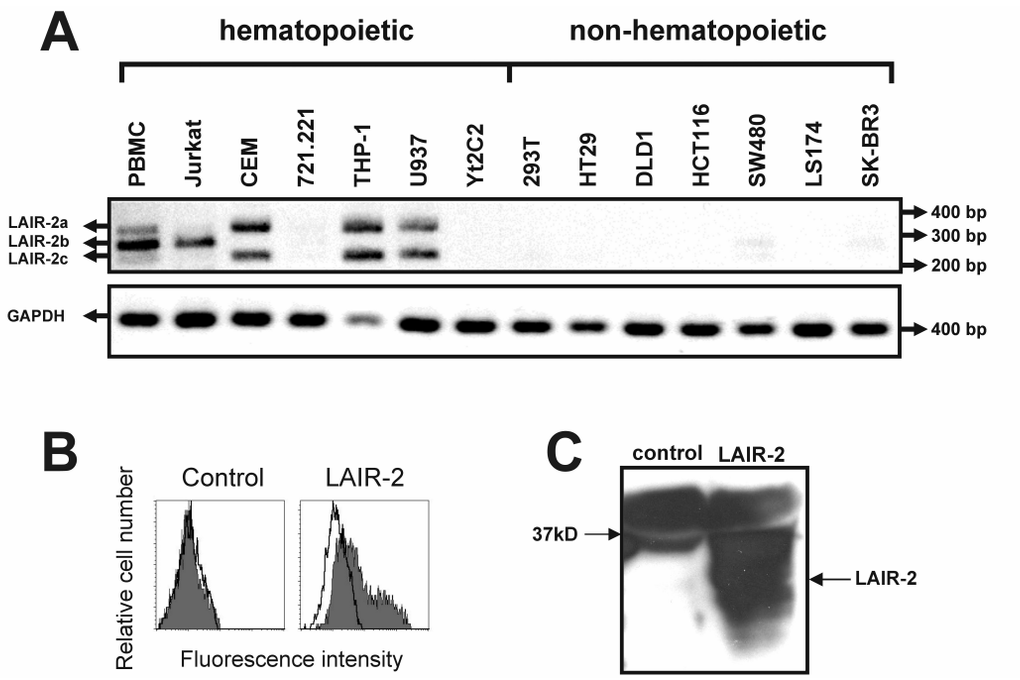

Figure 1. LAIR-2 is expressed as a soluble protein by various hematopoietic cell types. *A*, RNA samples obtained from human PBMCs and various human cell lines were converted to cDNA by RT PCR. The cDNA fragments were amplified using LAIR-2 (top panel) or GAPDH (bottom panel) specific primers. RT PCR analysis was performed on RNA isolated from the following cells: peripheral blood mononuclear cells (PBMCs), 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*, 293T cells transiently transfected with cDNA encoding LAIR-2 (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*, Reducing Western blot analysis of supernatant from wild-type (left lane) and LAIR-2 transfected 293T cells (right lane) for presence of LAIR-2.

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[9]. In order 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 1B). The antibody did not cross-react with human LAIR-1 (data not shown). 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 1C). The protein was present as a smear on reducing Western blot, ranging from an apparent molecular mass of ~25 to ~37 kD. Monomeric LAIR-2a has a predicted molecular mass of ~16.3 kD and the protein contains 10 putative O-linked glycosylation sites but no N-linked glycosylation sites, whereas LAIR-2b has a predicted molecular mass of ~14.7 kD containing 4 putative O-linked glycosylation sites (data not shown). Hence, the higher molecular weight as seen on Western blot may be caused by various states of O-linked glycosylation of the LAIR-2 protein. We conclude that the *LAIR-2* gene encodes a secreted protein.

hLAIR-2 is a high affinity collagen-receptor

Recently, we identified collagens as high affinity ligands for the LAIR-1 molecules[15]. The collagen super family is a large family of trimeric molecules composed of three polypeptide α chains, which contain the sequence repeat $(Gly\text{-}X\text{-}Y)_n$, X being frequently proline (P) and, after post-translational modification, Y being hydroxyproline (O)[23]. Besides LAIR-1, LAIR-2-IgG fusion proteins interacted with transmembrane collagens XIII, XVII and XXIII[15], suggesting that all LAIR-molecules bind the same collagen molecules as ligand. In order to determine whether LAIR-2 has the potential to serve as a competitor for LAIR-1 binding by blocking binding sites on human collagens, we measured binding and affinity of hLAIR-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 (+/- 3.5) and 41.4 nM (+/- 3.7) to collagen I and III, respectively (Fig. 2A). Each collagen molecule has ~10 bindingsites for LAIR-2 (Fig. 2A). The dissociation rate of hLAIR-2 is relatively fast and is characterized by a rapid initial phase and a slower secondary phase (Fig 2B).

The GPO triplet is almost exclusively present in collagenous molecules and allows the formation of a triple helix, which is the main characteristic feature of collagens[23]. Like hLAIR-1, LAIR-2 binds common collagen motifs in a hydroxyproline-dependent manner, since it interacts with trimeric peptides containing 10 repeated GPO triplets ($(GPO)_{10}$[24]), but not with the control $(GPP)_{10}$ trimeric peptide (Fig. 2C).

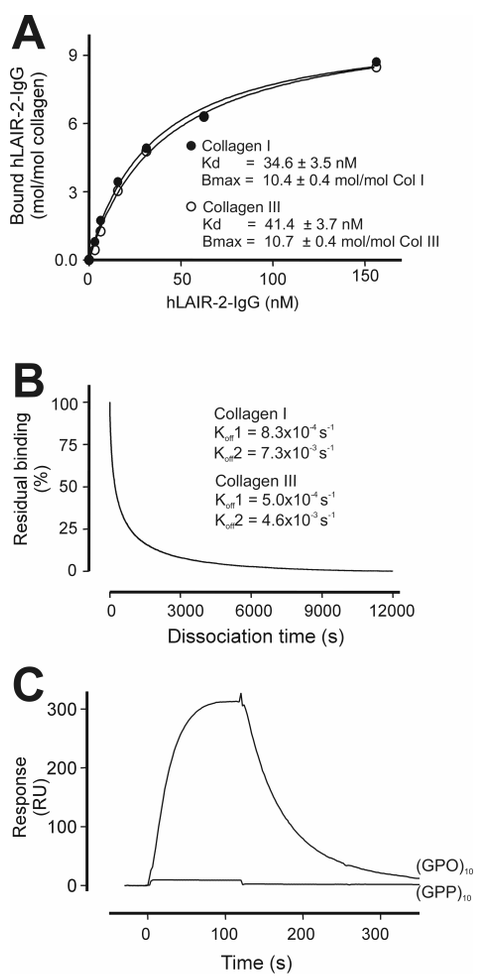

Figure 2. LAIR-2 is a high affinity collagen receptor. *A*, Binding of hLAIR-2 to collagen I and III as indicated by surface plasmon resonance. hLAIR-2-IgG concentrations were injected at 20 μl/min sequentially through a BIAcore flow cell containing ~2000-3000 RU of immobilized collagen I (•) or III (o). Each symbol represents the resonance at equilibrium and the corresponding concentration of the LAIR-2 fusion protein. Calculated affinities (Kd) and number of maximal binding sites (Bmax) are indicated. *B*, Rate of dissociation of hLAIR-2-IgG from collagen III as determined by surface plasmon resonance. The dissociation curve of hLAIR-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.

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

Since 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 LAIR-1 ligand(s)[15;20;21]. Incubation of HT29 cells with biotinylated hLAIR-1-IgG fusion proteins resulted in specific staining of the cells, whereas pre-incubation of these cells with unlabeled hLAIR-2-IgG decreased the biotinylated hLAIR-1-IgG staining (Fig 3A, top panel). In the reverse experiment, pre-incubation of HT29 cells with hLAIR-1-IgG reduced the staining with biotinylated hLAIR-2-IgG (Figure 3A, 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. 3B, upper panel). This interaction was efficiently prevented by pre-incubation of the fluorescent collagen IV with soluble hLAIR-2-IgG fusion proteins (Fig 3B).

As documented before, hLAIR-1 transfected K562 cells bind firmly to plate-bound collagens I and III[15]. Pre-incubation of plate-bound collagens I and III with hLAIR-2-IgG fusion proteins blocked the binding of hLAIR-1 expressing K562 cells to collagens in a dose-dependent manner (Fig. 3C, left panel), while incubation with an irrelevant fusion protein had no effect (Fig. 3C, right panel). 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*.

LAIR-2 prevents LAIR-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[15]. We used this system to investigate whether hLAIR-2-IgG can interfere with the activation of hLAIR-1-CD3ζ. Receptor engagement of cells expressing the hLAIR-1-CD3ζ chimera, but not the parental cells, via plate bound collagens I and III resulted in expression of GFP (Fig. 3D). Pre-incubation of plate-bound collagen I and III with hLAIR-2-IgG fusion proteins, but not isotype matched control IgG, abrogated the NFAT-GFP activation (Fig. 3D), demonstrating that hLAIR-2-IgG is also capable of blocking the functional interaction between hLAIR-1 and collagens.

To assess whether wild-type LAIR-2 protein is capable of interfering with the LAIR-1/collagen interaction, we concentrated supernatant from hLAIR-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, left panel), but not control supernatant (Figure 4, right panel), blocked binding of stable human LAIR-1 expressing K562 cells to plate-bound collagen I and III. In conclusion, five independent assays confirmed that LAIR-2 has the capacity to interfere with human LAIR-1 binding to

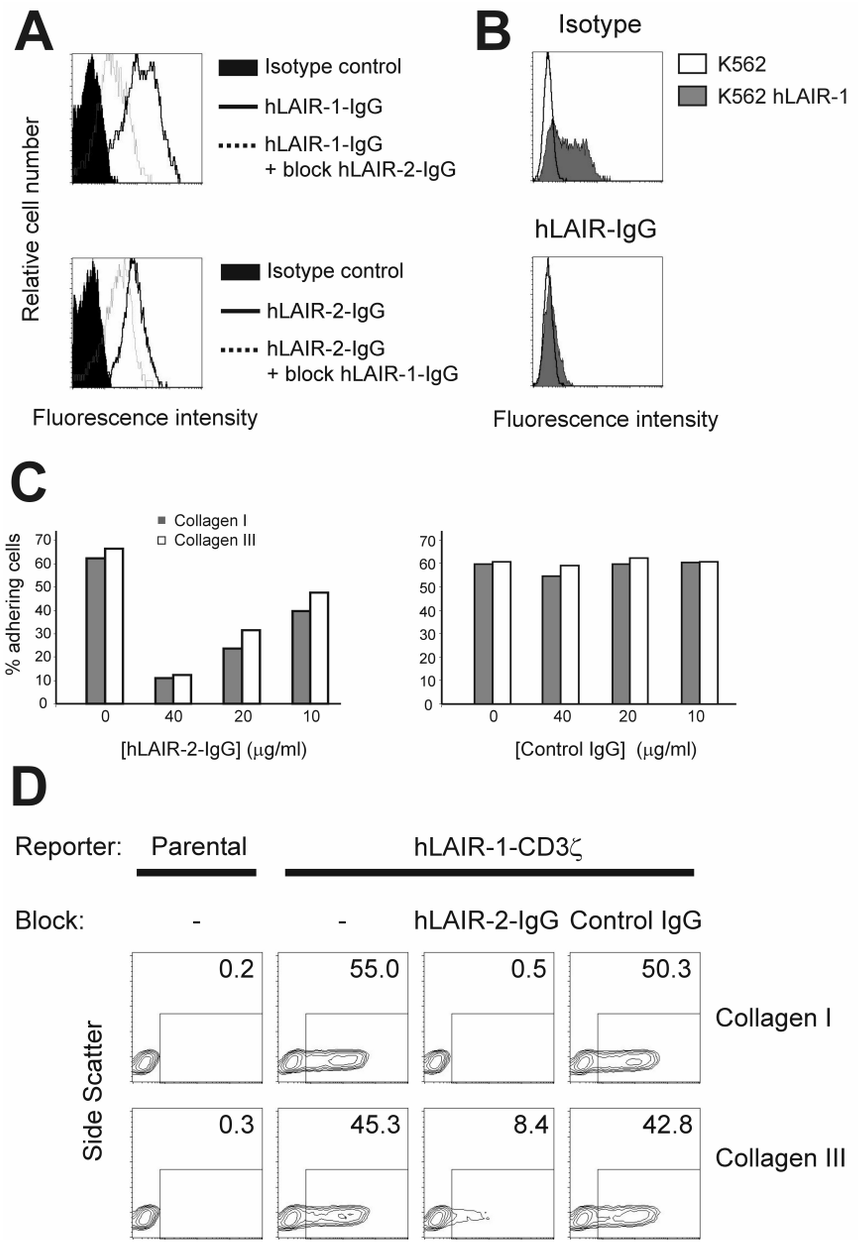

Figure 3. hLAIR-2-IgG blocks the interaction of hLAIR-1 to collagens I and III. *A*, human colon carcinoma HT29 cells were pre-incubated for 30 min with hLAIR-2-IgG (left panel) or hLAIR-1-IgG (right panel) and subsequently stained with biotin-conjugated human LAIR fusion proteins. Specific binding of the biotinylated fusion proteins was detected with APC 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 pre-incubated with 40 μg/ml hLAIR-2-IgG (lower panel) or control Ig fusion proteins (isotype, top panel). Cells were analyzed by flow cytometry. *C*, 96-well plates were coated with collagen I (gray bars) or collagen III (open bars). 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 hours. Percentage of adhering cells relative to the input signal is shown. Cells did not adhere to wells coated with BSA. *D*, NFAT-GFP reporter cells[22] transfected with (right 3 panels) or without hLAIR-1-CD3ζ chimeric molecules (left panels) were incubated with immobilized collagen I (top panels) or III (lower panels) that were pre-incubated with or without hLAIR-2-IgG or control fusion proteins. After approximately 20 hours, GFP expression was analyzed by flow cytometry. Percentage of GFP-positive cells is indicated in each plot.

various classes of collagen molecules, suggesting that the protein may have a similar function *in vivo*.

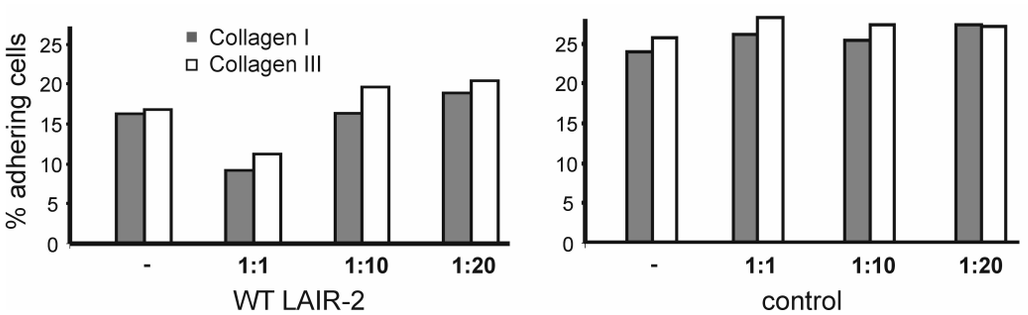

Figure 4. Wild-type LAIR-2 blocks the interaction of hLAIR-1 to human collagen I and III. *A.* Concentrated supernatants of 293T cells transfected with LAIR-2 (WT LAIR-2, left panel) or control cDNA (right panel), were analyzed for their capacity to block adhesion of hLAIR-1 transfected K562 cells to immobilized collagens I and III, as described in figure 3A.

hLAIR-2 is present in urine of pregnant women

To allow detection of wild-type hLAIR-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. 5A), 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). 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 antigen was not detected in urine of man and non-pregnant women (average: 209 pg/ml, range: 140 (below detection level)-695 pg/ml) (Fig. 5B). Although high levels of LAIR-2 were detected in urine, LAIR-2 levels in serum of pregnant woman were still below detection level of our ELISA system (data not shown). Hence we conclude that hLAIR-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 presence of the molecule in synovial fluid (SF) of patients with rheumatoid arthritis (RA) and compared these with SF from patients suffering from osteoarthritis (OA). LAIR-2 concentrations in SF from RA patients was elevated (average: 229 pg/ml, range: 150-318 pg/ml) as compared to that in OA patients (average: 165 pg/ml, range: 150-216 pg/ml). Thus, LAIR-2 presence in SF correlates with the local infection status of the patient.

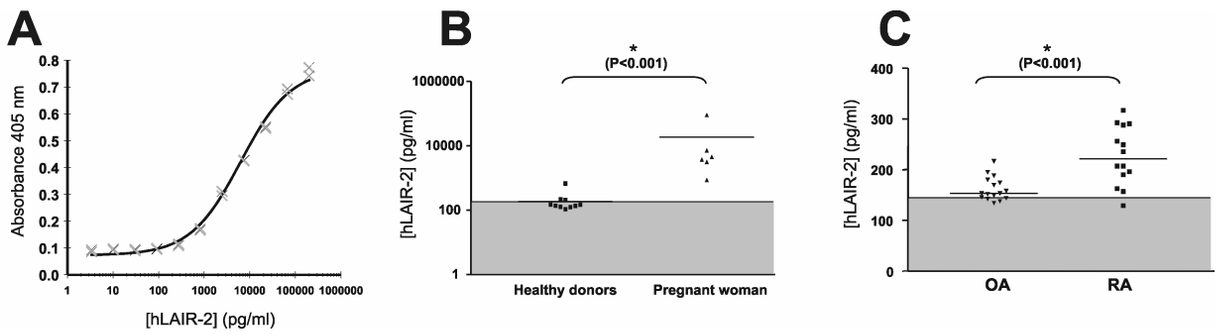

Figure 5. LAIR-2 is present in urine of pregnant women and in synovial fluid of RA patients. *A.* Calibration curve of LAIR-2 ELISA. The plotted values were obtained with 3, 10, 30, 91, 274, 823, 2.5x10^3, 7.4x10^3, 2.2x10^4, 6.7x10^4 and 2.0x10^5 pg/ml recombinant LAIR-2 proteins (R&D systems). Detection limit is approximately 150 pg/ml. *B*, concentration of LAIR-2 in urine of non-pregnant donors (10 males and 6 females) and pregnant women (16-37 weeks of gestation). Measurements were performed by ELISA as described in material and methods. A Mann-Whitney test indicated a statistical significant elevation of LAIR-2 levels in urine of pregnant women as compared to healthy controls (P=0.001). The detection limit is indicated in the figure. *C,* Concentration of LAIR-2 in synovial fluid of patients with rheumatoid arthritis (RA) or osteoarthritis (OA). Measurements were performed by ELISA as described in material and methods. Identical ELISA measurements using an isotype-matched control capturing mAb did not give specific signals above the background (data not shown). A Mann-Whitney test indicated a statistical significant elevation of LAIR-2 levels in synovial fluid of RA patients as compared to OA patients (P<0.001). The detection limit is indicated in the figure. One representative experiment of two is shown.

DISCUSSION

Given the broad expression profile of LAIR-1 on immune cells[9] and the high abundance of collagen molecules in the human body[16], strict regulation of the collagen-LAIR interaction is needed to allow proper inhibitory signaling of the LAIR-1 receptor. In this paper we show that wild-type and chimeric LAIR-2 molecules bind the same sites on collagen molecules as the transmembrane inhibitory receptor LAIR-1 and that this soluble receptor can block the interaction of LAIR-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[7;25].

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 [7;25]). 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 [7]), this is less common for inhibitory

ITIM-bearing immune receptors. A single member of the LILR-family of immune receptors (LILR3, also known as CD85e, ILT6 or LIR-4) lacks cytoplasmic or transmembrane domains and is thus a putative secreted molecule[26-28]. Additionally, KIR3DP1 is a potential soluble member of the 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[29]. 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[29;30]. Hence, our study on LAIR-2 is the first proof of a homologue of an inhibitory immune receptor expressed in humans that has the potential to bind the same ligands as its membrane-bound counterpart.

Since human LAIR-1 and LAIR-2 bind the same sites on 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 hLAIR-2 *in vivo* could greatly impact the binding capacity of LAIR-1 to collagens. By increasing the concentration of soluble LAIR-2 at a site of infection, the inhibitory capacity of membrane bound LAIR-1 may be subsided due to a blockage of the LAIR-1 binding to collagens. Vice versa, a reduced concentration of LAIR-2 may result in LAIR-1 binding to collagens, thereby allowing the inhibitory receptor to signal at sites where immune activation should be avoided. In addition, we found that LAIR-2 dissociates at a somewhat higher rate from collagen than hLAIR-1 does. This may indicate that binding of LAIR-2 to collagens is a dynamic process, and that dissociation of LAIR-2 from collagens can occur swiftly when the concentration of this molecule decreases providing this system with a sensitive regulatory mechanism.

Interestingly, LAIR-2 levels were elevated in the joints of patients suffering from RA as compared to 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[31]. In contrast, OA diseases are 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[32]. Increased LAIR-2 levels in the joints of RA patients suggest that this molecule is associated with a higher inflammatory state. Thus, LAIR-2 may function as a pro-inflammatory mediator by decreasing the inhibitory potential of the immune inhibitor LAIR-1, resulting in more highly activated immune cells, a hallmark for autoimmune diseases such as RA.

We demonstrate that pregnant women secrete high amounts of LAIR-2 in their urine. Although the glomerular filtration rate increases during pregnancy[33], 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) IL13 binding protein[34], sTNF receptors[35], sCD58[36], sIL6 receptor[37], sIFNγ receptor[37], sIL2 receptor[38], and sCD27[39]. Like for LAIR-2, urine levels of sCD58 and sTNF receptors in healthy pregnant women are significantly elevated compared to that of non pregnant, female controls[35;40]. Since many inflammatory factors are enhanced in pregnant women, it has been postulated that pregnancy represents a state of controlled systemic inflammation (reviewed in [41]). 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 however, humans excrete a more or less constant amount of hydroxyproline-containing peptides in their urine[42], which are derived from degraded collagens[43]. During pregnancy, collagen content in the uterus increases more than 8-fold[44], and the amount of hydroxyproline peptides present in urine increases accordingly[45;46]. Since LAIR-2 specifically binds hydroxyproline trimeric peptides, co-localization of LAIR-2 and hydroxyproline peptides in urine may be caused by the increased content and excretion of such peptides in complex with LAIR-2.

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 LAIR-1 via competing for the same ligands.

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REFERENCES

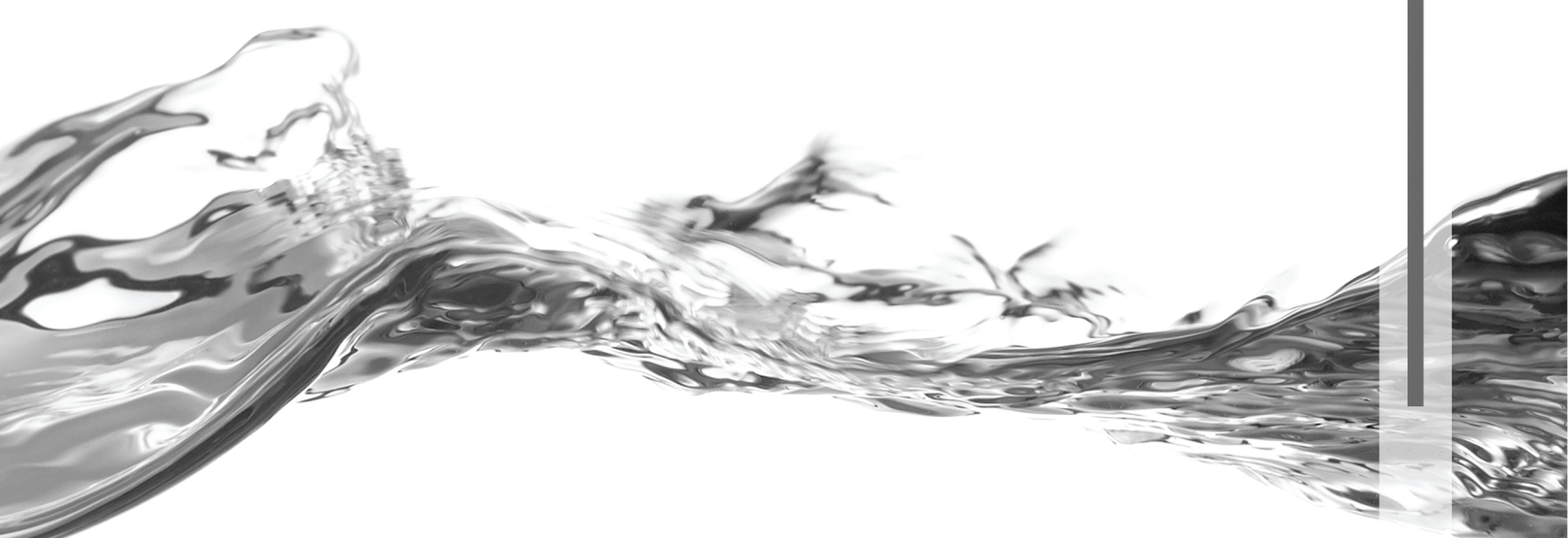
1. Lanier,L.L. 2005. NK Cell Recognition. *Annu. Rev. Immunol.*225-274.
2. Ravetch,J.V. and L.L.Lanier. 2000. Immune inhibitory receptors. *Science* 290:84-89.
3. Verbrugge,A., T.de Ruiter, C.Geest, P.J.Coffer, and L.Meyaard. 2006. Differential expression of leukocyte-associated Ig-like receptor-1 during neutrophil differentiation and activation. *J. Leukoc. Biol.* 79:828-836.
4. Maasho,K., M.Masilamani, R.Valas, S.Basu, J.E.Coligan, and F.Borrego. 2005. The inhibitory leukocyte-associated Ig-like receptor-1 (LAIR-1) is expressed at high levels by human naive T cells and inhibits TCR mediated activation. *Mol. Immunol.* 42:1521-1530.
5. Bennett,F., D.Luxenberg, V.Ling, I.M.Wang, K.Marquette, D.Lowe, N.Khan, G.Veldman, K.A.Jacobs, V.E.Valge-Archer, M.Collins, and B.M.Carreno. 2003. Program death-1 engagement upon TCR activation has distinct effects on costimulation and cytokine-driven proliferation: attenuation of ICOS, IL-4, and IL-21, but not CD28, IL-7, and IL-15 responses. *J. Immunol.* 170:711-718.
6. Moller,M.J., R.Kammerer, F.Grunert, and S.von Kleist. 1996. Biliary glycoprotein (BGP) expression on T cells and on a natural-killer-cell sub-population. *Int. J. Cancer.* 65:740-745.
7. Levine,S.J. 2004. Mechanisms of soluble cytokine receptor generation. *J Immunol.* 173:5343-5348.
8. Ilan,N. and J.A.Madri. 2003. PECAM-1: old friend, new partners. *Curr. Opin. Cell Biol.* 15:515-524.
9. Meyaard,L., G.J.Adema, C.Chang, E.Woollatt, G.R.Sutherland, L.L.Lanier, and J.H.Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
10. Poggi,A., E.Tomasello, E.Ferrero, M.R.Zocchi, and L.Moretta. 1998. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. *Eur. J. Immunol.* 28:2086-2091.
11. van der Vuurst de Vries AR, H.Clevers, T.Logtenberg, and L.Meyaard. 1999. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur. J. Immunol.* 29:3160-3167.
12. Poggi,A., N.Pella, L.Morelli, F.Spada, V.Revello, S.Sivori, R.Augugliaro, L.Moretta, and A.Moretta. 1995. p40, a novel surface molecule involved in the regulation of the non-major histocompatibility complex-restricted cytolytic activity in humans. *Eur. J. Immunol.* 25:369-376.
13. Meyaard,L., J.Hurenkamp, H.Clevers, L.L.Lanier, and J.H.Phillips. 1999. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. *J. Immunol.* 162:5800-5804.
14. Saverino,D., M.Fabbi, A.Merlo, G.Ravera, C.E.Grossi, and E.Ciccone. 2002. Surface density expression of the leukocyte-associated Ig-like receptor-1 is directly related to inhibition of human T-cell functions. *Hum. Immunol.* 63:534-546.
15. Lebbink,R.J., T.de Ruiter, J.Adelmeijer, A.B.Brenkman, J.M.van Helvoort, M.Koch, R.W.Farndale, T.Lisman, A.Sonnenberg, P.J.Lenting, and L.Meyaard. 2006. Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. *J. Exp. Med.* 203:1419-1425.
16. Myllyharju,J. and K.I.Kivirikko. 2004. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet.* 20:33-43.
17. Jansen, C. A., Cruijsen, C., de Ruiter, T., Nanlohy, N., Willens, N, Janssens-Korpela, P. L, and Meyaard, L. Regulated expression of the inhibitory receptor LAIR-1 during human T-cell activation and differentiation. 2006. Submitted for publication.
18. Verbrugge,A., T.de Ruiter, C.Geest, P.J.Coffer, and L.Meyaard. 2006. Differential expression of Leukocyte Associated Ig-like Receptor-1 during neutrophil differentiation and activation. *J. Leukoc. Biol.* 79:828-836.

19. Ouyang,W., J.Xue, J.Liu, W.Jia, Z.Li, X.Xie, X.Liu, J.Jian, Q.Li, Y.Zhu, A.Yang, and B.Jin. 2004. Establishment of an ELISA system for determining soluble LAIR-1 levels in sera of patients with HFRS and kidney transplant. *J Immunol. Methods* 292:109-117.
20. Lebbink,R.J., T.de Ruiter, A.Verbrugge, W.S.Bril, and L.Meyaard. 2004. The mouse homologue of the leukocyte-associated Ig-like receptor-1 is an inhibitory receptor that recruits Src homology region 2-containing protein tyrosine phosphatase (SHP)-2, but not SHP-1. *J. Immunol.* 172:5535-5543.
21. Lebbink,R.J., T.de Ruiter, G.J.Kaptijn, and L.Meyaard. 2005. Identification and characterization of the rat homologue of LAIR-1. *Immunogenetics* 57:344-351.
22. Voehringer,D., D.B.Rosen, L.L.Lanier, and R.M.Locksley. 2004. CD200 receptor family members represent novel DAP12-associated activating receptors on basophils and mast cells. *J. Biol. Chem.* 279:54117-54123.
23. Ricard-Blum,S. and F.Ruggiero. 2005. The collagen superfamily: from the extracellular matrix to the cell membrane. *Pathol. Biol. (Paris)* 53:430-442.
24. Knight,C.G., L.F.Morton, D.J.Onley, A.R.Peachey, T.Ichinohe, M.Okuma, R.W.Farndale, and M.J.Barnes. 1999. Collagen-platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen. *Cardiovasc. Res.* 41:450-457.
25. Heaney,M.L. and D.W.Golde. 1996. Soluble cytokine receptors. *Blood* 87:847-857.
26. Colonna,M., F.Navarro, T.Bellon, M.Llano, P.Garcia, J.Samaridis, L.Angman, M.Cella, and M.Lopez-Botet. 1997. A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. *J. Exp. Med.* 186:1809-1818.
27. Arm,J.P., C.Nwankwo, and K.F.Austen. 1997. Molecular identification of a novel family of human Ig superfamily members that possess immunoreceptor tyrosine-based inhibition motifs and homology to the mouse gp49B1 inhibitory receptor. *J Immunol.* 159:2342-2349.
28. Borges,L., M.L.Hsu, N.Fanger, M.Kubin, and D.Cosman. 1997. A family of human lymphoid and myeloid Ig-like receptors, some of which bind to MHC class I molecules. *J Immunol.* 159:5192-5196.
29. Gomez-Lozano,N., E.Estefania, F.Williams, I.Halfpenny, D.Middleton, R.Solis, and C.Vilches. 2005. The silent KIR3DP1 gene (CD158c) is transcribed and might encode a secreted receptor in a minority of humans, in whom the KIR3DP1, KIR2DL4 and KIR3DL1/KIR3DS1 genes are duplicated. *Eur. J Immunol.* 35:16-24.
30. Borges,L., M.Kubin, and T.Kuhlman. 2003. LIR9, an immunoglobulin-superfamily-activating receptor, is expressed as a transmembrane and as a secreted molecule. *Blood* 101:1484-1486.
31. Feldmann,M., F.M.Brennan, and R.N.Maini. 1996. Rheumatoid arthritis. *Cell.* 85:307-310.
32. Hunter,D.J. and D.T.Felson. 2006. Osteoarthritis. *BMJ.* 332:639-642.
33. Baylis,C. 1999. Glomerular filtration rate in normal and abnormal pregnancies. *Semin. Nephrol.* 19:133-139.
34. Zhang,J.G., D.J.Hilton, T.A.Willson, C.McFarlane, B.A.Roberts, R.L.Moritz, R.J.Simpson, W.S.Alexander, D.Metcalf, and N.A.Nicola. 1997. Identification, purification, and characterization of a soluble interleukin (IL)-13-binding protein. Evidence that it is distinct from the cloned Il-13 receptor and Il-4 receptor alpha-chains. *J. Biol. Chem.* 272:9474-9480.
35. Arntzen,K.J., N.B.Liabakk, G.Jacobsen, T.Espevik, and R.Austgulen. 1995. Soluble tumor necrosis factor receptor in serum and urine throughout normal pregnancy and at delivery. *Am. J Reprod. Immunol.* 34:163-169.
36. Hoffmann,J.C., T.J.Dengler, P.A.Knolle, M.Albert-Wolf, M.Roux, R.Wallich, and S.C.Meuer. 1993. A soluble form of the adhesion receptor CD58 (LFA-3) is present in human body fluids. *Eur. J. Immunol.* 23:3003-3010.
37. Novick,D., H.Engelmann, D.Wallach, and M.Rubinstein. 1989. Soluble cytokine receptors are present in normal human urine. *J. Exp. Med.* 170:1409-1414.

38. Marcon,L., M.E.Fritz, C.C.Kurman, J.C.Jensen, and D.L.Nelson. 1988. Soluble Tac peptide is present in the urine of normal individuals and at elevated levels in patients with adult T cell leukaemia (ATL). *Clin. Exp. Immunol.* 73:29-33.
39. Hintzen,R.Q., R.de Jong, C.E.Hack, M.Chamuleau, E.F.de Vries, I.J.ten Berge, J.Borst, and R.A.van Lier. 1991. A soluble form of the human T cell differentiation antigen CD27 is released after triggering of the TCR/CD3 complex. *J. Immunol.* 147:29-35.
40. Hefler,L., C.Kainz, H.Zeisler, G.Heinze, C.Schatten, P.Husslein, S.Leodolter, and C.Tempfer. 1999. Serum levels of leukocyte functional antigen-3 in pregnancy and preeclampsia. *Acta Obstet. Gynecol. Scand.* 78:580-585.
41. Sargent,I.L., A.M.Borzychowski, and C.W.Redman. 2006. NK cells and human pregnancy - an inflammatory view. *Trends Immunol.*
42. Meilman,E., M.M.Uriveztzky, and Rapoport C.M. 1963. Urinary Hydroxyproline Peptides. *J Clin Invest* 42:40-50.
43. Kivirikko,K.I. 1970. Urinary excretion of hydroxyproline in health and disease. *Int. Rev. Connect. Tissue Res.* 5:93-163.
44. Morrione,T.G. and S.Seifter. 1962. Alteration in the collagen content of the human uterus during pregnancy and post partum involution. *J Exp. Med.* 115:357-365.
45. Szymanovicz,A., P.M.Joubert, A.Garbe, A.Randoux, and J.P.Borel. 1980. An automatic technique for the routine fractionation of the urinary hydroxyproline containing peptides. *Clin Chim. Acta* 100:155-164.
46. Klein,L.R. and S.S.Yen. 1970. Urinary peptide hydroxyproline before and during postpartum involution of human uterus. *Metabolism* 19:19-23.

CHAPTER 9

Summarizing discussion



In this thesis, function of the inhibitory leukocyte-associated Ig-like receptor (LAIR)-1 was investigated. Inhibitory immune receptors are important molecules in the control of immune responses and prevention of autoimmunity. This thesis describes the identification and characterization of a rat and mouse LAIR-1 orthologue, identification and biochemical characterization of the natural ligands for LAIR-1, and studies on a soluble member of the LAIR-family in humans, LAIR-2. Here, the implications of these studies for future research and applications are discussed.

Implications of LAIR/collagen interaction for immune regulation

As reviewed in chapter 1, human LAIR-1 (CD305) is expressed on the majority of peripheral blood mononuclear cells and thymocytes[1;2], and its inhibitory capacity has been documented for many different cell types in *in vitro* cross-linking experiments with monoclonal antibodies (mAbs)[1-7]. In chapter 2 and 3 of this thesis the expression profile of the natural ligand for human LAIR-1 was mapped to adherent cells, and in chapter 4 transmembrane and extracellular matrix collagens were identified as the counter-molecules for this receptor. In chapter 5 requirements for the collagen/LAIR-1 interaction were examined by using synthetic trimeric collagen peptides, which led to the identification of several potent new LAIR-1 agonists.

The broad expression profile of LAIR-1 on immune cells[1] and the high abundance of collagen molecules in the human body[8], suggests that interactions of both molecules may occur in many stages of an immune response and provide an increased threshold for immune cell activation. Why is an increased threshold relevant for immune cell function? When immune cells migrate from the bloodstream into the tissues, they become exposed to multiple activating signals that may trigger cellular responses. To ensure that these responses are specifically targeted against molecules alien tot the organism while sparing the organism itself, a fine-tuned system is required that discriminates between the two. Hence, immune cells should only respond to activating stimuli, when the causative entity poses a real threat (provides a strong activating signal) to the organism and needs to be eradicated. To prevent immune cells from responding to e.g. self antigens on healthy cells, immune cells require a basal level of inhibition (threshold) which is provided by inhibitory immune receptors[9]. The immune cell will only become activated, if it receives potent activating signals that override the inhibitory signaling.

LAIR-1 is expressed on many different immune cells, suggesting a role for the collagen/LAIR-1 interaction in a broad spectrum of immune responses. Indeed, *in vitro* cross-linking of LAIR-1 by mAbs delivers a potent inhibitory signal that is capable of inhibiting cellular functions of NK cells[1;2], T cells[3-5], B cells[6], and dendritic cell precursors[7]. In chapter 4, 5, and 7 we describe that LAIR-1 cross-linking via natural collagen ligands also induces inhibition of CD3-mediated T cell activation and FcεRI-induced degranulation of RBL-2H3 cells. This suggests that the natural ligands and antibodies have the same functional effect on LAIR-1. Furthermore, collagens could

inhibit CD3-induced IFNγ production by primary human T cells (unpublished data), indicating that endogenous LAIR-1 may also be functionally cross-linked by this ligand *in vivo*. Nevertheless, although the inhibitory potential of the collagen/LAIR-1 interaction in some cell types is clearly established, this needs to be formally proven for other LAIR-1 expressing cells.

Regulation of the collagen/LAIR-1 interaction

A widely distributed ligand like collagens implicates that the interaction between ligand and receptor needs to be tightly regulated. One means of regulation may occur by modulating LAIR-1 expression at different stages of differentiation/activation of immune cells, as was demonstrated for B cells[6], T cells[5;10], neutrophils[11], and dendritic cells (unpublished observations). Another system to regulate the interaction between collagens and LAIR-1 may be provided by the putative secreted homologue LAIR-2, which we identified and described in chapter 8. LAIR-2 binds with high affinity to the same binding sites in collagens as LAIR-1. Furthermore, the soluble protein is capable of blocking LAIR-1 interactions with collagens I and III, suggesting that the protein has an immuno-regulatory function *in vivo*. However, is LAIR-2 actually produced *in vivo*? And how is LAIR-2 expression regulated? In chapter 8 we showed that LAIR-2 is present in urine of pregnant women and that LAIR-2-levels are elevated in synovial fluid (SF) of patients suffering from rheumatoid arthritis (RA) as compared to control SF samples. Hence, LAIR-2 may function as a pro-inflammatory mediator by decreasing the inhibitory potential of the immune inhibitor LAIR-1, resulting in more highly activated immune cells, a hallmark for autoimmune diseases such as RA.

Next to LAIR-2, soluble LAIR-1 (sLAIR-1) can be shed from immune cells upon cellular activation[12]. This allows a rapid downregulation of the inhibitory potential of the receptor not only due to reduced membrane-bound LAIR-1 expression but also by occupation of LAIR-1 binding sites on collagens in analogy to our findings for LAIR-2. Intriguingly, also sLAIR-1 was significantly elevated in SF of RA patients (unpublished data), providing rationale to assess the prognostic value of LAIR-2 and sLAIR-1 levels in clinical samples of patients suffering from autoimmune-like diseases.

LAIR-1 and hematopoiesis

The presence of collagens in many sites of the body, implicates that LAIR-1 signaling may regulate various aspects of the immune response in both normal and diseased tissue. Is LAIR-1 also involved in the actual production of blood cells? As a prerequisite, CD34+ hematopoietic progenitor cells in the bone marrow and cord blood indeed express high amount of LAIR-1 ([13;14]) and they are exposed to collagens present in stroma of lymphoid organs[15]. Hence, collagen/LAIR-1 interactions in primary immunological organs may impact hematopoiesis. In favor of this hypothesis, high LAIR-1 expression decreased dramatically during myeloid maturation, suggesting that LAIR-1 may regulate the production and proliferation of myeloid precursors[14]. Additionally, collagen X null mice,

show defects in hematopoiesis[16], which could be caused by impaired signaling via LAIR-1.

In addition, preliminary studies revealed that LAIR-1 is expressed on several human megakaryocytic cell lines (unpublished data). Intriguingly, glycoprotein VI (GPVI), an activating collagen receptor involved in platelet–collagen adhesive interactions leading to thrombus formation[17], is also expressed on these cell types. It is conceivable that a single megakaryocyte simultaneously expresses activating and inhibitory collagen receptors. Since LAIR-1 and GPVI have similar binding sites on collagen molecules (discussed in chapter 5), both receptors may compete for these ligands thereby providing these cells with a sensitive switch between collagen-induced activation (via GPVI) and inhibition (via LAIR-1). Furthermore, LAIR-1 may generate intracellular signals that can directly inhibit GpVI-mediated activation signals. This paired expression of activating and inhibiting receptors interacting with identical ligands has been reported for several other receptor pairs (reviewed in [18;19]), and may represent a sensitive system to regulate immune cell function.

Additional LAIR-1 ligands

In addition to the collagen family members, several multimeric soluble proteins contain collagenous domains[8]. Surfactant proteins A and D (SP-A and SP-D) for example, are multimeric proteins abundantly present in the lung that contain globular C-type carbohydrate-recognition (lectin) domains coupled to collagen-like tails[20]. It is conceivable that SP-A and SP-D can interact via their collagenous domains with LAIR-1 expressed on macrophages and as such down regulate immune responses in healthy lungs (further discussed in chapter 6). Besides SP-A and SP-D, collagenous domains are also present in other pattern-recognition molecules such as mannan-binding lectin (MBL), components of the complement system (C1q) and in ficolins[8]. All these molecules are humoral proteins that play important roles in the innate defense against pathogens such as bacteria and viruses[21]. Hypothetically, the collagenous domains of these proteins can interact with LAIR-1 thereby modulating immune responses.

LAIR-1 as adhesion molecule?

Besides functioning as an inhibitory immune receptor, it is interesting to speculate that LAIR-1 may also act as a heterophilic adhesion molecule involved in cell-cell contact by interacting with transmembrane collagens or in cell-matrix contact via extracellular matrix collagens. In support of this hypothesis: overexpression of LAIR-1 on K562 cells allows cells to strongly adhere to collagen III under sheer flow conditions (chapter 4). Furthermore, upon steady state contact with immobilized collagen molecules, LAIR-1 expressing K562 cells show a remarkable change in morphology and adhere strongly to the immobilized collagen matrix (chapter 4). Hence, LAIR-1 may evoke adhesive interactions with collagen surfaces or collagen expressing cells *in vivo*. Therefore, besides functioning as an inhibitory immune receptor, LAIR-1 may also be involved in regulation

of immune cell trafficking. Correspondingly, several other inhibitory immune receptors display this same dual functionality (as discussed in chapter 6).

How can we study the collagen/LAIR-1 interaction *in vivo*?

Studies on the *in vivo* role of LAIR-1 were hampered by the lack of murine orthologues to study the role of the receptor in normal physiology and disease. In chapter 2 and 3 we identified and characterized the mouse and rat LAIR-1 orthologues of LAIR-1 respectively, which may help pave the way to study the biological role of the receptor by using animal models. The rat and mouse LAIR-1 proteins show moderate protein sequence identity to human LAIR-1 (~40%) and are encoded in a region of the chromosome syntenic to human chromosome 19q13.4 where human LAIR-1 is encoded. Mouse and human LAIR-1 have similar expression profiles and share a potent inhibitory potential (chapter 2 and 7). Furthermore, both receptors are functionally triggered by intact collagens and synthetic trimeric collagen peptides (chapter 4, 5 and 7). Hence, the structural and functional overlap between both receptors justifies studies on mouse LAIR-1 as a model for human LAIR-1. Nevertheless, since rats and mice lack direct orthologues of the soluble LAIR-2 (chapter 2 and 3), studies on the biological interplay between LAIR-1 and LAIR-2 in normal physiology are hampered.

The remaining challenge is to determine in which *in vivo* immunological processes LAIR-1 is involved. To be able to understand the LAIR-1 interaction with its ligand in normal physiology, the generation of a LAIR-1 knock-out mouse will be informative. This mouse may show signs of enhanced immune activation or even autoimmunity due to a reduced threshold for immune activation. Furthermore, if above speculations are correct, mLAIR-1 knock-out animals may show aberrant hematopoiesis and immune cell trafficking, or may display enhanced immunepathology in response to infections.

Role for collagen/LAIR-1 interaction in tumor immunology

Cytotoxic lymphocytes, such as natural killer (NK) cells and CD8+ T cells, provide an essential defense against tumors. Interestingly, numerous studies suggest that up regulation of ligands for inhibitory immune receptors represents a mechanism by which tumor or virus-infected cells acquire resistance to cytotoxic immune cell-mediated lysis (reviewed in chapter 6). Increased expression of collagens by tumors is related to malignancy, metastic capacity, and/or tumor progression[22-27]. Until now, the relation between collagen expression and tumor malignancy was thought to be mainly related to the capacity of tumor cells to remodel the extracellular matrix in order to create a suitable microenvironment for tumor cell growth. However, also the collagen/LAIR-1 interaction may provide tumor cells with a mechanism to defend themselves against attacks by the immune system, thereby allowing rapid tumor growth. Future studies using tumor models in mice should aim at dissecting a role for LAIR-1 in tumor biology which may provide rationale for the development of therapeutic reagents that can interfere with the collagen/LAIR-1 interaction as a means to abrogate LAIR-1-induced immune evasion.

Can we exploit collagen/LAIR-1 interactions for therapy?

As discussed above, the collagen/LAIR-1 interaction could play an important role in immune cell regulation and may be implicated in the development of human disease. Can we exploit LAIR-collagen interactions to treat human diseases? First, by stimulating the inhibitory function of LAIR-1, immune cell function may be dampened which could be valuable to treat e.g. autoimmune-like diseases. Second, by blocking the inhibitory function of LAIR-1, immune cells may become more activated and promote anti-tumor responses against malignant cells that display enhanced collagen expression. Third, LAIR could be engineered to serve as an anti-thrombotic reagent. Since the platelet activator GPVI binds similar sites in collagen molecules as LAIR-1 (chapter 5), high affinity soluble LAIR variants (sLAIR-1 or LAIR-2) may be used to block GPVI-binding sites on collagens thereby preventing GPVI-induced blood clotting. A drawback of this approach is that it will also affect LAIR-1 functioning thereby inducing a potentially dangerous state of enhanced immune cell activation. Thus, detailed knowledge of the LAIR-1 and GPVI binding sites might allow a rational design of agonistic or antagonistic trimeric peptide therapeutics that may discriminate between effects on either thrombosis (via GPVI) or the immune system (via LAIR-1).

Concluding remarks

Genetic analysis in human disease and studies in knock-out mice have indicated that inhibitory immune receptors play essential, non-redundant roles in the control of the immune system. Although studies dissecting the expression profiles and signal transduction pathways of human LAIR-1 have broadened our understanding of this receptor, the actual biological function(s) of LAIR-1 remains unknown. With the identification of murine orthologues, the cloning of collagens as ligands for LAIR-1, and the generation of potent research tools in the form of synthetic trimeric collagen peptides, the way is paved to assess the true function of LAIR-1 in immune regulation. Therefore, further studies should aim at dissecting the *in vivo* role of the protein in normal physiology and disease. Furthermore, detailed studies on the biochemical interaction between collagens and LAIR-1 may allow rational design of new and potent research tools and could result in the development of therapeutics which exploits collagen/LAIR-1 interactions.

REFERENCES

1. Meyaard,L., G.J.Adema, C.Chang, E.Woollatt, G.R.Sutherland, L.L.Lanier, and J.H.Phillips. 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
2. Poggi,A., N.Pella, L.Morelli, F.Spada, V.Revello, S.Sivori, R.Augugliaro, L.Moretta, and A.Moretta. 1995. p40, a novel surface molecule involved in the regulation of the non-major histocompatibility complex-restricted cytolytic activity in humans. *Eur. J. Immunol.* 25:369-376.
3. Meyaard,L., J.Hurenkamp, H.Clevers, L.L.Lanier, and J.H.Phillips. 1999. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. *J. Immunol.* 162:5800-5804.
4. Saverino,D., M.Fabbi, A.Merlo, G.Ravera, C.E.Grossi, and E.Ciccone. 2002. Surface density expression of the leukocyte-associated Ig-like receptor-1 is directly related to inhibition of human T-cell functions. *Hum. Immunol.* 63:534-546.
5. Maasho,K., M.Masilamani, R.Valas, S.Basu, J.E.Coligan, and F.Borrego. 2005. The inhibitory leukocyte-associated Ig-like receptor-1 (LAIR-1) is expressed at high levels by human naive T cells and inhibits TCR mediated activation. *Mol. Immunol.* 42:1521-1530.
6. van der Vuurst de Vries AR, H.Clevers, T.Logtenberg, and L.Meyaard. 1999. Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur. J. Immunol.* 29:3160-3167.
7. Poggi,A., E.Tomasello, E.Ferrero, M.R.Zocchi, and L.Moretta. 1998. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. *Eur. J. Immunol.* 28:2086-2091.
8. Myllyharju,J. and K.I.Kivirikko. 2004. Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet.* 20:33-43.
9. Ravetch,J.V. and L.L.Lanier. 2000. Immune inhibitory receptors. *Science* 290:84-89.
10. Jansen, C. A., Cruijsen, C., de Ruiter, T., Nanlohy, N., Willens, N, Janssens-Korpela, P. L, and Meyaard, L. Regulated expression of the inhibitory receptor LAIR-1 during human T-cell activation and differentiation. 2006. Submitted for publication.
11. Verbrugge,A., T.de Ruiter, C.Geest, P.J.Coffer, and L.Meyaard. 2006. Differential expression of Leukocyte Associated Ig-like Receptor-1 during neutrophil differentiation and activation. *J. Leukoc. Biol.* 79:828-836.
12. Ouyang,W., J.Xue, J.Liu, W.Jia, Z.Li, X.Xie, X.Liu, J.Jian, Q.Li, Y.Zhu, A.Yang, and B.Jin. 2004. Establishment of an ELISA system for determining soluble LAIR-1 levels in sera of patients with HFRS and kidney transplant. *J Immunol. Methods* 292:109-117.
13. Ouyang,W., D.Ma, D.Lin, Y.Sun, X.Liu, Q.Li, W.Jia, Y.Cao, Y.Zhu, and B.Jin. 2003. 9.1C3 is identical to LAIR-1, which is expressed on hematopoietic progenitors. *Biochem. Biophys. Res. Commun.* 310:1236-1240.
14. Verbrugge,A., T.de Ruiter, C.Geest, P.J.Coffer, and L.Meyaard. 2006. Differential expression of leukocyte-associated Ig-like receptor-1 during neutrophil differentiation and activation. *J. Leukoc. Biol.* 79:828-836.
15. Gelse,K., E.Poschl, and T.Aigner. 2003. Collagens--structure, function, and biosynthesis. *Adv. Drug Deliv. Rev.* 55:1531-1546.
16. Gress,C.J. and O.Jacenko. 2000. Growth plate compressions and altered hematopoiesis in collagen X null mice. *J. Cell Biol.* 149:983-993.
17. Moroi,M. and S.M.Jung. 2004. Platelet glycoprotein VI: its structure and function. *Thromb. Res.* 114:221-233.
18. Lanier,L.L. 2001. Face off--the interplay between activating and inhibitory immune receptors. *Curr. Opin. Immunol.* 13:326-331.

19. Arase,H. and L.L.Lanier. 2004. Specific recognition of virus-infected cells by paired NK receptors. *Rev. Med. Virol.* 14:83-93.
20. Barclay,A.N. and M.H.Brown. 2006. The SIRP family of receptors and immune regulation. *Nat. Rev. Immunol.* 6:457-464.
21. Holmskov,U., S.Thiel, and J.C.Jensenius. 2003. Collections and ficolins: humoral lectins of the innate immune defense. *Annu. Rev. Immunol.* 21:547-578.
22. Parikka,M., T.Kainulainen, K.Tasanen, A.Vaananen, L.Bruckner-Tuderman, and T.Salo. 2003. Alterations of collagen XVII expression during transformation of oral epithelium to dysplasia and carcinoma. *J. Histochem. Cytochem.* 51:921-929.
23. Roepman,P., L.F.Wessels, N.Kettelarij, P.Kemmeren, A.J.Miles, P.Lijnzaad, M.G.Tilanus, R.Koole, G.J.Hordijk, P.C.van der Vliet, M.J.Reinders, P.J.Slootweg, and F.C.Holstege. 2005. An expression profile for diagnosis of lymph node metastases from primary head and neck squamous cell carcinomas. *Nat. Genet.* 37:182-186.
24. Vaisanen,T., M.R.Vaisanen, H.Autio-Harmainen, and T.Pihlajaniemi. 2005. Type XIII collagen expression is induced during malignant transformation in various epithelial and mesenchymal tumours. *J. Pathol.* 207:324-335.
25. Iizasa,T., H.Chang, M.Suzuki, M.Otsuji, S.Yokoi, M.Chiyo, S.Motohashi, K.Yasufuku, Y.Sekine, A.Iyoda, K.Shibuya, K.Hiroshima, and T.Fujisawa. 2004. Overexpression of collagen XVIII is associated with poor outcome and elevated levels of circulating serum endostatin in non-small cell lung cancer. *Clin. Cancer Res.* 10:5361-5366.
26. Nielsen,G.P., J.X.O'Connell, B.M.Wehrli, and A.E.Rosenberg. 2003. Collagen-rich tumors of soft tissues: an overview. *Adv. Anat. Pathol.* 10:179-199.
27. Banyard,J., L.Bao, and B.R.Zetter. 2003. Type XXIII collagen, a new transmembrane collagen identified in metastatic tumor cells. *J. Biol. Chem.* 278:20989-20994.

Nederlandse samenvatting voor niet ingewijden

Inleiding

Het immuunsysteem (afweersysteem) is een systeem in het lichaam dat werkt om infecties en ziekten af te weren. Het Latijnse woord *immunis* betekent 'vrijgesteld', een term die verwijst naar de bescherming tegen indringers van buiten. Naast het afweren van virussen, bacteriën en parasieten (ook wel pathogenen genoemd) is het immuunsysteem ook in staat zieke lichaamscellen zoals kankercellen op te ruimen. Witte bloedcellen spelen een centrale rol in het immuunsysteem. Deze produceren antistoffen die binden aan de indringers, of functioneren als aaseters door bijvoorbeeld bacteriën en dode cellen op te ruimen. De samenwerking tussen verschillende soorten immuuncellen tijdens een immuunreactie zorgt ervoor dat pathogenen en 'zieke' lichaamscellen aangevallen en verwijderd worden.

Immuuncellen worden aangezet (geactiveerd) zodra bepaalde eiwitten op de celoppervlakte (receptoren) binden aan pathogenen, geïnfecteerde cellen of kankercellen. Deze stimulerende (activerende) receptoren geven een seintje aan de immuuncel dat er gevaar aanwezig is. Dit signaal wordt via de receptor van buiten de cel naar binnen doorgegeven (signaal-transductie), wat zorgt voor activatie van de immuuncel en uiteindelijk eliminatie van 'het gevaar'. Echter, voor het adequaat functioneren van het immuunsysteem is niet alleen activatie van immuuncellen belangrijk, maar ook de remming daarvan. Wanneer cellen in het immuunsysteem te veel of op het verkeerde moment worden geactiveerd, kan de immuuncel het eigen gezonde lichaam aanvallen en beschadigen (auto-immuunziekte). Voorbeelden van dergelijke auto-immuunziekten zijn: diabetes mellitus (suikerziekte) en reumatoïde artritis. Om deze ongewenste aanvallen te voorkomen bevatten immuuncellen remmende (inhibitoire) receptoren op het celoppervlak. Deze inhibitoire receptoren kunnen binden aan lichaamseigen eiwitten aanwezig op andere cellen/weefsels (een ligand), en zo voorkomen dat immuuncellen geactiveerd worden door ongevaarlijke lichaamseigen cellen/weefsels. Dat doen deze receptoren door het signaal van activerende receptoren te verstoren, waardoor immuuncellen niet (of minder) geactiveerd worden. Activerende en inhibitoire receptoren zijn tegelijk aanwezig op de oppervlakte van immuuncellen en kunnen ook simultaan signalen geven; de balans tussen beide receptoren (activatie versus inhibitie) bepaalt wat de immuuncel zal doen: aanvallen of toekijken.

Dit proefschrift

Onze kennis van inhibitoire receptoren is beperkt. Tijdens de start van het hier beschreven promotie onderzoek waren veel inhibitoire receptoren geïdentificeerd maar was de functie van veel van deze moleculen onbekend. Een van deze receptoren is LAIR-1 (Leukocyte-associated Ig-like receptor)-1. LAIR-1 is een menselijke (humane) inhibitoire receptor welke voorkomt op de meeste immuuncellen in het bloed. Voorafgaande studies hebben aangetoond dat LAIR-1 immuuncellen kan remmen (samengevat in hoofdstuk 1).

Echter, aangezien het ligand van de receptor onbekend was en geen homologen in andere organismen beschreven waren, was het lastig de exacte rol van de receptor in regulatie van

het immuunsysteem te bepalen. De doelstelling van dit promotieonderzoek was de biologie van LAIR-1 verder op te helderen, met de nadruk op het identificeren van de homologen en liganden.

In hoofdstuk 2 beschrijven we de ontdekking van een muis familielid (homoloog) van LAIR-1. Net als humaan LAIR-1, bevindt muis LAIR-1 zich op immuuncellen en is in staat immuuncellen te remmen. Het heeft een vergelijkbare signaal-transductie route als humaan LAIR-1, hoewel enkele verschillen op te merken zijn. Naast een homoloog in de muis bevat ook de rat een LAIR-1 receptor, wat beschreven staat in hoofdstuk 3. De identificatie van muis en rat homologen maken het mogelijk de biologie van LAIR-1 te bestuderen in de "modeldieren" rat en muis. Deze dieren staan model voor de mens, aangezien ze in biologisch opzicht erg op elkaar lijken; in veel gevallen werken muis/rat receptoren hetzelfde als de humane familieleden. Het bestuderen van homologen in deze dieren kan daardoor informatie verschaffen over de rol van de receptor in regulatie van humane immuunreacties. Echter, om muis/rat LAIR-1 als model te kunnen gebruiken, is het belangrijk in kaart te brengen of de receptoren ook daadwerkelijk dezelfde eigenschappen hebben. In hoofdstuk 7 vergelijken we daarom muis LAIR-1 en humaan LAIR-1 in meer detail. Zoals verwacht hebben beide receptoren vergelijkbare eigenschappen: ze zien er gelijk uit, ze worden door dezelfde immuuncellen aangemaakt, ze werken beiden inhiberend en hun aanmaak wordt op gelijke wijze gereguleerd. Muis LAIR-1 kan daarom goed als model gebruikt worden om de functie van de receptor te doorgronden met behulp van muizen studies.

Hoewel eerdere experimenten uitwezen dat LAIR-1 functioneert als inhibitoire receptor, was het ligand van de receptor nog onbekend. In hoofdstuk 4 beschrijven we de identificatie van collageen als ligand voor LAIR-1. Collageen moleculen zijn grote moleculen die zeer veel in het lichaam voorkomen, ~25 tot 50 % van al het eiwit in het menselijke lichaam is collageen. Deze moleculen vormen een belangrijk onderdeel van bindweefsel, bot en bloedvaten. Daarnaast is collageen onmisbaar voor de bloedstolling. Collageen moleculen zijn celoppervlak gebonden of worden uitgescheiden door cellen. LAIR-1 kan aan beide typen collageen binden, wat resulteert in remming van de immuuncel waarop LAIR-1 aanwezig is. Daarnaast hebben we laten zien dat LAIR-1 specifiek bindt aan een gebied op het eiwit dat enkel in collageen voorkomt. Daarom verwachten we dat LAIR-1 aan elk eiwit kan binden dat dergelijke 'collageen-domeinen' bevat. Om dit verder te bestuderen hebben we in hoofdstuk 5 synthetische stukjes van collageen II en III gemaakt (peptiden), door deze moleculen in ~56 stukken te hakken. Vervolgens hebben we bekeken aan welke stukken LAIR-1 kan binden en aan welke niet, waardoor we nu weten wat de exacte plaats is waar LAIR-1 bindt op deze moleculen. Daarnaast heeft deze studie enkele interessante peptiden opgeleverd die zeer goed in staat zijn LAIR-1 aan te zetten en zo immuuncellen te remmen. Deze peptiden zijn waardevolle studiemiddelen om de functie van LAIR verder te onderzoeken in het

laboratorium, wat er in de toekomst misschien voor zorgt dat de peptiden gebruikt kunnen worden als medicatie om het immuunsysteem te remmen.

Aangezien collageen zeer veel in ons lichaam voorkomt en LAIR-1 op het celoppervlak van bijna alle immuuncellen aanwezig is, is een strikte regulatie van de interactie tussen beide moleculen noodzakelijk om te voorkomen dat het immuunsysteem continue geremd wordt. Deze regulatie kan op verschillende niveaus plaatsvinden. De immuuncel kan bijvoorbeeld LAIR-1 van het celoppervlak weghalen, en zo de 'rem' opheffen. In hoofdstuk 8 beschrijven we de karakterisering van een oplosbare variant van LAIR-1 (LAIR-2), welke de remmende functie van LAIR-1 kan reguleren. LAIR-2 wordt uitgescheiden door cellen en is in staat de interactie tussen LAIR-1 en collageen te blokkeren. LAIR-2 doet dat door aan dezelfde collageen moleculen te binden als LAIR-1 Doordat de LAIR-1/collageen binding verbroken wordt door de concurrende binding van LAIR-2/collageen wordt de immuuncel niet meer geremd. Het gevolg is dat immuuncellen eerder geactiveerd raken en dus iets gevoeliger worden voor immuunactivatie.

Conclusies

In dit proefschrift beschrijven we de karakterisatie van muis en rat LAIR-1 en hebben we collageen als ligand voor deze receptor geïdentificeerd. Deze bevindingen leggen een basis om de exacte biologische rol van de receptor te bestuderen. LAIR-1 bindt aan verschillende collageen moleculen en de interactie resulteert in inhibitie van immuunresponsen. Naast LAIR-1 bindt ook LAIR-2 aan deze collageen moleculen en is derhalve in staat de interactie tussen LAIR-1 en collageen te blokkeren om zo inhibitie via LAIR-1 te reguleren. Om de functie van LAIR-1 en LAIR-2 volledig te begrijpen is verder onderzoek noodzakelijk. Dit vervolg onderzoek moet gericht zijn op de verdere karakterisering van de LAIR/collageen interactie, hetgeen wellicht de ontwikkeling van immuun-remmers mogelijk maakt. Verder dient de rol van de collageen/LAIR-1 interactie bestudeerd te worden in het ontstaan van kanker. De hier beschreven muis homoloog van LAIR-1 kan hierbij als model worden gebruikt. Tenslotte: inzicht in de functie van inhibitoire receptoren zal leiden tot een verbeterde kennis over de regulatie van het immuunsysteem en maakt wellicht ontwikkeling van specifieke therapeutica mogelijk.

Dankwoord
Curriculum Vitae
List of publications
Abbreviations
Supplemental data

DANKWOORD

Zo, dat is af!!! Lekker. Maar dat is natuurlijk niet geheel mijn verdienste. De afgelopen 5 jaar heeft een grote groep mensen direct of indirect bijgedragen aan dit proefschrift. Daarom wil ik al deze mensen van harte bedanken. Helaas is het onmogelijk iedereen bij naam te noemen, maar ik zal toch een poging doen…

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'Lanier-Lab' Unfortunately the MCMV results were not all that great, but it was fun working with you. Many thanks.

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Robert Jan

CURRICULUM VITAE

Robert Jan Lebbink werd geboren op 11 mei 1978 te Doesburg. Na het behalen van zijn VWO diploma aan het Ulenhof College in Doetinchem, begon hij in 1996 aan de studie Moleculaire wetenschappen aan de Universiteit Wageningen. Als onderdeel van zijn studie liep hij onderzoeksstages bij de afdeling Moleculaire Biologie onder supervisie van Prof. Dr. T. Bisseling, de afdeling Microbiologie onder supervisie van Prof. Dr. J. van der Oost en Dr. A. Brinkman en de afdeling Virologie onder supervisie van Prof. Dr. R.W. Goldbach en Dr. W.F.J. IJkel aan bovengenoemde universiteit. Ter afsluiting van zijn studie heeft hij stage gelopen onder begeleiding van Prof. F.A. Arnold en Dr. H. Berk bij the Department of Chemical Engineering gevestigd aan de California Institute of Technology in Pasadena, USA. In 2001 behaalde hij het doctoraal examen en begon hij als promovendus bij de afdeling Immunologie van het Universitair Medisch Centrum Utrecht, onder begeleiding van Dr. L. Meyaard. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.
Per 1 februari 2007 zal hij werkzaam zijn in the Department of Microbiology and Immunology aan de University of California San Francisco onder begeleiding van Dr. M.T. McManus, alwaar hij de rol van microRNAs in hematopoiese zal bestuderen.

LIST OF PUBLICATIONS

W.F. IJkel, R.J. Lebbink, M.L. Op den Brouw, R.W. Goldbach, J.M. Vlak and D. Zuidema. 2001. Identification of a novel occlusion derived virus-specific protein in Spodoptera exigua multicapsid nucleopolyhedrovirus. *Virology. 284(2):170-181.*

A.B. Brinkman, S.D. Bell, R.J. Lebbink, W.M. de Vos and J. van der Oost. 2002. The Sulfolobus solfataricus Lrp-like protein LysM regulates lysine biosynthesis in response to lysine availability. *J Biol Chem. 277(33): 29537-29549.*

H. Berk and R.J. Lebbink. 2003. High-throughput screening of mutant alpha-amylase libraries for increased activity at 129^{o}C. *Methods Mol Biol. 230:127-135.*

R.J. Lebbink, T. de Ruiter, A. Verbrugge, W.S. Bril and L. Meyaard. 2004. The mouse homologue of the leukocyte-associated immunoglobulin-like receptor-1 is an inhibitory receptor that recruits SHP-2 but not SHP-1. *J Immunol. 179: 5535-5543.*

R.J. Lebbink, T. de Ruiter, G.J.A. Kaptijn, and L. Meyaard. 2005. Identification and characterization of the rat homologue of LAIR-1. *Immunogenetics. 57:344-351.*

R.J. Lebbink, T. de Ruiter, J. Adelmeijer, A.B. Brenkman, J.M. van Helvoort, M. Koch, R.W. Farndale, T. Lisman, A. Sonnenberg, P.J. Lenting, and L. Meyaard. 2006. Collagens are functional, high-affinity ligands for the inhibitory immune receptor LAIR-1. *J Exp Med. 203:1419-25.*

R.J. Lebbink and L. Meyaard. 2006. Review: Non-MHC ligands for inhibitory immune receptors: novel insights and implications for immune regulation. *Mol Immunol, in press.*

R.J. Lebbink, G.J.A. Kaptijn, T. de Ruiter, D.G. Bihan, C.A. Jansen, P.J. Lenting, L. Meyaard. 2006. Mouse Leukocyte-associated Ig-like receptor-1 (LAIR-1) functions as an inhibitory collagen-binding receptor on immune cells. *Submitted.*

R.J. Lebbink, M.C.W. van den Berg, T. de Ruiter, N. Raynal, J.A.G. van Roon, P.J. Lenting, B. Jin, and L. Meyaard. 2006. LAIR-2 is a soluble competitor of the collagen/LAIR-1 inhibitory immune interaction. *Submitted.*

ABBREVIATIONS

-/-	knock out
aa	amino acid
APC	allophycocyanine
B2M	beta-2-microglobulin
BCR	B cell receptor
Bmax	maximum bindingsites
BMDC	bone-marrow derived DC
BORG-3	Binder of Rho GTPase 3
BSA	bovine serum albumin
BTLA	B and T lymphocyte attenuator
C	carboxy
CAM	cellular adhesion molecule
CD	cluster of differentiation
CLECSF	C-type lectin superfamily
CMV	cytomegalovirus
CRP	collagen-related peptide
Csk	C-terminal src kinase
CTLA-4	cytotoxic T lymphocyte antigen-4
Da	Dalton
DC	dendritic cell
DDR	discoidin domain receptor
ELISA	enzyme-linked immunosorbent assay
Ep-CAM	epithelial cell adhesion molecule
FACS	fluorescence activated cell sorter
FcαR	IgA receptor
FCS	fetal calf serum
FcγR	IgG receptor
FcεR	IgE receptor
FITC	fluorescein isothiocyanate
GAPDH	gylceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony stimulating factor
GFP	green fluorescent protein
GM-CSF	granulocyte/macrophage colony stimulating factor
GPI	glycophosphatidylinositol
GP-VI	glycoprotein VI
h	human
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HVEM	herpes virus entry mediator
i.p.	intraperitoneal
IFN	interferon
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
ILT	Ig-like transcript
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibitory motif

ITSM	immuno-receptor tyrosine-based switch motifs
KIR	killer cell Ig-like receptor
KLRG1	killer cell lectin-like receptor G1
LAIR	leukocyte-associated Ig-like receptor
LENG	LRC-encoded novel gene
LILR	leukocyte Ig-like receptor
LLT1	lectin-like transcript 1
LRC	leukocyte receptor complex
Ly-94	lymphocyte antigen 94
m	mouse
mAb	monoclonal antibody
MHC	major histocompatibility complex
MIR	monocyte-macrophage inhibitory receptor
N	amino
NCBI	National Center of Biotechnology Information
NCR-1	natural cytotoxicity triggering receptor 1
NFAT	nuclear factor of activated T cells
NK	natural killer
OA	osteoarthritis
PAMP	pathogen associated molecular pattern
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	programmed cell death
PD-L	programmed cell death ligand
PE	phycoerythrin
PECAM	platelet endothelial cell adhesion molecule
PIR	paired Ig-like receptor
r	rat
RA	rheumatoid arthritis
RBL	rat basophilic leukaemia
RBS9	ribosomal protein S9
RT	reverse transcriptase
RU	response units
SAP	SLAM-associated protein
SF	synovial fluid
SH	src homology
SHIP	SH2 domain-containing 5'-inositol phosphatase
SHP	SH2 domain-containing protein tyrosine phosphatase
Siglec	sialic acid binding immunoglobulin-like lectin
SIRP	signal regulatory protein
SLAM	signaling lymphocyte activation molecule
SP	surfactant protein
TNP	2,4,6-trinitrophen
TtyhI	tweety homologue 1
VWF	von Willebrand Factor
wt	wild type

SUPPLEMENTAL DATA

Table I: Peptide sequences of the collagen II Toolkit. GPO residues are shown in boldface.

#	*Sequences*
1	GPC(GPP)$_5$-GPMGPMGPR**GPO**GPAGAOGPQGFQGNO-(GPP)$_5$GPC-NH$_2$
2	GPC(GPP)$_5$-GPQGFQGNOGEOGEOGVSGPMGPR**GPO**-(GPP)$_5$GPC-NH$_2$
3	GPC(GPP)$_5$-GPMGPR**GPOGPO**GKOGDDGEAGKOGKA-(GPP)$_5$GPC-NH$_2$
4	GPC(GPP)$_5$-GEAGKOGKAGER**GPO**GPQGARGFOGTO-(GPP)$_5$GPC-NH$_2$
5	GPC(GPP)$_5$-GARGFOGTOGLOGVKGHRGYOGLDGAK-(GPP)$_5$GPC-NH$_2$
6	GPC(GPP)$_5$-GYOGLDGAKGEAGAOGVKGESGSOGEN-(GPP)$_5$GPC-NH$_2$
7	GPC(GPP)$_5$-GESGSOGENGSOGPMGPRGLOGERGRT-(GPP)$_5$GPC-NH$_2$
8	GPC(GPP)$_5$-GLOGERGRTGPAGAAGARGNDGQOGPA-(GPP)$_5$GPC-NH$_2$
9	GPC(GPP)$_5$-GNDGQOGPA**GPO**GPVGPAGGOGFOGAO-(GPP)$_5$GPC-NH$_2$
10	GPC(GPP)$_5$-GGOGFOGAOGAKGEAGPTGARGPEGAQ-(GPP)$_5$GPC-NH$_2$
11	GPC(GPP)$_5$-GARGPEGAQGPRGEOGTOGSOGPAGAS-(GPP)$_5$GPC-NH$_2$
12	GPC(GPP)$_5$-GSOGPAGASGNOGTDGIOGAKGSAGAO-(GPP)$_5$GPC-NH$_2$
13	GPC(GPP)$_5$-GAKGSAGAOGIAGAOGFOGPR**GPO**GPQ-(GPP)$_5$GPC-NH$_2$
14	GPC(GPP)$_5$-GPR**GPO**GPQGATGPLGPKGQTGEOGIA-(GPP)$_5$GPC-NH$_2$
15	GPC(GPP)$_5$-GQTGEOGIAGFKGEQGPKGEOGPAGPQ-(GPP)$_5$GPC-NH$_2$
16	GPC(GPP)$_5$-GEOGPAGPQGAOGPAGEEGKRGARGEO-(GPP)$_5$GPC-NH$_2$
17	GPC(GPP)$_5$-GKRGARGEOGGVGPI**GPO**GERGAOGNR-(GPP)$_5$GPC-NH$_2$
18	GPC(GPP)$_5$-GERGAOGNRGFOGQDGLAGPKGAOGER-(GPP)$_5$GPC-NH$_2$
19	GPC(GPP)$_5$-GPKGAOGERGPSGLAGPKGANGDOGRO-(GPP)$_5$GPC-NH$_2$
20	GPC(GPP)$_5$-GANGDOGROGEOGLOGARGLTGROGDA-(GPP)$_5$GPC-NH$_2$
21	GPC(GPP)$_5$-GLTGROGDAGPQGKVGPSGAOGEDGRO-(GPP)$_5$GPC-NH$_2$
22	GPC(GPP)$_5$-GAOGEDGRO**GPO**GPQGARGQOGVMGFO-(GPP)$_5$GPC-NH$_2$
23	GPC(GPP)$_5$-GQOGVMGFOGPKGANGEOGKAGEKGLO-(GPP)$_5$GPC-NH$_2$
24	GPC(GPP)$_5$-GKAGEKGLOGAOGLRGLOGKDGETGAA-(GPP)$_5$GPC-NH$_2$
25	GPC(GPP)$_5$-GKDGETGAA**GPO**GPAGPAGERGEQGAO-(GPP)$_5$GPC-NH$_2$
26	GPC(GPP)$_5$-GERGEQGAOGPSGFQGLO**GPOGPO**GEG-(GPP)$_5$GPC-NH$_2$
27	GPC(GPP)$_5$-**GPOGPO**GEGGKOGDQGVOGEAGAOGLV-(GPP)$_5$GPC-NH$_2$
28	GPC(GPP)$_5$-GEAGAOGLVGPRGERGFOGERGSOGAQ-(GPP)$_5$GPC-NH$_2$
29	GPC(GPP)$_5$-GERGSOGAQGLQGPRGLOGTOGTDGPK-(GPP)$_5$GPC-NH$_2$
30	GPC(GPP)$_5$-GTOGTDGPKGASGPA**GPO**GAQ**GPO**GLQ-(GPP)$_5$GPC-NH$_2$
31	GPC(GPP)$_5$-GAQ**GPO**GLQGMOGERGAAGIAGPKGDR-(GPP)$_5$GPC-NH$_2$
32	GPC(GPP)$_5$-GIAGPKGDRGDVGEKGPEGAOGKDGGR-(GPP)$_5$GPC-NH$_2$
33	GPC(GPP)$_5$-GAOGKDGGRGLTGPI**GPO**GPAGANGEK-(GPP)$_5$GPC-NH$_2$
34	GPC(GPP)$_5$-GPAGANGEKGEV**GPO**GPAGSAGARGAO-(GPP)$_5$GPC-NH$_2$
35	GPC(GPP)$_5$-GSAGARGAOGERGET**GPO**GPAGFA**GPO**-(GPP)$_5$GPC-NH$_2$

36	GPC(GPP)$_5$-GPAGFA**GPO**GADGQOGAKGEQGEAGQK-(GPP)$_5$GPC-NH$_2$
37	GPC(GPP)$_5$-GEQGEAGQKGDAGAOGPQGPSGAOGPQ-(GPP)$_5$GPC-NH$_2$
38	GPC(GPP)$_5$-GPSGAOGPQGPTGVTGPKGARGAQ**GPO**-(GPP)$_5$GPC-NH$_2$
39	GPC(GPP)$_5$-GARGAQ**GPO**GATGFOGAAGRV**GPO**GSN-(GPP)$_5$GPC-NH$_2$
40	GPC(GPP)$_5$-GRV**GPO**GSNGNO**GPOGPO**GPSGKDGPK-(GPP)$_5$GPC-NH$_2$
41	GPC(GPP)$_5$-GPSGKDGPKGARGDS**GPO**GRAGEOGLQ-(GPP)$_5$GPC-NH$_2$
42	GPC(GPP)$_5$-GRAGEOGLQGPA**GPO**GEKGEOGDDGPS-(GPP)$_5$GPC-NH$_2$
43	GPC(GPP)$_5$-GEOGDDGPSGAE**GPO**GPQGLAGQRGIV-(GPP)$_5$GPC-NH$_2$
44	GPC(GPP)$_5$-GLAGQRGIVGLOGQRGERGFOGLOGPS-(GPP)$_5$GPC-NH$_2$
45	GPC(GPP)$_5$-GFOGLOGPSGEOGKQGAOGASGDR**GPO**-(GPP)$_5$GPC-NH$_2$
46	GPC(GPP)$_5$-GASGDR**GPO**GPV**GPO**GLTGPAGEOGRE-(GPP)$_5$GPC-NH$_2$
47	GPC(GPP)$_5$-GPAGEOGREGSOGAD**GPO**GRDGAAGVK-(GPP)$_5$GPC-NH$_2$
48	GPC(GPP)$_5$-GRDGAAGVKGDRGETGAVGAOGAO**GPO**-(GPP)$_5$GPC-NH$_2$
49	GPC(GPP)$_5$-GAOGAO**GPO**GSOGPAGPTGKQGDRGEA-(GPP)$_5$GPC-NH$_2$
50	GPC(GPP)$_5$-GKQGDRGEAGAQGPMGPSGPAGARGIQ-(GPP)$_5$GPC-NH$_2$
51	GPC(GPP)$_5$-GPAGARGIQGPQGPRGDKGEAGEOGER-(GPP)$_5$GPC-NH$_2$
52	GPC(GPP)$_5$-GEAGEOGERGLKGHRGFTGLQGLO**GPO**-(GPP)$_5$GPC-NH$_2$
53	GPC(GPP)$_5$-GLQGLO**GPO**GPSGDQGASGPAGPSGPR-(GPP)$_5$GPC-NH$_2$
54	GPC(GPP)$_5$-GPAGPSGPR**GPO**GPVGPSGKDGANGIO-(GPP)$_5$GPC-NH$_2$
55	GPC(GPP)$_5$-GKDGANGIOGPI**GPO**GPRGRSGETGPA-(GPP)$_5$GPC-NH$_2$
56	GPC(GPP)$_5$-GPRGRSGETGPA**GPO**GNO**GPOGPOGPO**-(GPP)$_5$GPC-NH$_2$

Table II: Peptide sequences of the collagen III Toolkit. GPO residues are shown in boldface.

#	*Sequences*
1	GPC(GPP)$_5$-GLAGYOGPA**GPOGPOGPO**GTSGHOGSO-(GPP)$_5$GPC-NH$_2$
2	GPC(GPP)$_5$-GTSGHOGSOGSOGYQ**GPO**GEOGQAGPS-(GPP)$_5$GPC-NH$_2$
3	GPC(GPP)$_5$-GEOGQAGPS**GPOGPO**GAIGPSGPAGKD-(GPP)$_5$GPC-NH$_2$
4	GPC(GPP)$_5$-GPSGPAGKDGESGROGROGERGLO**GPO**-(GPP)$_5$GPC-NH$_2$
5	GPC(GPP)$_5$-GERGLO**GPO**GIKGPAGIOGFOGMKGHR-(GPP)$_5$GPC-NH$_2$
6	GPC(GPP)$_5$-GFOGMKGHRGFDGRNGEKGETGAOGLK-(GPP)$_5$GPC-NH$_2$
7	GPC(GPP)$_5$-GETGAOGLKGENGLOGENGAOGPMGPR-(GPP)$_5$GPC-NH$_2$
8	GPC(GPP)$_5$-GAOGPMGPRGAOGERGROGLOGAAGAR-(GPP)$_5$GPC-NH$_2$
9	GPC(GPP)$_5$-GLOGAAGARGNDGARGSDGQO**GPOGPO**-(GPP)$_5$GPC-NH$_2$
10	GPC(GPP)$_5$-GQO**GPOGPO**GTAGFOGSOGAKGEVGPA-(GPP)$_5$GPC-NH$_2$
11	GPC(GPP)$_5$-GAKGEVGPAGSOGSNGAOGQRGEOGPQ-(GPP)$_5$GPC-NH$_2$
12	GPC(GPP)$_5$-GQRGEOGPQGHAGAQ**GPOGPO**GINGSO-(GPP)$_5$GPC-NH$_2$
13	GPC(GPP)$_5$-**GPO**GINGSOGGKGEMGPAGIOGAOGLM-(GPP)$_5$GPC-NH$_2$
14	GPC(GPP)$_5$-GIOGAOGLMGAR**GPO**GPAGANGAOGLR-(GPP)$_5$GPC-NH$_2$
15	GPC(GPP)$_5$-GANGAOGLRGGAGEOGKNGAKGEOGPR-(GPP)$_5$GPC-NH$_2$
16	GPC(GPP)$_5$-GAKGEOGPRGERGEAGIOGVOGAKGED-(GPP)$_5$GPC-NH$_2$
17	GPC(GPP)$_5$-GVOGAKGEDGKDGSOGEOGANGLOGAA-(GPP)$_5$GPC-NH$_2$
18	GPC(GPP)$_5$-GANGLOGAAGERGAOGFRGPAGPNGIO-(GPP)$_5$GPC-NH$_2$
19	GPC(GPP)$_5$-GPAGPNGIOGEKGPAGERGAOGPAGPR-(GPP)$_5$GPC-NH$_2$
20	GPC(GPP)$_5$-GAOGPAGPRGAAGEOGRDGVOGGOGMR-(GPP)$_5$GPC-NH$_2$
21	GPC(GPP)$_5$-GVOGGOGMRGMOGSOGGOGSDGKO**GPO**-(GPP)$_5$GPC-NH$_2$
22	GPC(GPP)$_5$-GSDGKO**GPO**GSQGESGRO**GPO**GPSGPR-(GPP)$_5$GPC-NH$_2$
23	GPC(GPP)$_5$-**GPO**GPSGPRGQOGVMGFOGPKGNDGAO-(GPP)$_5$GPC-NH$_2$
24	GPC(GPP)$_5$-GPKGNDGAOGKNGERGGOGGOGPQ**GPO**-(GPP)$_5$GPC-NH$_2$
25	GPC(GPP)$_5$-GGOGPQ**GPO**GKNGETGPQ**GPO**GPTGPG-(GPP)$_5$GPC-NH$_2$
26	GPC(GPP)$_5$-**GPO**GPTGPGGDKGDT**GPO**GPQGLQGLO-(GPP)$_5$GPC-NH$_2$
27	GPC(GPP)$_5$-GPQGLQGLOGTG**GPO**GENGKOGEOGPK-(GPP)$_5$GPC-NH$_2$
28	GPC(GPP)$_5$-GKOGEOGPKGDAGAOGAOGGKGDAGAO-(GPP)$_5$GPC-NH$_2$
29	GPC(GPP)$_5$-GGKGDAGAOGER**GPO**GLAGAOGLRGGA-(GPP)$_5$GPC-NH$_2$
30	GPC(GPP)$_5$-GAOGLRGGA**GPO**GPEGGKGAA**GPOGPO**-(GPP)$_5$GPC-NH$_2$
31	GPC(GPP)$_5$-GAA**GPOGPO**GAAGTOGLQGMOGERGGL-(GPP)$_5$GPC-NH$_2$
32	GPC(GPP)$_5$-GMOGERGGLGSOGPKGDKGEOGGOGAD-(GPP)$_5$GPC-NH$_2$
33	GPC(GPP)$_5$-GEOGGOGADGVOGKDGPRGPTGPI**GPO**-(GPP)$_5$GPC-NH$_2$
34	GPC(GPP)$_5$-GPTGPI**GPO**GPAGQOGDKGEGGAOGLO-(GPP)$_5$GPC-NH$_2$
35	GPC(GPP)$_5$-GEGGAOGLOGIAGPRGSOGERGET**GPO**-(GPP)$_5$GPC-NH$_2$
36	GPC(GPP)$_5$-GERGET**GPO**GPAGFOGAOGQNGEOGGK-(GPP)$_5$GPC-NH$_2$
37	GPC(GPP)$_5$-GQNGEOGGKGERGAOGEKGEG**GPO**GVA-(GPP)$_5$GPC-NH$_2$

38	GPC(GPP)$_5$-GEG**GPO**GVA**GPO**GGSGPA**GPO**GPQGVK-(GPP)$_5$GPC-NH$_2$
39	GPC(GPP)$_5$-**GPO**GPQGVKGERGSOGGOGAAGFOGAR-(GPP)$_5$GPC-NH$_2$
40	GPC(GPP)$_5$-GAAGFOGARGLO**GPO**GSNGNO**GPO**GPS-(GPP)$_5$GPC-NH$_2$
41	GPC(GPP)$_5$-GNO**GPO**GPSGSOGKD**GPO**GPAGNTGAO-(GPP)$_5$GPC-NH$_2$
42	GPC(GPP)$_5$-GPAGNTGAOGSOGVSGPKGDAGQOGEK-(GPP)$_5$GPC-NH$_2$
43	GPC(GPP)$_5$-GDAGQOGEKGSOGAQ**GPO**GAOGPLGIA-(GPP)$_5$GPC-NH$_2$
44	GPC(GPP)$_5$-GAOGPLGIAGITGARGLA**GPO**GMOGPR-(GPP)$_5$GPC-NH$_2$
45	GPC(GPP)$_5$-**GPO**GMOGPRGSOGPQGVKGESGKOGAN-(GPP)$_5$GPC-NH$_2$
46	GPC(GPP)$_5$-GESGKOGANGLSGER**GPO**GPQGLOGLA-(GPP)$_5$GPC-NH$_2$
47	GPC(GPP)$_5$-GPQGLOGLAGTAGEOGRDGNOGSDGLO-(GPP)$_5$GPC-NH$_2$
48	GPC(GPP)$_5$-GNOGSDGLOGRDGSOGGKGDRGENGSO-(GPP)$_5$GPC-NH$_2$
49	GPC(GPP)$_5$-GDRGENGSOGAOGAOGHO**GPO**GPVGPA-(GPP)$_5$GPC-NH$_2$
50	GPC(GPP)$_5$-**GPO**GPVGPAGKSGDRGESGPAGPAGAO-(GPP)$_5$GPC-NH$_2$
51	GPC(GPP)$_5$-GPAGPAGAOGPAGSRGAOGPQGPRGDK-(GPP)$_5$GPC-NH$_2$
52	GPC(GPP)$_5$-GPQGPRGDKGETGERGAAGIKGHRGFO-(GPP)$_5$GPC-NH$_2$
53	GPC(GPP)$_5$-GIKGHRGFOGNOGAOGSOGPAGQQGAI-(GPP)$_5$GPC-NH$_2$
54	GPC(GPP)$_5$-GPAGQQGAIGSOGPAGPRGPVGPS**GPO**-(GPP)$_5$GPC-NH$_2$
55	GPC(GPP)$_5$-GPVGPS**GPO**GKDGTSGHOGPI**GPO**GPR-(GPP)$_5$GPC-NH$_2$
56	GPC(GPP)$_5$-GPI**GPO**GPRGNRGERGSEGSOGHOGQO-(GPP)$_5$GPC-NH$_2$
57	GPC(GPP)$_5$-GERGSEGSOGHOGQO**GPOGPO**GAOGPC-(GPP)$_5$GPC-NH$_2$

Table III: Peptide sequences of Toolbox and collagen III peptide 30 variants. GPO residues are shown in boldface. Top panel represents Toolbox peptides, bottom panel represent III-30 variants.

#	*Sequences*
$(GPP)_{10}$	GPC-GPPGPPGPPGPPGPPGPPGPPGPPGPPGPP-GPC-NH_2
CRP	GPC-**GPOGPOGPOGPOGPOGPOGPOGPOGPOGPO**-GPC-NH_2
$(GPO)_1$	GPC-GPPGPPGPPGPPGPP**GPO**GPPGPPGPPGPP-GPC-NH_2
$(GPO)_2$	GPC-GPPGPPGPPGPP**GPOGPO**GPPGPPGPPGPP-GPC-NH_2
$(GPO)_4$	GPC-GPPGPPGPP**GPOGPOGPOGPO**GPPGPPGPP-GPC-NH_2
$(GPO)_6$	GPC-GPPGPP**GPOGPOGPOGPOGPOGPO**GPPGPP-GPC-NH_2
III-30	GPC$(GPP)_5$-GAOGLRGGA**GPO**GPEGGKGAA**GPOGPO**-$(GPP)_5$GPC-NH_2
III-30a	GPC$(GPP)_5$-GAOGLRGGAGPPGPEGGKGAA**GPOGPO**-$(GPP)_5$GPC-NH_2
III-30b	GPC$(GPP)_5$-GAOGLRGGA**GPO**GPEGGKGAAGPP**GPO**-$(GPP)_5$GPC-NH_2
III-30c	GPC$(GPP)_5$-GAOGLRGGA**GPO**GPEGGKGAA**GPO**GPP-$(GPP)_5$GPC-NH_2
III-30d	GPC$(GPP)_5$-GGA**GPO**GPEGGKGAA**GPOGPO**-$(GPP)_5$GPC-NH_2
III-30e	GPC$(GPP)_5$-GAOGLRGGA**GPO**GPEGGKGAA-$(GPP)_5$GPC-NH_2
III-30f	GPC$(GPP)_5$-GGA**GPO**GPEGGKGAA-$(GPP)_5$GPC-NH_2