IMMUNE REGULATION BY LAIR PROTEINS

Marloes Olde Nordkamp

Commissie:

Prof. dr. A.B.J. Prakken

Prof. dr. H.P. Haagsman

Prof. dr. C. van Kooten

Dr. T.K. van den Berg

Prof. dr. T.R.D.J. Radstake

Paranimfen:

Margreet Westerlaken

Michel Olde Nordkamp

ISBN: 978-90-393-5989-1

Layout & Printing: Off Page, Amsterdam, the Netherlands

The research described in this thesis was performed at the Department of Immunology, Laboratory of Translational Immunology from the University Medical Centre Utrecht, the Netherlands.

Printing of this thesis was financially supported by: Infection and Immunity Center Utrecht

IMMUNE REGULATION
BY
LAIR PROTEINS

Immuun regulatie door LAIR eiwitten

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 5 september 2013 des middags te 12.45 uur

door

Marloes Josefina Maria Olde Nordkamp

geboren op 12 november 1983 te Hengelo

Promoter: Prof. dr. L. Meyaard

The studies in this thesis were financially supported by the Dutch Arthritis Foundation (grant 06-1-403)

Aan mijn ouders

CONTENTS

1

GENERAL INTRODUCTION AND THE SCOPE OF THIS THESIS

INTRODUCTION

This thesis is about immune regulation by the inhibitory immune receptor leukocyte associated immunoglobulin-like receptor-1 (LAIR-1) and proteins derived thereof.

The immune system plays a pivotal role in the defense of our body against pathogens. Not only does it protect us against numerous viruses, bacteria and fungi on a daily basis, it is also capable of eliminating tumor cells. To do so, the immune system must recognize microbes and cells that are infected or abnormal. Dying, stressed or injured cells are recognized because they release danger associated molecular patterns (DAMPs) or express them at their cell surface (1). These DAMPs can function either as a trigger for the immune system, or as an adjuvant in an already ongoing immune response (2). Pathogen recognition receptors (PRRs) recognize pathogen associated molecular patterns (PAMPs) such as LPS, flaggelin, single stranded RNA or zymosan (3). There are four major classes of PRRs, namely Toll like receptors, C-type lectin receptors, nucleotide-binding domain leucine-rich repeat-containing receptors and the RigI helicases (3). Besides being recognized by PRRs, pathogens can also be bound by antibodies in a process called opsonization. These bound antibodies can form immune complexes which can be recognized by Fc receptors which are involved in cellular processes such as phagocytosis, degranulation and antibody dependent cell mediated cytotoxicity (4;5) or by the complement system (Figure 1).

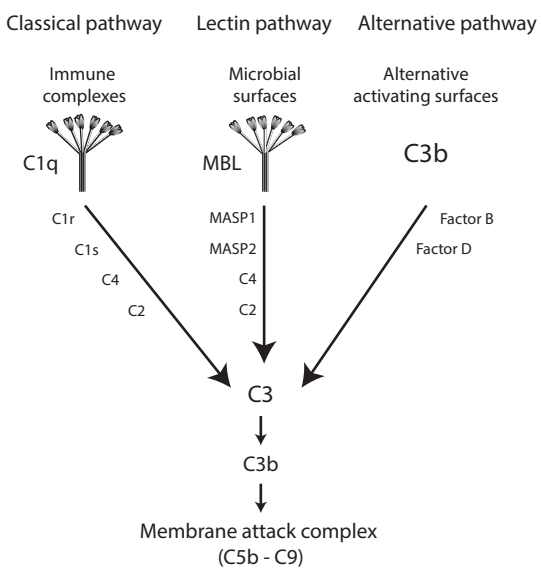


Figure 1: The complement pathway. Schematic and simplified representation of the three pathways of the complement system.

The complement system

The classical pathway of the complement system is often referred to as the antibody dependent pathway and can be activated by IgM or IgG immune complexes (6). C1q, the key initiator of the classical pathway, can also activate the classical pathway by direct recognition of distinct structures on microbial, or even apoptotic cell surfaces (7). Mannose binding lectin (MBL), the key initiator of the lectin pathway, which is another major complement pathway, can activate the complement system by binding to carbohydrate patterns (8). Activation of the complement system does not only lead to lysis of the pathogen or apoptotic cell, it also orchestrates the immune system by releasing for instance chemoattractants and anaphylatoxins (6).

Generally, the immune system is capable of performing its task without inflicting damage upon one self, but to do so, it needs to be tightly regulated.

Immune regulation

Multiple layers of immune regulation need to be in place to ensure that no inappropriate or excess immune activation takes place, since that could lead to severe tissue damage. Furthermore, the immune response needs to be terminated at the appropriate time to restore homeostasis. One of the ways to accomplish this is by the release of anti-inflammatory cytokines such as interleukin-10 or transforming growth factor-β. Regulated apoptosis of immune cells can control an immune response in another way. Finally, the expression of inhibitory immune receptors can provide the necessary fine tuning of an immune response to ensure that the threat is eliminated while there is no damage to 'self'.

So far, about 60 inhibitory immune receptors have been described, while a genome wide screen has shown that there can be as many as 300 expressed in the human genome (9).

Although there are many inhibitory receptors, there does not seem to be redundancy. Inhibitory receptor knockout mice are often prone to autoimmune disease, and may develop autoimmunity upon stimulation of the immune system, or develop uncontrolled inflammation in response to infection. Depending on the background, many inhibitory receptor deficient mice are more susceptible to developing for instance SLE-like and rheumatoid arthritis like disease. For instance, CD22-deficient mice are prone to develop high affinity isotype switched auto antibodies to double stranded DNA (10). The $CD200^{-/-}$ mouse, which has defective CD200R signaling, as well as the platelet endothelial cell adhesion molecule $(PECAM)^{-/-}$ mouse show an increased sensitivity to the collagen induced arthritis model, which is often used as a mouse model for arthritis (11;12).

Programmed death-1 (PD-1) deficient mice on a 129svEv-Brd background have an increased susceptibility for experimental autoimmune encephalomyelitis (EAE), which is a commonly used mouse model for multiple sclerosis (MS) while PD-1 deficient mice on a C57BL/6 background develop auto antibodies and IgG3 immune complex mediated glomerulonephritis which is similar to that seen in humans suffering from systemic lupus erythematosus (SLE) (13;14).

Most inhibitory immune receptors express an intracellular immunoreceptor tyrosine-based inhibitory motif (ITIM) of which the prototype amino-acid sequence is (I/V/L/S)xYxx(L/V), or immunoreceptor tyrosine-based switch motif (ITSM) of which the prototype amino-acid sequence is TxYxx(V/I) where x denotes any amino acid (15;16). Upon ligation of the receptor with their ligand, the tyrosines get phosphorylated by Src family tyrosine kinases and recruit SH-2 domain containing phosphatases such as SHP-1, SHP-2 or SHIP (9). These phosphatases also get phosphorylated and activated by the Src family tyrosine kinases and suppress cellular activation by dephosphorylating effector molecules (17). Although the recruitment of phosphastases is the most common manner of mediating inhibition, it has been shown that the recruitment of C-terminal Src kinase (CSK) can also inhibit cellular activation (18-20).

Regulation of inhibitory receptors

The signaling of these inhibitory immune receptors, in turn, needs to be regulated. Firstly, these receptors are selectively expressed by different cell subsets. While some receptors, such as CD22 are only expressed by one cell type, others such as PD-1 are expressed by multiple cell types (21). Secondly, the expression of these receptors can also be regulated during the immune response. It is known for instance, that the expression of LAIR-1 is down regulated on activated T cells and maturing neutrophils in such a way that blood neutrophils do not express LAIR-1(22;23). Upon stimulation with G-CSF, LAIR-1 expression on neutrophils is induced (23). Another inhibitory receptor, signal *inhibitory receptor* on leukocytes-1 (SIRL-1) is expressed on blood neutrophils and down regulated upon activation (24;25). Thirdly, the availability of the ligand plays a role in the regulation of the immune response. This can be availability by location; in the bloodstream for instance, is no readily available collagen for the inhibitory collagen receptor LAIR-1. Upon migration to damaged or inflamed tissue, collagen becomes available to act as a ligand. Furthermore, the availability of the ligand can be regulated by the production of competitors for the binding sites on the ligand. There are four soluble variants of PD-1 that are obtained by alternative splicing which can bind PD-L1 and PD-L2, and therefore can compete for ligand binding sites (26). Soluble cytotoxic T-lymphocyte antigen-4 (sCTLA-4), is formed by alternative splicing and is capable of binding the CTLA-4 ligand B7 (27). Soluble leukocyte Ig-like receptor (LILR)B1, which can be formed by alternative splicing, antagonizes the effect of membrane bound LILRB1 on NK-cells in vitro (28) LAIR-2 is a soluble protein which is produced by various hemopoietic cell types which can block the collagen interaction of LAIR-1 (29).

Interestingly, the occurrence of these soluble inhibitory receptors can be linked to disease. One of the four soluble variants of PD-1 is elevated in the serum of aplastic anaemia patients and is correlated significantly with tumor necrosis factor-alpha concentrations in synovial fluid derived from rheumatoid arthritis (RA) patients (26;30). sCTLA-4 can be measured in sera of healthy individuals and elevated levels are observed in patients with Graves disease, autoimmune thyroiditis, myasthenia gravis, SLE,

spondylarthropathy and systemic sclerosis (31-37). Another soluble inhibitory receptor, sLILRB4, was found in high concentrations in patients with melanoma and carcinomas of the colon, rectum and pancreas (38). sLAIR-1 levels in the urine of hemorrhagic fever with renal syndrome patients, or patient 3-7 days after renal transplantation, were higher than sLAIR-1 levels of healthy controls. Moreover, sLAIR-1 levels in the serum of transplant patients with rejection were higher than those without rejection (39). LAIR-2 levels were elevated in urine of pregnant women, as compared to healthy controls and in the synovial fluid of RA patients as compared to osteo-arthritis patients (29). Thus, these soluble inhibitory receptors may function as biomarkers for disease.

LAIR-1

In this thesis we focus on the inhibitory immune receptor LAIR-1 (Figure 2). LAIR-1 is a transmembrane receptor containing an extracellular C2-type Ig-like domain and two ITIMs in its cytoplasmic tail which was first cloned in 1997 (40). Mouse LAIR-1 and rat LAIR-1 were discovered in later years (41;42). A duplication event in the human genome led to the rise of *LAIR-2*, which is ~84% homologous to *LAIR-1*, but lacks the transmembrane

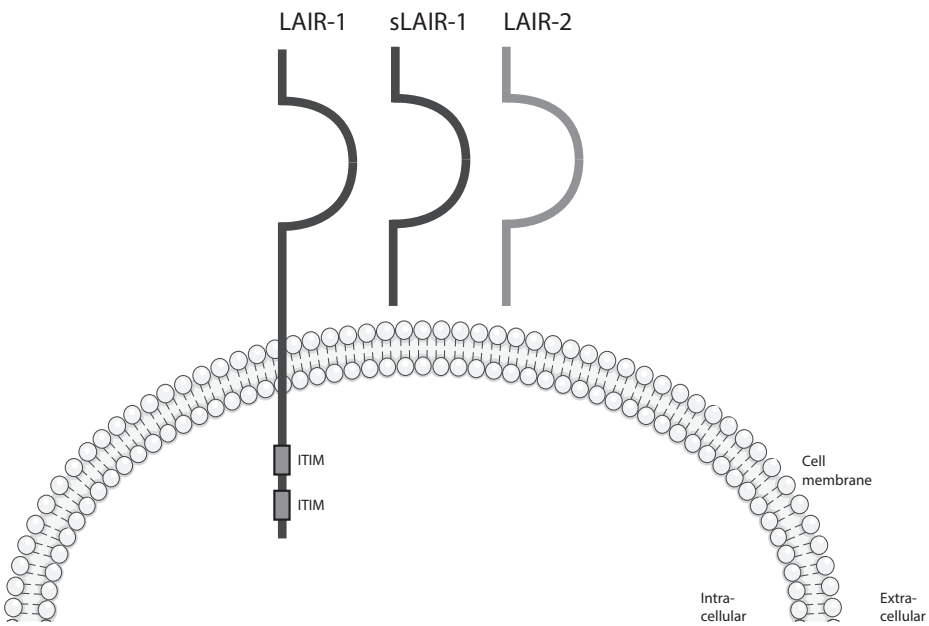


Figure 2: Schematic representation of LAIR-1, sLAIR-1 and LAIR-2. LAIR -1 is a transmembrane protein with an Ig-like domain in the extracellular space, and two ITIMs in its cytoplasmic domain. sLAIR-1 is presumably shed from the cell surface and consists of the extracellular domain of LAIR-1. LAIR-2 is a homologue of LAIR-1, which consists of an Ig-domain. It lacks the transmembrane and intracellular domains and is therefore a secreted protein. This figure is not drawn to scale and was produced using Servier Medical Art.

and intracellular domains of *LAIR-1* and is therefore a secreted protein (43;44). LAIR-1 is expressed on the majority of immune cells, including NK cells, T cells, B cells, monocytes (45;46) and monocyte-derived dendritic cells (47), eosinophils (23), basophils, mast cells (48) $CD34^+$ hematopoietic progenitor cells (23;49) and the majority of thymocytes (40;46). LAIR-1 inhibitory function was demonstrated by inhibiting cytotoxicity *in vitro* in NK and T cells, inhibition of calcium mobilization and down regulation of Ig and cytokine production in B cells, and inhibition of maturation in dendritic cells (40;43;47;50;51). Collagens were discovered as ligands for LAIR-1 and LAIR-2 (29;52). These trimeric extracellular martix and membrane molecules are composed of three polypeptide α chains containing the sequence repeat $(Gly\text{-}x\text{-}Y)_n$, x frequently being Proline (P) and Y being hydroxyproline after post-translational modification (53). Interaction of LAIR-1 was shown to be dependent of the presence of hydroxiproline in the Gly-Pro-Hyp collagen repeats (52). Collagens are the most abundant proteins in the body and so far, LAIR-1 seems to interact with all types of collagen tested (52). Mapping of the collagen binding sites showed that there are several binding sites on collagen for LAIR-1 and LAIR-2, although LAIR-2 seems to bind more different sites than LAIR-1 (54). Since both collagen and LAIR-1 are widely expressed, the regulation of the interaction needs to be tightly controlled by regulation of expression of the receptor and the production of soluble competitors for binding sites, as discussed above.

Collagenous domain containing proteins

Besides collagens there are multiple proteins that contain collagenous domains, such as the immune regulatory surfactant protein-D (SP-D) and the key initiators of the classical and lectin pathway of the complement system, C1q and MBL (Figure 3). SP-D possesses a 177-aa-long collagenous domain (55), and is abundantly expressed at mucosal surfaces where it functions as an opsonin (reviewed in (56)). It is known to have

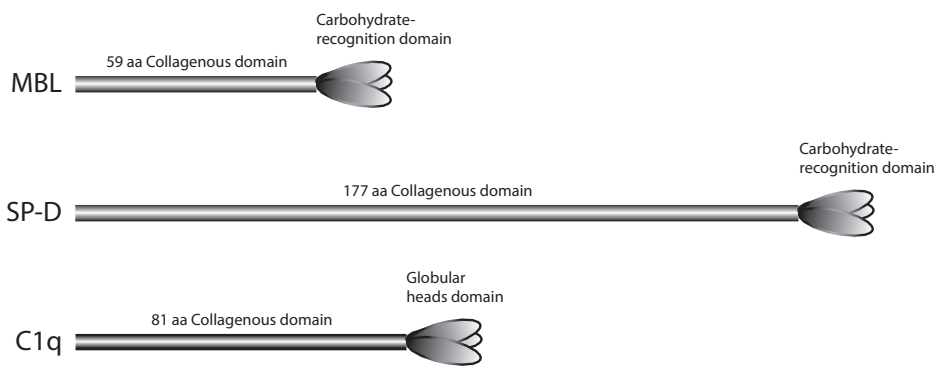


Figure 3: Schematic representation of the collagenous domain containing proteins MBL, SPD and C1q. Schematic overview of subunits of MBL, SP-D and C1q, showing the length of the collagenous domains. This representation is not drawn to scale. aa: amino acid.

immune regulatory functions, of which the mechanisms are incompletely understood. Both C1q and MBL are abundantly present in blood. They are oligomeric proteins made up of up to 6 subunits which each consist of a homo-trimer of the same polypeptide chain. These chains all contain an N-terminal collagen-like region (57). The quantity of C1q can be as much as 113±40 µg/ml serum (58). The general hypothesis of immune regulation by LAIR-1 was based on the assumption that there is no ligand available in steady state conditions in the bloodstream, and injury or inflammation leads to tissue damage, thereby exposing collagen as a ligand and ensuring proper immune regulation (59). The binding of LAIR proteins to collagenous domains of for instance SP-D or C1q needs to be investigated to further elucidate LAIR-1 mediated immune regulation.

SCOPE OF THIS THESIS

The immune system needs to be strictly regulated in order to function properly. Inhibitory immune receptors play an important role in fine-tuning the immune response. The general assumption is that too much inhibition leads to immune deficiencies and opportunistic infections of pathogens, while to little inhibition leads to an exaggerated immune response, leading to damage to self or autoimmune disorders. In Chapter 2 we review whether mutations in inhibitory immune receptors actually do predispose to autoimmune disease.

Further regulation of the immune response by the shed extracellular part of LAIR-1 or the production of LAIR-2, and whether these proteins can be used as biomarkers is elucidated in Chapter 3. In Chapter 4 we investigated the origin of sLAIR-1 in amniotic fluid, as well as the potential role of sLAIR-1 as a biomarker for fetal lung maturity.

In Chapter 5 we reveal that the immunoregulatory collectin surfactant protein-D, which contains a collagen like domain, can exert its inhibitory functions via LAIR-1. After showing that LAIR proteins can bind molecules which express a collagenous domain, we describe in Chapter 6 that LAIR-2 fusion protein can inhibit the lectin and classical pathway of the complement system, thereby posing it as a novel potential therapy to prevent rejection after organ transplantation or to prevent myocardial damage following reperfusion of coronary arteries. Finally, the implications of these findings are discussed in Chapter 7.

Taken together, this thesis provides further insight in the regulation of the immune system via inhibitory immune receptors.

REFERENCE LIST

1. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol 1994;12:991-1045.
2. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. Nat Rev Cancer 2012 Dec;12(12):860-75.
3. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006 Feb 24;124(4):783-801.
4. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. Annu Rev Immunol 1999;17:593-623.
5. Ravetch JV, Bolland S. IgG Fc receptors. Annu Rev Immunol 2001;19:275-90.
6. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010 Sep;11(9):785-97.
7. Wallis R, Mitchell DA, Schmid R, Schwaeble WJ, Keeble AH. Paths reunited: Initiation of the classical and lectin pathways of complement activation. Immunobiology 2010;215(1):1-11.
8. Chen CB, Wallis R. Two mechanisms for mannose-binding protein modulation of the activity of its associated serine proteases. J Biol Chem 2004 Jun 18;279(25):26058-65.
9. Daeron M, Jaeger S, Du Pasquier L, Vivier E. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev 2008;224:11-43.
10. O'Keefe TL, Williams GT, Batista FD, Neuberger MS. Deficiency in CD22, a B cell-specific inhibitory receptor, is sufficient to predispose to development of high affinity autoantibodies. J Exp Med 1999 Apr 19;189(8):1307-13.
11. Wong MX, Hayball JD, Hogarth PM, Jackson DE. The inhibitory co-receptor, PECAM-1 provides a protective effect in suppression of collagen-induced arthritis. J Clin Immunol 2005 Jan;25(1):19-28.
12. Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, *et al.* Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science 2000;290:1768-71.
13. Carter LL, Leach MW, Azoitei ML, Cui J, Pelker JW, Jussif J, *et al.* PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. J Neuroimmunol 2007 Jan;182(1-2):124-34.
14. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999 Aug;11(2):141-51.
15. Daeron M, Latour S, Malbec O, Espinosa E, Pina P, Pasmans S, *et al.* The same tyrosine-based inhibition motif, in the intracytoplasmic domain of FCgammaRIIB, regulates negatively BCR-, TCR-, and FcR-dependent cell activation. Immunity 1995;3:635-46.
16. Shlapatska LM, Mikhalap SV, Berdova AG, Zelensky OM, Yun TJ, Nichols KE, *et al.* CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. J Immunol 2001 May 1;166:5480-7.
17. Long EO. Negative signaling by inhibitory receptors: the NK cell paradigm. Immunol Rev 2008 Aug;224:70-84.
18. Verbrugge A, Rijkers ESK, De Ruiter T, Meyaard L. Leukocyte-associated Ig-like receptor-1 has SH-2 domain-containing phosphatase-independent function and recruits C-terminal Src kinase. Eur J Immmunol 2006 Jan;36:190-8.
19. Veillette A, Thibaudeau E, Latour S. High expression of inhibitory receptor SHPS-1 and its association with protein-tyrosine phosphatase SHP-1 in macrophages. J Biol Chem 1998;273:22719-28.
20. Sayos J, Martinez-Barriocanal A, Kitzig F, Bellon T, Lopez-Botet M. Recruitment of C-terminal Src kinase by the leukocyte inhibitory receptor CD85j. Biochem Biophys Res Commun 2004 Nov 12;324:640-7.

21. Pritchard NR, Smith KG. B cell inhibitory receptors and autoimmunity. Immunology 2003 Mar;108:263-73.
22. Jansen CA, Cruijsen C, De Ruiter T, Nanlohy N, Willems N, Janssens-Corpela P, *et al.* Regulated expression of the inhibitory receptor LAIR-1 on human peripheral T cells during T cell activation and differentiation. Eur J Immmunol 2007 May 1;37:914-24.
23. Verbrugge A, De Ruiter T, Geest C, Coffer PJ, Meyaard L. Differential expression of Leukocyte Associated Ig-like Receptor-1 during neutrophil differentiation and activation. J Leukoc Biol 2006;79:828-36.
24. Steevels TAM, Lebbink RJ, Westerlaken GHA, Coffer PJ, Meyaard L. Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) is a novel functional inhibitory immune receptor expressed on human phagocytes. J Immunol 2010;184:4741-8.
25. Steevels TA, Van AK, Westerlaken GH, Stalpers F, Walk J, Bont L, *et al.* Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) negatively regulates the oxidative burst in human phagocytes. Eur J Immunol 2013 Feb 22.
26. Wu H, Miao M, Zhang G, Hu Y, Ming Z, Zhang X. Soluble PD-1 is associated with aberrant regulation of T cells activation in aplastic anemia. Immunol Invest 2009;38(5):408-21.
27. Magistrelli G, Jeannin P, Herbault N, oit De CA, Gauchat JF, Bonnefoy JY, *et al.* A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. Eur J Immunol 1999 Nov;29(11):3596-602.
28. Jones DC, Roghanian A, Brown DP, Chang C, Allen RL, Trowsdale J, *et al.* Alternative mRNA splicing creates transcripts encoding soluble proteins from most LILR genes. Eur J Immunol 2009 Nov;39(11):3195-206.
29. Lebbink RJ, van den Berg MCW, De Ruiter T, Raynal N, van Roon JAG, Lenting PJ, *et al.* The soluble LAIR-2 antagonizes the collagen/LAIR-1 inhibitory immune interaction. J Immunol 2008;180:1662-9.
30. Wan B, Nie H, Liu A, Feng G, He D, Xu R, *et al.* Aberrant regulation of synovial T cell activation by soluble costimulatory molecules in rheumatoid arthritis. J Immunol 2006 Dec 15;177(12):8844-50.
31. Daroszewski J, Pawlak E, Karabon L, Frydecka I, Jonkisz A, Slowik M, *et al.* Soluble CTLA-4 receptor an immunological marker of Graves' disease and severity of ophthalmopathy is associated with CTLA-4 Jo31 and CT60 gene polymorphisms. Eur J Endocrinol 2009 Nov;161(5):787-93.
32. Saverino D, Brizzolara R, Simone R, Chiappori A, Milintenda-Floriani F, Pesce G, *et al.* Soluble CTLA-4 in autoimmune thyroid diseases: relationship with clinical status and possible role in the immune response dysregulation. Clin Immunol 2007 May;123(2):190-8.
33. Wang XB, Kakoulidou M, Giscombe R, Qiu Q, Huang D, Pirskanen R, *et al.* Abnormal expression of CTLA-4 by T cells from patients with myasthenia gravis: effect of an AT-rich gene sequence. J Neuroimmunol 2002 Sep;130(1-2):224-32.
34. Liu MF, Wang CR, Chen PC, Fung LL. Increased expression of soluble cytotoxic T-lymphocyte-associated antigen-4 molecule in patients with systemic lupus erythematosus. Scand J Immunol 2003 Jun;57(6):568-72.
35. Wong CK, Lit LC, Tam LS, Li EK, Lam CW. Aberrant production of soluble costimulatory molecules CTLA-4, CD28, CD80 and CD86 in patients with systemic lupus erythematosus. Rheumatology (Oxford) 2005 Aug;44(8):989-94.
36. Toussirot E, Saas P, Deschamps M, Pouthier F, Perrot L, Perruche S, *et al.* Increased production of soluble CTLA-4 in patients with spondylarthropathies correlates with disease activity. Arthritis Res Ther 2009;11(4):R101.
37. Sato S, Fujimoto M, Hasegawa M, Komura K, Yanaba K, Hayakawa I, *et al.* Serum soluble CTLA-4 levels are increased in diffuse cutaneous systemic sclerosis. Rheumatology (Oxford) 2004 Oct;43(10):1261-6.

38. Suciu-Foca N, Feirt N, Zhang QY, Vlad G, Liu Z, Lin H, *et al.* Soluble Ig-like transcript 3 inhibits tumor allograft rejection in humanized SCID mice and T cell responses in cancer patients. J Immunol 2007 Jun 1;178(11):7432-41.
39. Ouyang W, Xue J, Liu J, Jia W, Li Z, Xie X, *et al.* Establishment of an ELISA system for determining soluble LAIR-1 levels in sera of patients with HFRS and kidney transplant. J Immunol Methods 2004 Sep;292(1-2):109-17.
40. Meyaard L, Adema GJ, Chang C, Woollatt E, Sutherland GR, Lanier LL, *et al.* LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. Immunity 1997 Aug;7(2):283-90.
41. Lebbink RJ, De Ruiter T, Verbrugge A, Bril WS, Meyaard L. 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 2004 May 1;172:5535-43.
42. Lebbink RJ, De Ruiter T, Kaptijn GJA, Meyaard L. Identification and characterization of the rat homologue of LAIR-1. Immunogenetics 2005;57:344-51.
43. Meyaard L, Hurenkamp J, Clevers H, Lanier LL, Phillips JH. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. J Immunol 1999;162:5800-4.
44. Wende H, Volz A, Ziegler A. Extensive gene duplications and a large inversion characterize the human leukocyte receptor cluster. Immunogenetics 2000;51:703-13.
45. Meyaard L, Adema GJ, Chang C, Woollatt E, Sutherland GR, Lanier LL, *et al.* LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. Immunity 1997;7:283-90.
46. Poggi A, Pella N, Morelli L, Spada F, Revello V, Sivori S, *et al.* p40, a novel surface molecule involved in the regulation of the non-major histocompatibility complex-restricted cytolytic activity in humans. Eur J Immunol 1995;25:369-76.
47. Poggi A, Tomasello E, Ferrero E, Zocchi MR, Moretta L. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. Eur J Immunol 1998;28:2086-91.
48. Florian S, Sonneck K, Czerny M, Hennersdorf F, Hauswirth AW, Buhring HJ, *et al.* Detection of novel leukocyte differentiation antigens on basophils and mast cells by HLDA8 antibodies. Allergy 2006 Sep;61(9):1054-62.
49. Ouyang WM, Ma DC, Lin D, Sun YH, Liu XS, Li Q, *et al.* 9.1C3 is identical to LAIR-1, which is expressed on hematopoietic progenitors. Biochemical and Biophysical Research Communications 2003 Oct 31;310(4):1236-40.
50. Van der Vuurst de Vries A, Clevers H, Logtenberg T, Meyaard L. Leukocyte Associated Ig-like Receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. Eur J Immunol 1999;29:3160-7.
51. Merlo A, Tenca C, Fais F, Battini L, Ciccone E, Grossi CE, *et al.* Inhibitory Receptors CD85j, LAIR-1, and CD152 Down-Regulate Immunoglobulin and Cytokine Production by Human B Lymphocytes. Clin Diagn Lab Immunol 2005 Jun;12:705-12.
52. Lebbink RJ, De Ruiter T, Adelmeijer J, Brenkman AB, van Helvoort JM, Koch M, *et al.* Collagens are functional, high-affinity ligands for the inhibitory immune receptor LAIR-1. J Exp Med 2006;203:1419-25.
53. Ricard-Blum S, Ruggiero F. The collagen superfamily: from the extracellular matrix to the cell membrane. Pathol Biol (Paris) 2005 Sep;53(7):430-42.
54. Lebbink RJ, Raynal N, De Ruiter T, Bihan D, Farndale RW, Meyaard L. Identification of multiple potent binding sites for human leukocyte associated Ig-like receptor LAIR on collagens II and III. Matrix Biol 2009 May;28:202-10.
55. Haagsman HP, Hogenkamp A, van EM, Veldhuizen EJ. Surfactant collectins and innate immunity. Neonatology 2008;93(4):288-94.

56. Wright JR. Immunoregulatory functions of surfactant proteins. Nat Rev Immunol 2005 Jan;5(1):58-68.
57. Fujita T. Evolution of the lectin-complement pathway and its role in innate immunity. Nat Rev Immunol 2002 May;2(5):346-53.
58. Dillon SP, D'Souza A, Kurien BT, Scofield RH. Systemic lupus erythematosus and C1q: A quantitative ELISA for determining C1q levels in serum. Biotechnol J 2009 Aug;4(8):1210-4.
59. Meyaard L. The inhibitory collagen receptor LAIR-1 (CD305). J Leukoc Biol 2008 Apr;83(4):799-803.

2

DO INHIBITORY IMMUNE RECEPTORS PLAY A ROLE IN THE ETIOLOGY OF AUTOIMMUNE DISEASE?

Marloes J.M. Olde Nordkamp[1], Bobby P. Koeleman[2], Linde Meyaard[1]

Submitted

[1] Laboratory of Translational Immunology, Department of Immunology, [2] Section Complex Genetics, Department of Medical Genetics, University Medical Centre Utrecht, the Netherlands

ABSTRACT

Inhibitory receptors are thought to be important in balancing immune responses. The general assumption is that lack of inhibition predisposes for autoimmune diseases caused by an overactive immune system. Here, we review the various experimental and patient data that seem to support this assumption. Individual inhibitory immune receptor knockout mice often display an autoimmune phenotype. In humans, disturbed inhibitory receptor expression and function can be found in autoimmune patients. However, in humans genetic evidence implicates only a limited number of inhibitory receptors. Large Genome-Wide Association Studies (GWAS) have established common variation in a few inhibitory receptor genes as risk factors for different autoimmune diseases. For example, genetic variation in *FCγRIIB*, *PD-1* and *CTLA-4* has been detected in various, yet not all, autoimmune diseases.

The question arises whether inhibitory receptor function is a major determinant of autoimmune disease in general. In this respect, the finding that *CSK* and *PTPN22* are strongly associated with multiple autoimmune diseases is of interest. We propose a model in which these molecules are involved in inhibitory receptor signaling. We conclude that although on individual level malfunctioning of inhibitory receptors may be of great influence, common genetic variation of inhibitory receptors, with few exceptions, is not a determining factor for autoimmunity in humans. However, common downstream signaling pathways are.

INHIBITORY IMMUNE RECEPTORS

The proper functioning of the immune system is based on a fine balance between activation and inhibition. Too much inhibition leads to the threat of infection by pathogens and immunodeficiency, while insufficient inhibition leads to damage to self or autoimmunity (1).

Inhibitory immune receptors play a crucial role in this balance. Most inhibitory immune receptors contain one or more immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tail. An ITIM is formed by a six amino acid consensus sequence (V/L/I/S)-x-Y-x-x-(L/V/I) in which x represents any amino acid (2). Ligation of the receptor bearing the ITIM leads to phosphorylation of the tyrosine which recruits SH-2 domain containing phosphatases such as SHP-1, SHP-2 or SHIP (3) or the inhibitory C-terminal Src kinase (CSK) (4). Although most inhibitory receptors exert their immune regulating function via ITIMs, some receptors such as CD200R and CTLA-4 signal via other signaling motifs. So far, about 60 inhibitory immune receptors have been characterized, while a genome wide analysis indicated that there are as many as 300 genes encoding for putative ITIM-bearing transmembrane proteins in the human genome (3), which probably all have different ligands.

The inhibitory immune receptors discussed in this review, along with their expression patterns and ligands are depicted in Table 1. The expression pattern of inhibitory receptors varies greatly. While some receptors, such as Siglec-2 on B cells are only expressed by one cell type, others such as PD-1 are expressed by multiple cell types (1). Furthermore, the expression of these receptors varies with the activation or differentiation state of the cell. The expression of leukocyte-associated immunoglobulin-like receptor -1 (LAIR-1) is down regulated on activated T cells and during neutrophil maturation, resulting in absence of LAIR-1 on blood neutrophils (5;6). Ligands for inhibitory receptor vary greatly in nature and distribution, being cell-expressed ligands (CD47 for SIRP-α), or soluble ligands (immune complexes for FCγRIIB) or very abundantly expressed ligands (collagens for LAIR-1). Thus each inhibitory receptor is thought to have its specific role in a distinct phase or place during the immune response.

Many papers claim a connection between malfunction of an inhibitory receptor and the predisposition for autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), type 1diabetes (T1D) and multiple sclerosis (MS).

In this review we discuss the literature on immune inhibitory receptors and autoimmune disease, based on data in inhibitory receptor knockout mice, functional studies with human cells and large genome wide association studies (GWAS) and META-analysis studies.

INHIBITORY RECEPTOR DEFICIENT MICE

Genetically modified mice that are deficient for a specific inhibitory receptor often display autoimmune like diseases. Depending on the background, many inhibitory receptor

Table 1: Overview of the inhibitory immune receptors discussed in this review, with their expression patterns and ligands

Receptor	Expression	Ligand	
Ig super family			
FCγRIIB	B cells	Immune complexes	(97)
TIGIT	T and NK cells	poliovirus receptor and PVRL2	(98;99)
PECAM-1	platelets, monocytes, neutrophils, T and B cells and endothelial cells	PECAM-1, CD177	(100)
ILT-2	T cells (during development), monocytes, B cells, DCs, NK cells	HLA-G	(101)
PD-1	Activated T and B cells and monocytes, NKT cells, NK cells and subsets DCs	PDL-1 and PDL-2	(102)
BTLA	B cells, T_h1 cells, NK cells, macrophages, and DCs	Herpesvirus entry mediator (HVEM)	(103)
IREM-1	macrophages, DCs, granulocytes, mast cells, and a subset of B cells	Unknown	(22;104)
TIM3	T_h1, naive DCs and monocytes	galectin-9	(105)
CEACAM-1	epithelial cells, T cells, B cells, NK cells and neutrophils	CEACAM-1	(106)
SIRPα	Myeloid and neuronal cells	CD47	(107)
LAIR-1	NK cells, T cells, B cells, monocytes, DCs, eosinophils, basophils, mast cells, $CD34^+$ hematopoietic progenitor cells, subset of megakaryocytes	Collagen	(108)
2B4	NK cells, CD8 T cells, γδT cells, eosinophils, basophils, monocytes, $CD34^+$ hematopoietic progenitors	CD48	(109)
KIRs			
KIR3DL2	NK cells, effector memory T cells	HLA-C	(110)
Siglecs			
Siglec-2	B cells	Sialic acid	(111)
C-type lectin			
DCIR	monocytes, granulocytes, DC, B cells.	HIV-1 (gp120); HepC Virus (E2)	(112)
CD94 / NKG2a	NK cells, activated T cells	HLA-E	(113)
Non-ITIM			
CTLA-4	T cells	CD80 and CD86	(114)
CD200R	Mast cells, neutrophils, macrophages, dendritic cells, T and B cells, neurons, cardiomyocytes, endothelial cells, trophoblasts, retinal and optical nerve cells, and keratinocytes	CD200	(115)

DCs = dendritic cells; NK = natural killer cells, NKT = Natural Killer T cel

knockout mice are more susceptible to develop for instance SLE-like and rheumatoid arthritis-like disease. Most commonly, studies with inhibitory receptor knockout mice are using experimental models for arthritis, MS and SLE. An overview of the involvement of different inhibitory receptors in mouse autoimmune models is depicted in figure 1.

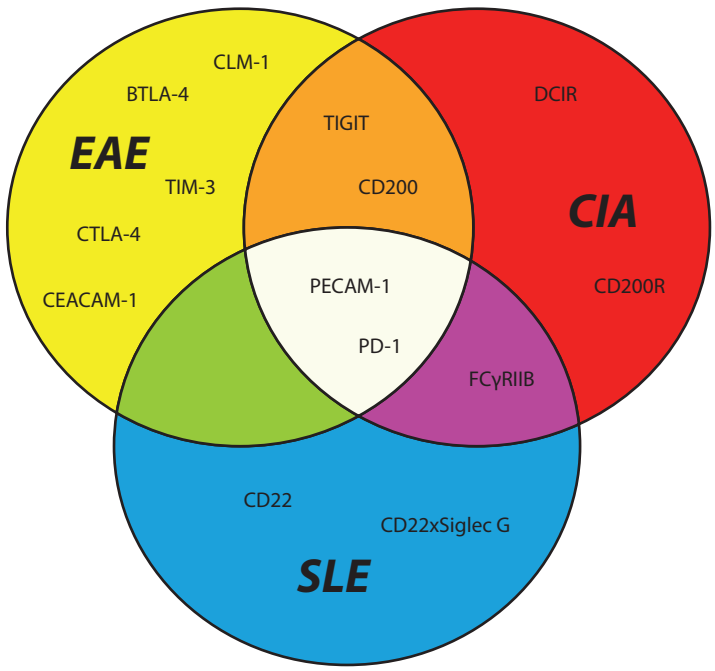


Figure 1: Involvement of inhibitory receptors in mouse autoimmune disease models. Schematic representation of different inhibitory receptor knockout mice, and their involvement in the autoimmune disease models for collagen induced arthritis (CIA), experimental autoimmune encephalomyelitis (EAE) and systemic lupus erythematosus (SLE).

Collagen induced arthritis (CIA) is a widely used mouse model for RA. As early as in 1977, Trentham *et al.* showed that immunization of rats with an emulsion from human, chick or rat collagen type II, which is the major constituent protein in cartilage, in complete Freund's adjuvant led to the development of an immune response against cartilage (7). In later years similar protocols for induction of CIA in mice were described (8). The immune response renders collagen type II specific antibodies, which is also an aspect of RA (9). Several inhibitory receptor knockout mice show an increased susceptibility for this autoimmune mouse model. The H2q mouse is susceptible to collagen induced arthritis, while the H2b mouse is not. FCγRIIB is an inhibitory receptor for IgG, expressed on B cells. If *FCγRIIB*$^{-/-}$ mice are bred on the non-susceptible background, they become

susceptible for the collagen induced arthritis model (10), thereby showing a pivotal role for this receptor in the onset of the disease. Additionally, deletion of *PECAM-1*, which is thought to serve an important role in the modulation of B cells and regulation of peripheral tolerance, renders the non-permissive H2b mouse strain susceptible to CIA in an active CIA model (11). CD200R and its ligand CD200 are thought to be essential for myeloid cell regulation. *CD200R1*$^{-/-}$and *CD200*$^{-/-}$mice, which lack the ligand, also show increased sensitivity to the CIA model, thereby showing a disease limiting role of these receptors (11;12). Furthermore, CD200-Fc fusion proteins have a therapeutic effect in this model (12). DCIR (*CLEC4A2*)$^{-/-}$ mice also show enhanced susceptibility to CIA, while they also develop a typical form of Sjögrens syndrome named sialadenitis, and enthesisis which is a characteristic feature of a group of inflammatory rheumatic diseases (13;14). TIGIT is a novel inhibitory immune receptor which is expressed on NK and T cells. It is a member of the CD28 family, which regulates T cell responses. Levin *et al.* showed that the administration of TIGIT-Fc fusion protein or TIGIT tetramers inhibits the severity of CIA (15). PD-1 is thought to play a role in the regulation of T cells, as well as in the development of B cells, regulating the selection in the germinal centers and affecting the longevity of plasma cells (16). *PD-1*$^{-/-}$ mice spontaneously develop a deforming arthritis with age, which histologically resembles rheumatoid arthritis (17).Taken together, these data show that various cell types are involved in the pathogenesis of CIA, as the expression of all of the above mentioned inhibitory receptors is very diverse.

Experimental autoimmune encephalomyelitis (EAE), a mouse model for the human autoimmune disease MS, was introduced by Rivers *et al.* in 1933, who immunized primates with rabbit brain emulsion (18). Currently, mice are a well used model system for EAE. Depending on the genetic background, the protocol, and the different antigens such as central nervous system (CNS) homogenate, myelin proteins and peptides and fusion proteins, differences in clinical outcome, pathology and effector cells can be induced (19). Many inhibitory receptor knockout mice prove to be hypersensitive for this model, by showing increased susceptibility or early onset of symptoms. For instance, absence of PECAM-1 expression, which is also involved in CIA, is associated with an early onset of clinical symptoms. During EAE, mononuclear cell extravasation and infiltration of the CNS occur at earlier time points in *PECAM*$^{-/-}$ mice than in wild-type mice (20). Significantly increased nitric oxide and pro-inflammatory cytokine production, along with increased demyelination and worsened clinical scores were observed in *CLM-1*$^{-/-}$ mice (21). CLM is the mouse orthologue of human CD300f, also named IREM-1, which is mainly expressed on myeloid cells (22). Furthermore, *PD-1*$^{-/-}$ mice, *CD200*$^{-/-}$ mice, both also sensitive for CIA, and *BTLA*$^{-/-}$ mice, which regulates T cell responses, also have an increased susceptibility for EAE (23-25). *TIGIT*$^{-/-}$ mice show a more severe EAE phenotype (15), which is in agreement with the finding that TIGIT blocking antibodies lead to a more severe phenotype of CIA.

Blockade of the inhibitory receptor by blocking antibodies or fusion proteins can lead to a similar phenotype as knockout mice. TIM-3 regulates T_h1 responses and affects

macrophage activation. Blockade of TIM-3 by blocking antibodies enhances the clinical and pathological severity of EAE (26). CEACAM-1 is important in cell - cell adhesion and is involved in regulation of immune responses as well as maintaining epithelial integrity. CEACAM-1 can exert its function by binding other members of the CEA family, or by homophilic ligation. Blocking of CEACAM-1 by a blocking antibody leads to an increased severity of EAE, while ligation of the receptor by CEACAM-1 Fc fusion proteins leads to reduced myelin oligodendrocyte glycoprotein-derived peptide reactive interferon-γ and interleukin-17 production (27). CTLA-4 is expressed on T cells upon activation and inhibits their function. CTLA-4 blocking antibodies lead to a significantly more severe clinical course and more inflammatory and demyelinating lesions in the CNS (28). Hurwitz *et al.* further show that CTLA-4 engagement also controls disease susceptibility in BALB/c mice, a strain considered to be resistant to EAE induction (29). Similar to the CIA model, the inhibitory receptors involved in EAE are very diverse, expressed on a wide range of cells. There are a number of receptors whose absence enhances the severity of both CIA and EAE, indicating that the cells expressing them are involved in the pathogenesis of both autoimmune models.

Mouse models prone to develop spontaneous lupus like symptoms can contribute to the elucidation of the pathogenesis of SLE. Most mouse models develop high levels of circulating IgG autoantibodies to nuclear antigens, including chromatin and dsDNA, and an immune-complex mediated glomerulonephritis that resembles severe lupus nephritis in SLE patients (30). Inhibitory receptor knockout mice are often prone to the development of these antibodies, particularly for the receptors that are expressed by B cells.

FCγRIIB, mainly expressed by B cells and involved in the pathogenesis of CIA, also plays a major role in the mouse model of SLE. *FCγRIIB*$^{-/-}$ mice bred on a C57BL/6 background develop an SLE-like immune complex mediated autoimmune disease with spontaneous development of glomerulonephritis, while129SSv/C57BL/6 mice do not develop SLE-like immune complex mediated autoimmune disease (31). In 1999 Pritchard *et al.* sequenced the *FCγRIIB* gene, encoding FCγRIIB, and identified a haplotype defined by deletions in the promoter region, which was shared by the major SLE-prone mouse strains (NZB, BXSB, SB/Le, MRL, 129) and was absent in the control strains (BALB/c, C57BL/6, DBA/2 C57BL/10). The haplotype was associated with reduced expression of FCγRIIB and hyper reactive macrophages which resembled *FCγRIIB*$^{-/-}$ mice (32). Another type of mice prone to develop high affinity isotype switched autoantibodies to double stranded DNA are *CD22*$^{-/-}$ mice (33), CD22, also known as Siglec 2, is exclusively expressed by B cells and binds sialic acid. Although *CD22* deficient mice do not develop overt autoimmune disease, *CD22* x *Siglec G* double deficient mice, show spontaneous development of anti-DNA and antinuclear auto antibodies, resulting in a moderate form of immune complex glomerulonephritis (34). Siglec G is another member of the Siglec family which is exclusively expressed by B cells.

PECAM-1$^{-/-}$ mice, which also showed increased sensitivity to CIA and EAE, develop antinuclear antibodies and lupus-like autoimmune disease with age (35). *PD-1*$^{-/-}$ mice on a C57BL/6 background spontaneously develop autoantibodies and IgG3 immune

complex mediated glomerulonephritis which is similar to that seen in humans suffering from SLE (17). Some inhibitory receptors such as PD-1 and PECAM-1 are involved in all models of autoimmunity, indicating that they are crucial to a proper functioning of the immune system. Since the development of the lupus like symptoms is dependent on the presence of IgG autoantibodies, it is not surprising that all receptors studied in the SLE models are expressed on B cells. However, it would be interesting to speculate about involvement of inhibitory receptors that are not expressed on B cells in this model. There is a possibility that these receptors have not been properly investigated in the lupus like model because they are not expected to play a role. Careful reconsideration of specific inhibitory knockout mice and the models in which they are thoroughly tested might render new possibilities in understanding the complexity of autoimmune diseases.

Not all inhibitory receptor knockout mice have an altered phenotype in autoimmune models. *LAIR-1* knockout mice showed no signs of altered immune function in an experimental colitis model and in the EAE model (36). Moreover, *SIRP-α* knockout mice show increased resistance to CIA and EAE (37;38). The most extreme phenotype of an inhibitory receptor knockout mouse is the *CTLA-4* deficient mouse. These mice die within 3-4 weeks of birth, due to the rapid development of lymphoproliferative disease with multi-organ lymphocytic infiltration and tissue destruction, with particularly severe myocarditis and pancreatitis (39;40), which shows the importance of this receptor in immunological homeostasis.

The studies in knockout mice give numerous indications that the loss of a functional inhibitory receptor leads to sensitivity for, or the spontaneous development of, autoimmune disease. However, these studies must be reviewed with care. For instance, the background of mice, or the back-crossing of mice to a different background can account for part of the phenotype. Boross *et al.* investigated the hyper active phenotype and lethal lupus of *FCγRIIB* deficient mice in a 129 background, which are back crossed into C57BL/6. They found that the lethal lupus phenotype was partially mediated by a 129 derived flanking region *Sle16*. They conclude that *FCγRIIB* deficiency only amplifies spontaneous autoimmunity determined by other loci, and is not a cause of lethal lupus in itself (41).

Still, we can conclude that in mice, absence of inhibitory receptors predisposes to autoimmunity in the most commonly used mice models.

ASSOCIATION WITH AUTOIMMUNITY: FUNCTIONAL STUDIES IN HUMANS

Given the importance of inhibitory receptors for balancing the immune system, it is not surprising that many patient studies show abnormal expression or function of these receptors.

One example is Ig-like transcript-2 (ILT-2) that is expressed in some lymphocytic subsets and all myelomonocytic cells (42-45). $CD19^+$ B lymphocytes from SLE patients have significantly lower ILT-2 expression than controls. Furthermore, *in vitro*, ILT-2

engagement in peripheral blood mononuclear cells (PBMCs) of SLE patients is less capable of inhibiting cellular proliferation in response to CD3 stimulation than ILT-2 engagement in PBMC of healthy controls (42).

Two papers studying PBMCs from SLE patients *ex vivo* show decreased expression of FCγRIIB on memory B cells from SLE patients. They show altered signaling in B cells in response to intact IgG or $F(ab')_2$ fragments of anti –γ antibody as compared to healthy controls. Furthermore, they show that monocyte-derived dendritic cells (DCs) of SLE patients have an altered receptor expression pattern for the activating FCgRIIA and the inhibitory FCγRIIB (46;47). Additionally, DCs from patients with active RA showed an increased expression of FCγRIIB, as compared to patients with inactive RA or healthy controls (48).

LAIR-1, which is expressed on many immune cells, was found to be decreased on B cells of SLE patients, but not of RA or systemic sclerosis patients as compared to healthy controls (49). Moreover, we previously showed significantly elevated levels of sLAIR-1 and LAIR-2 in urine of RA patients as compared to healthy controls, and in synovial fluid of RA patients as compared to osteo artritis patients. LAIR-2 can function as a competitor for binding sites to LAIR-1, while shedding of LAIR-1 lowers the receptor expression. This leads to a higher activation status of the immune system (50;51). The expression of CD94/NKG2A on γδ T cells of SLE patients is decreased as compared to healthy controls (52) while in RA low expression of CD94/NKG2A is associated with disease activity (53).

The signaling lymphocyte activation molecule (SLAM) family member CD244 (also known as 2B4) is expressed at a wide variety of cells (Table 1, reviewed in (54)). Kim *et al.* found that the proportion of NK cells and monocytes expressing CD244 on their surface was significantly lower in patients with SLE compared to healthy controls, which also suggests involvement of the receptor in the pathogenesis of SLE (55). Another receptor involved in the pathogenesis of SLE might be CD200R. The number of $CD200^+$ cells and the level of soluble CD200 were significantly higher in SLE patients than in healthy controls, whereas the expression of CD200R by $CD4^+$ T cells and DCs was decreased (56).

TIM-3 was first is expressed by $CD4^+$ T_h1 and T_h17 cells (26;57). A study examining the functional differences in TIM-3 by using blocking antibodies on T cells of MS patients and healthy controls showed that blockage during T cell stimulation significantly enhanced IFN-γ secretion in control subjects but had no effect in untreated patients with MS. These data indicate a defect in immunoregulation by TIM-3 in MS (58). Jury *et al.* found an abnormal CTLA-4 expression and function in responder T cells from SLE patients. CTLA-4 was unable to inhibit T cell proliferation and the induction of intracellular pathways induced by T cell activation and CTLA-4 was displaced from membrane micro domains (59).

Blocking antibodies for inhibitory receptors are now entering the clinic and have been proven to be effective in the treatment of cancer (60). The side-effects of these treatments give insight into the important role of these inhibitory receptors on human immune homeostasis. In patients suffering from metastatic melanoma CTLA-4 is

blocked with Ipilimumab, a fully human monoclonal antibody (IgG1), to promote anti-tumor immunity. However, the treatment with Ipilimumab can cause severe side effects which resemble an autoimmune phenotype and are related to the continuous immune activation caused by the blockage of the inhibitory signal of CTLA-4 (61). Similarly, clinical trials involving PD-1 blockade show adverse events related to treatment, although less than in the CTLA-4 clinical trial (62).

The expression levels of many of the above discussed receptors changes during activation of the immune system. Whether the defective expression and function in patients is causal to the autoimmune disease, or a consequence of the continued immune activation, remains to be elucidated. In most cases, the abnormal expression of these receptors in autoimmune patients directly affects the functioning of the receptor. All these data combined indicate that the expression and function of inhibitory immune receptors is often defective in patients suffering from autoimmune diseases.

EVIDENCE FROM GWAS

Genetic association studies of autoimmune diseases have provided important evidence that illuminate the underlying mechanism and cause of disease. In general, association studies use indirect association of neutral genetic markers to tag unknown genetic disease variation. Unambiguous association of genetic variation in close proximity or in a certain gene is taken as evidence that first, the function of the protein encoded by that gene must play a critical role in the disease mechanism, and second, that genetic variation of the gene affects "normal" function and is, in part, causal for disease. For many individual inhibitory receptors genetic variation has been reported to be associated with autoimmune disease in relatively small case-control cohorts. Unfortunately, association studies of cohorts consisting of a few hundred subjects have only sufficient power to detect genetic risk variation that confer strong susceptibility for disease with relative risk of two or more. For relative common diseases such as most autoimmune diseases, such high risks are rare and unlikely, apart from a few exceptions. It is therefore not surprising that most reported associations detected in smaller cohorts are rarely confirmed by independent studies and currently are believed to be spurious associations. Even more so, in the last decade very large case control GWAS have been performed in cohorts of thousands of subjects, powerful enough to detect genetic variation with relative risk below 1.2. GWAS is firmly based on the common-variant common disease hypothesis (63), which states that multiple common genetic variations, each conferring a low risk for disease, collectively determine disease. Therefore, a complete genetic scan of the whole human genome is necessary to obtain the complete genetic profile. This scan is performed by genotyping more than 500,000 genetic markers; single nucleotide polymorphisms (SNP). The analysis entails association testing of each single marker, which provides a complete coverage of the human genome. However, this approach also introduces a multiple testing issue, requiring a more stringent cut-off level for

significance of $5x10^{-08}$ to control Type I error rate (64;65). GWAS studies have detected more than a hundred different genes associated to different autoimmune diseases, most of which confirmed by similar equally power independent studies. Nevertheless, previous inhibitory receptor genes associations are rarely confirmed by such studies, making it unlikely that these associations are important determinants for disease.

However, a few exceptions have been detected. More than 1537 genetic loci to date have been detected by GWAS for many common diseases, among which autoimmune diseases, and catalogued in the GWAS catalogue (66).

To answer the question whether genetic variations in inhibitory receptors or downstream effectors are important determinants for autoimmune disease, we reviewed these GWAS studies as leading evidence for their involvement. Taking into account the phenotypes of the knockout mice and the many functional or expression studies pointing to a role for inhibitory receptors in the etiology of autoimmune diseases, surprisingly few, yet strong associations have been detected for this group of genes. Robust association is detected for three out of the ~60 currently characterized inhibitory receptors; *CTLA-4*, the cluster of *FCγR* genes, and *CD244*.

Association of *CTLA-4* has been reported by many studies including those preceding the GWAS era. The interest was mainly focused on a functional polymorphism, yet fine mapping of the association signal in a large cohort of T1D patients pointed to variation in the 3'-UTR as the most likely location of the causal variation (67;68). *CTLA-4* is consistently associated with T1D, Graves' disease, Celiac Disease, and RA (67;69-72), yet is not associated to HLA class I associated autoimmune diseases such as Ankylosis Spondilytis and Behcet's Disease. The cluster of *FCγRIIB* includes both activating and inhibiting Fc-gamma receptors. A first publication on copy number variation of *FCgRIIIB* showed significant association with SLE (73). Unfortunately, reliable determination of copy numbers of this gene has been difficult and hampered confirmation by GWAS. Nevertheless, significantly associated of *FCγRIIB* has been demonstrated for inflammatory bowel disease and ulcerative colitis (74;75). The question remains which variation or copy number variation is driving the disease association, or if a functional defect is associated with disease. The known functional T232 mutation (rs1050501) leads to exclusion of the receptor from lipid rafts by a mutation within the transmembrane region, resulting in reduced phosphorylation and reduced inhibitory function (76;77). A decrease in the inhibition by FcγRIIB of phagocytosis and superoxide production in macrophages and on proliferation in B cells was shown *in vitro* (76). It remains to be investigated what the effect of this mutation is on the pathogenesis of autoimmune disease.

A large GWAS study that performed a META-analysis of different GWAS of Crohn's disease and Ulcerative Colitis of more than 75.000 cases and controls further added some evidence for association of inhibitory receptors with these diseases. This study reported association of variation linked to *CD244*, but also reported association close to *KIR3DL2*. Interestingly, *KIR3DL2* is located in a genomic region that contains a cluster of KIR genes and *LILRB1*, *LILRB4*, and both *LAIR-1* and *LAIR-2* (75).

This association is interesting, since GWAS may not be the suitable method for determining gene copy number variation such as described for *FCγRIIIB* receptor gene. The KIR family of genes displays a high level of variation of presence or absence of specific KIR genes organized on chromosome 19. One main KIR haplotype lacks all functional 2DS-activating genes, and is therefore referred to as an inhibitory haplotype. Interestingly, a study directly determined presence or absence of activating or inhibiting KIR genes in T1D and found a significant increase in the number of activating KIR genes (78). Several other studies have shown some evidence for association of KIR genes in autoimmune disease (79).

Finally, Armstrong *et al.* took a different approach because of the downsides of the GWAS, and defined *KLRG-1* as a gene predisposing for SLE in a two-step Bayesian study (80). This finding needs to be confirmed by others, but they raise an interesting alternative for GWAS by concentrating on a collection of candidate pathways rather than specific genes.

GWAS can not pick up relative rare (less than 1% population frequency) risk variation. It remains possible that multiple rare genetic variations exist in the different inhibitory receptors. When such factors have modest risk for disease, such variation will at best contribute a very minute proportion to the population attributable fraction. However, rare genetic risk variations with higher risks for autoimmune disease have been reported. For instance, rare variants of *IFIH1*, a gene implicated in antiviral responses, protect against T1D (81).

Thus, few inhibitory receptors have detectable genetic variation that confers susceptibility for disease. However, strong association has been detected for a functional variant of LYP and multiple autoimmune diseases. The c-Src tyrosine kinase, CSK is a kinase that interacts with LYP, and can modify the activation state of downstream Src kinases in lymphocytes. Recently, *CSK* has been associated to systemic sclerosis (82) and SLE (83). The latter study also provided evidence for a functional variant affecting B-cell activation. *PTPN22*, which encodes the phosphatase LYP is a critical downstream regulator of T cell receptor signaling (84). A mutation in LYP, R620W, is significantly associated with autoimmunity (70-72;85). The R620W mutation interferes with the binding of LYP to CSK (86). Vang *et al.* recently showed that CSK regulates the capacity of wild type LYP to dephosphorylate proteins. R620W LYP shows decreased binding to CSK and might therefore not be properly regulated (87). Work to date, however, leads to conflicting conclusions whether the R620W mutation leads to a gain- or loss of function variant (88-92).

Here, we propose that LYP and CSK may be common effectors of ITIM-bearing receptors (Figure 2). We and others have shown that CSK is recruited to phosphorylated ITIMs in inhibitory receptors such as LAIR-1, SIRP-α and LILRB1 (93;94). Other ITIM bearing receptors may also recruit CSK, but to our knowledge, this has not been studied. CSK is thought to function as an effector in the inhibitory pathway downstream of these receptors, directly by inhibition of Src kinase activity, and indirectly by recruiting the

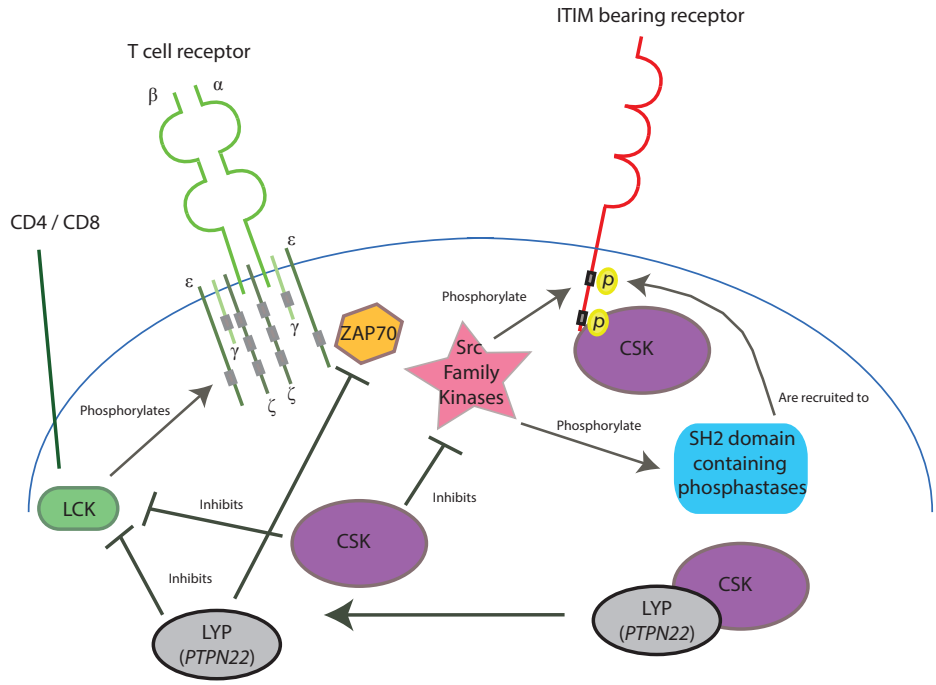


Figure 2: Inhibitory immune receptors and the T cell receptor both signal via CSK. Model of the proposed signaling pathway of the T cell receptor with intracellular ITAMs and an inhibitory receptor bearing ITIMs. Upon activation, LCK phosphorylates the ITAMs of the T cell receptor, which attract ZAP70. CSK, while bound to LYP, controls LYP. Upon release, LYP dephosphorylates, and thereby inhibits LCK and ZAP 70. Moreover, CSK has also been shown to inhibit Src family kinases (among which LCK). Upon ligation, the ITIMs of inhibitory receptors and SH2 domain containing phosphatases (such as SHP-1, SHP-2 and SHIP) are phosphorylated by Src family kinases. Phosphorylated ITIMs recruit these phosphorylated SH2 domain containing phosphatases and CSK.

phosphatase LYP (4). Impaired function of CSK and/or disruption of the binding of LYP to CSK, for instance due to the R620W mutation, could lead to impaired inhibitory function. Thus, genetic variation in the genes encoding for LYP and CSK may not only deregulate T cell receptor signaling, but also the signaling of these inhibitory receptors. Decreased signaling of inhibitory receptors may lead to an over activation of the immune system, thereby contributing to, or even being the cause of, autoimmune diseases. Indeed, introduction of the analogue of the R620W mutation in mice in mice results in lymphocyte hyper reactivity, consistent with a role for LYP inhibitory receptor signaling (95;96).

CONCLUDING REMARKS

There seems to be an abundance of studies showing that inhibitory immune receptors are predisposing for, or malfunctioning in, autoimmune diseases. However, when looking at large META-analyses or GWAS data, there is only significant association of three inhibitory immune receptors with autoimmune disease (*CTLA-4*, *FCγRIIB* and *CD244*). Therefore, we conclude that although on an individual level malfunctioning of an inhibitory receptor may be of great influence, no general effect of mutations in most inhibitory receptors on a predisposition for autoimmune diseases can be found.

There are more than 300 putative different inhibitory receptors with similar, yet not identical function, for which genetic variation may influence function and thus affect risk for autoimmune disease. To be detected by current GWAS, such variation needs to be relatively common, but can confer very modest risk for disease. Current large scale genome-wide screens of different autoimmune diseases are powered to detect modest genetic risk factors that are common and have relative risk for disease as low as 1.1 or even lower. Our review showed that given a few exceptions, such common risk variation is not abundant in inhibitory receptors. Future studies that use large scale new DNA sequencing techniques, i.e. Next Generation Sequencing, to detect rare variation in patients, followed by an association study of such variation in large cohorts, are needed to assess whether rare variation in other inhibitory receptors is present and affects risk for autoimmune disease.

Proteins involved in the downstream signaling pathways of inhibitory receptors do show significant association with autoimmunity. Further analysis of these pathways for a larger number of inhibitory immune receptors may render more insight in autoimmune pathology.

Lastly, inhibitory receptors could be targeted with agonists for therapeutic purposes. In mice, in vivo agonists for CD200, TIGIT, CTLA-4 and CEACAM-1 result in amelioration of disease (12;15;27;29). In patients where the downstream signaling pathway of those receptors is unaffected, this might be an interesting new treatment strategy.

REFERENCE LIST

1. Pritchard NR, Smith KG. B cell inhibitory receptors and autoimmunity. Immunology 2003 Mar;108:263-73.
2. Vivier E, Daeron M. Immunoreceptor tyrosine-based inhibition motifs. Immunol Today 1997 Jun;18:286-91.
3. Daeron M, Jaeger S, Du Pasquier L, Vivier E. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev 2008;224:11-43.
4. Verbrugge A, Rijkers ESK, De Ruiter T, Meyaard L. Leukocyte-associated Ig-like receptor-1 has SH-2 domain-containing phosphatase-independent function and recruits C-terminal Src kinase. Eur J Immmunol 2006 Jan;36:190-8.
5. Jansen CA, Cruijsen C, De Ruiter T, Nanlohy N, Willems N, Janssens-Corpela P, *et al.* Regulated expression of the inhibitory receptor LAIR-1 on human peripheral T cells during T cell activation and differentiation. Eur J Immmunol 2007 May 1;37:914-24.
6. Verbrugge A, De Ruiter T, Geest C, Coffer PJ, Meyaard L. Differential expression of Leukocyte Associated Ig-like Receptor-1 during neutrophil differentiation and activation. J Leukoc Biol 2006;79:828-36.
7. Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen an experimental model of arthritis. J Exp Med 1977 Sep 1;146(3):857-68.
8. Courtenay JS, Dallman MJ, Dayan AD, Martin A, Mosedale B. Immunisation against heterologous type II collagen induces arthritis in mice. Nature 1980 Feb 14;283(5748):666-8.
9. Nandakumar KS. Pathogenic antibody recognition of cartilage. Cell Tissue Res 2010 Jan;339(1):213-20.
10. Yuasa T, Kubo S, Yoshino T, Ujike A, Matsumura K, Ono M, *et al.* Deletion of fcgamma receptor IIB renders H-2(b) mice susceptible to collagen-induced arthritis. J Exp Med 1999 Jan 4;189(1):187-94.
11. Wong MX, Hayball JD, Hogarth PM, Jackson DE. The inhibitory co-receptor, PECAM-1 provides a protective effect in suppression of collagen-induced arthritis. J Clin Immunol 2005 Jan;25(1):19-28.
12. Simelyte E, Alzabin S, Boudakov I, Williams R. CD200R1 regulates the severity of arthritis but has minimal impact on the adaptive immune response. Clin Exp Immunol 2010 Oct;162(1):163-8.
13. Fujikado N, Saijo S, Yonezawa T, Shimamori K, Ishii A, Sugai S, *et al.* Dcir deficiency causes development of autoimmune diseases in mice due to excess expansion of dendritic cells. Nat Med 2008 Feb;14(2):176-80.
14. Helenius LM, Hietanen JH, Helenius I, Kautiainen H, Piirainen H, Paimela L, *et al.* Focal sialadenitis in patients with ankylosing spondylitis and spondyloarthropathy: a comparison with patients with rheumatoid arthritis or mixed connective tissue disease. Ann Rheum Dis 2001 Aug;60(8):744-9.
15. Levin SD, Taft DW, Brandt CS, Bucher C, Howard ED, Chadwick EM, *et al.* Vstm3 is a member of the CD28 family and an important modulator of T-cell function. Eur J Immunol 2011 Apr;41(4):902-15.
16. Good-Jacobson KL, Szumilas CG, Chen L, Sharpe AH, Tomayko MM, Shlomchik MJ. PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. Nat Immunol 2010 Jun;11(6):535-42.
17. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999 Aug;11(2):141-51.
18. Rivers TM, Sprunt DH, Berry GP. OBSERVATIONS ON ATTEMPTS TO PRODUCE ACUTE DISSEMINATED ENCEPHALOMYELITIS IN MONKEYS. J Exp Med 1933 Jun 30;58(1):39-53.

19. Batoulis H, Recks MS, Addicks K, Kuerten S. Experimental autoimmune encephalomyelitis--achievements and prospective advances. APMIS 2011 Dec;119(12):819-30.
20. Graesser D, Solowiej A, Bruckner M, Osterweil E, Juedes A, Davis S, *et al.* Altered vascular permeability and early onset of experimental autoimmune encephalomyelitis in PECAM-1-deficient mice. Journal of Clinical Investigation 2002 Feb;109(3):383-92.
21. Xi H, Katschke KJ, Jr., Helmy KY, Wark PA, Kljavin N, Clark H, *et al.* Negative regulation of autoimmune demyelination by the inhibitory receptor CLM-1. J Exp Med 2010 Jan 18;207(1):7-16.
22. Alvarez-Errico D, Aguilar H, Kitzig F, Brckalo T, Sayos J, Lopez-Botet M. IREM-1 is a novel inhibitory receptor expressed by myeloid cells. Eur J Immunol 2004 Dec;34(12):3690-701.
23. Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, *et al.* Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science 2000;290:1768-71.
24. Carter LL, Leach MW, Azoitei ML, Cui J, Pelker JW, Jussif J, *et al.* PD-1/PD-L1, but not PD-1/PD-L2, interactions regulate the severity of experimental autoimmune encephalomyelitis. J Neuroimmunol 2007 Jan;182(1-2):124-34.
25. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, *et al.* BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 2003 Jul;4:670-9.
26. Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, *et al.* Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature 2002 Jan 31;415(6871):536-41.
27. Fujita M, Otsuka T, Mizuno M, Tomi C, Yamamura T, Miyake S. Carcinoembryonic antigen-related cell adhesion molecule 1 modulates experimental autoimmune encephalomyelitis via an iNKT cell-dependent mechanism. Am J Pathol 2009 Sep;175(3):1116-23.
28. Hurwitz AA, Sullivan TJ, Krummel MF, Sobel RA, Allison JP. Specific blockade of CTLA-4/B7 interactions results in exacerbated clinical and histologic disease in an actively-induced model of experimental allergic encephalomyelitis. J Neuroimmunol 1997 Mar;73(1-2):57-62.
29. Hurwitz AA, Sullivan TJ, Sobel RA, Allison JP. Cytotoxic T lymphocyte antigen-4 (CTLA-4) limits the expansion of encephalitogenic T cells in experimental autoimmune encephalomyelitis (EAE)-resistant BALB/c mice. Proc Natl Acad Sci U S A 2002 Mar 5;99(5):3013-7.
30. Jorgensen TN, Gubbels MR, Kotzin BL. Links between type I interferons and the genetic basis of disease in mouse lupus. Autoimmunity 2003 Dec;36(8):491-502.
31. Bolland S, Ravetch JV. Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis. Immunity 2000 Aug;13(2):277-85.
32. Pritchard NR, Cutler AJ, Uribe S, Chadban SJ, Morley BJ, Smith KG. Autoimmune-prone mice share a promoter haplotype associated with reduced expression and function of the Fc receptor FcgammaRII. Curr Biol 2000 Feb 24;10(4):227-30.
33. O'Keefe TL, Williams GT, Batista FD, Neuberger MS. Deficiency in CD22, a B cell-specific inhibitory receptor, is sufficient to predispose to development of high affinity autoantibodies. J Exp Med 1999 Apr 19;189(8):1307-13.
34. Jellusova J, Wellmann U, Amann K, Winkler TH, Nitschke L. CD22 x Siglec-G double-deficient mice have massively increased B1 cell numbers and develop systemic autoimmunity. J Immunol 2010 Apr 1;184(7):3618-27.
35. Wilkinson R, Lyons AB, Roberts D, Wong MX, Bartley PA, Jackson DE. 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 2002 Jul 1;100:184-93.
36. Tang X, Tian L, Esteso G, Choi SC, Barrow AD, Colonna M, *et al.* Leukocyte-associated Ig-like receptor-1-deficient mice have an altered immune cell phenotype. J Immunol 2012 Jan 15;188(2):548-58.
37. Okuzawa C, Kaneko Y, Murata Y, Miyake A, Saito Y, Okajo J, *et al.* Resistance to collagen-induced arthritis in SHPS-1 mutant mice. Biochem Biophys Res Commun 2008 Jul 4;371(3):561-6.

38. Tomizawa T, Kaneko Y, Kaneko Y, Saito Y, Ohnishi H, Okajo J, *et al.* Resistance to experimental autoimmune encephalomyelitis and impaired T cell priming by dendritic cells in Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 mutant mice. J Immunol 2007 Jul 15;179(2):869-77.
39. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 1995 Nov;3(5):541-7.
40. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, *et al.* Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 1995 Nov 10;270:985-8.
41. Boross P, Arandhara VL, Martin-Ramirez J, Santiago-Raber ML, Carlucci F, Flierman R, *et al.* The inhibiting Fc receptor for IgG, FcgammaRIIB, is a modifier of autoimmune susceptibility. J Immunol 2011 Aug 1;187(3):1304-13.
42. Monsivais-Urenda A, Nino-Moreno P, bud-Mendoza C, Baranda L, Layseca-Espinosa E, Lopez-Botet M, *et al.* Analysis of expression and function of the inhibitory receptor ILT2 (CD85j/LILRB1/LIR-1) in peripheral blood mononuclear cells from patients with systemic lupus erythematosus (SLE). J Autoimmun 2007 Sep;29(2-3):97-105.
43. Young NT, Uhrberg M, Phillips JH, Lanier LL, Parham P. Differential expression of leukocyte receptor complex-encoded Ig-like receptors correlates with the transition from effector to memory CTL. J Immunol 2001 Mar 15;166(6):3933-41.
44. Colonna M, Navarro F, Lopez-Botet M. A novel family of inhibitory receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. Curr Top Microbiol Immunol 1999;244:115-22.
45. Saverino D, Fabbi M, Ghiotto F, Merlo A, Bruno S, Zarcone D, *et al.* The CD85/LIR-1/ILT2 inhibitory receptor is expressed by all human T lymphocytes and down-regulates their functions. J Immunol 2000 Oct 1;165(7):3742-55.
46. Mackay M, Stanevsky A, Wang T, Aranow C, Li M, Koenig S, *et al.* Selective dysregulation of the FcgammaIIB receptor on memory B cells in SLE. J Exp Med 2006 Sep 4;203(9):2157-64.
47. Carreno LJ, Pacheco R, Gutierrez MA, Jacobelli S, Kalergis AM. Disease activity in systemic lupus erythematosus is associated with an altered expression of low-affinity Fc gamma receptors and costimulatory molecules on dendritic cells. Immunology 2009 Nov;128(3):334-41.
48. Radstake TR, Blom AB, Sloetjes AW, van Gorselen EO, Pesman GJ, Engelen L, *et al.* Increased FcgammaRII expression and aberrant tumour necrosis factor alpha production by mature dendritic cells from patients with active rheumatoid arthritis. Ann Rheum Dis 2004 Dec;63(12):1556-63.
49. Colombo BM, Canevali P, Magnani O, Rossi E, Puppo F, Zocchi MR, *et al.* Defective expression and function of the leukocyte associated Ig-like receptor 1 in B lymphocytes from systemic lupus erythematosus patients. PLoS One 2012;7(2):e31903.
50. Olde Nordkamp MJM, van Roon JA, Douwes M, De Ruiter T, Urbanus RT, Meyaard L. Enhanced secretion of Leukocyte-Associated Immunoglobulin-like Receptor (LAIR)-2 and soluble LAIR-1 in rheumatoid arthritis: LAIR-2 is a more efficient antagonist of the LAIR-1-collagen inhibitory interaction than soluble LAIR -1. Arthritis Rheum 2011;63:3749-57.
51. Lebbink RJ, van den Berg MC, de RT, Raynal N, van Roon JA, Lenting PJ, *et al.* The soluble leukocyte-associated Ig-like receptor (LAIR)-2 antagonizes the collagen/LAIR-1 inhibitory immune interaction. J Immunol 2008 Feb 1;180(3):1662-9.
52. Wang L, Kang N, Zhou J, Guo Y, Zhang X, Cui L, *et al.* Downregulation of CD94/NKG2A inhibitory receptor on decreased gammadelta T cells in patients with systemic lupus erythematosus. Scand J Immunol 2012 Jul;76(1):62-9.
53. Walsh CE, Ryan EJ, O'Farrelly C, Golden-Mason L, FitzGerald O, Veale DJ, *et al.* Differential expression of NK receptors CD94 and NKG2A by T cells in rheumatoid arthritis patients in remission compared to active disease. PLoS One 2011;6(11):e27182.

54. Cannons JL, Tangye SG, Schwartzberg PL. SLAM family receptors and SAP adaptors in immunity. Annu Rev Immunol 2011;29:665-705.
55. Kim JR, Mathew SO, Patel RK, Pertusi RM, Mathew PA. Altered expression of signalling lymphocyte activation molecule (SLAM) family receptors CS1 (CD319) and 2B4 (CD244) in patients with systemic lupus erythematosus. Clin Exp Immunol 2010 Jun;160(3):348-58.
56. Li Y, Zhao LD, Tong LS, Qian SN, Ren Y, Zhang L, *et al.* Aberrant CD200/CD200R1 expression and function in systemic lupus erythematosus contributes to abnormal T-cell responsiveness and dendritic cell activity. Arthritis Res Ther 2012;14(3):R123.
57. Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC, *et al.* TIM-3 is expressed on activated human $CD4^+$ T cells and regulates Th1 and Th17 cytokines. Eur J Immunol 2009 Sep;39(9):2492-501.
58. Yang L, Anderson DE, Kuchroo J, Hafler DA. Lack of TIM-3 immunoregulation in multiple sclerosis. J Immunol 2008 Apr 1;180(7):4409-14.
59. Jury EC, Flores-Borja F, Kalsi HS, Lazarus M, Isenberg DA, Mauri C, *et al.* Abnormal CTLA-4 function in T cells from patients with systemic lupus erythematosus. Eur J Immunol 2010 Feb;40(2):569-78.
60. Meyaard L, Smyth MJ. Liberating tumor immunity. Curr Opin Immunol 2012 Apr;24(2):204-6.
61. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010 Aug 19;363(8):711-23.
62. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012 Jun 28;366(26):2443-54.
63. Pritchard JK, Cox NJ. The allelic architecture of human disease genes: common disease-common variant...or not? Hum Mol Genet 2002 Oct 1;11(20):2417-23.
64. Dudbridge F, Gusnanto A. Estimation of significance thresholds for genomewide association scans. Genet Epidemiol 2008 Apr;32(3):227-34.
65. Pe'er I, Yelensky R, Altshuler D, Daly MJ. Estimation of the multiple testing burden for genomewide association studies of nearly all common variants. Genet Epidemiol 2008 May;32(4):381-5.
66. Hindorff LA, MacArthur J (European Bioinformatics Institute), Morales J (European Bioinformatics Institute), Junkins HA, Hall PN, Klemm AK, *et al.* A Catalog of Published Genome-Wide Association Studies. 2013. 27-4-2013. Ref Type: Catalog
67. Barrett JC, Clayton DG, Concannon P, Akolkar B, Cooper JD, Erlich HA, *et al.* Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. Nat Genet 2009 Jun;41(6):703-7.
68. Qu HQ, Bradfield JP, Grant SF, Hakonarson H, Polychronakos C. Remapping the type I diabetes association of the CTLA4 locus. Genes Immun 2009 Dec;10 Suppl 1:S27-S32.
69. Cooper JD, Smyth DJ, Smiles AM, Plagnol V, Walker NM, Allen JE, *et al.* Meta-analysis of genome-wide association study data identifies additional type 1 diabetes risk loci. Nat Genet 2008 Dec;40(12):1399-401.
70. Gregersen PK, Amos CI, Lee AT, Lu Y, Remmers EF, Kastner DL, *et al.* REL, encoding a member of the NF-kappaB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. Nat Genet 2009 Jul;41(7):820-3.
71. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, *et al.* Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet 2010 Jun;42(6):508-14.
72. Plagnol V, Howson JM, Smyth DJ, Walker N, Hafler JP, Wallace C, *et al.* Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases. PLoS Genet 2011 Aug;7(8):e1002216.
73. Fanciulli M, Norsworthy PJ, Petretto E, Dong R, Harper L, Kamesh L, *et al.* FCGR3B copy number variation is associated with susceptibility to systemic, but not organ-specific, autoimmunity. Nat Genet 2007 Jun;39(6):721-3.

74. Anderson CA, Boucher G, Lees CW, Franke A, D'Amato M, Taylor KD, *et al.* Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. Nat Genet 2011 Mar;43(3):246-52.
75. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, *et al.* Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012 Nov 1;491(7422):119-24.
76. Floto RA, Clatworthy MR, Heilbronn KR, Rosner DR, MacAry PA, Rankin A, *et al.* Loss of function of a lupus-associated FcgammaRIIb polymorphism through exclusion from lipid rafts. Nat Med 2005 Oct;11(10):1056-8.
77. Kono H, Kyogoku C, Suzuki T, Tsuchiya N, Honda H, Yamamoto K, *et al.* FcgammaRIIB Ile232Thr transmembrane polymorphism associated with human systemic lupus erythematosus decreases affinity to lipid rafts and attenuates inhibitory effects on B cell receptor signaling. Hum Mol Genet 2005 Oct 1;14(19):2881-92.
78. van der Slik AR, Koeleman BP, Verduijn W, Bruining GJ, Roep BO, Giphart MJ. KIR in type 1 diabetes: disparate distribution of activating and inhibitory natural killer cell receptors in patients versus HLA-matched control subjects. Diabetes 2003 Oct;52(10):2639-42.
79. Espeli M, Niederer HA, Traherne JA, Trowsdale J, Smith KG. Genetic variation, Fcgamma receptors, KIRs and infection: the evolution of autoimmunity. Curr Opin Immunol 2010 Dec;22(6):715-22.
80. Armstrong DL, Reiff A, Myones BL, Quismorio FP, Jr., Klein-Gitelman M, McCurdy D, *et al.* Identification of new SLE-associated genes with a two-step Bayesian study design. Genes Immun 2009 Jul;10(5):446-56.
81. Nejentsev S, Walker N, Riches D, Egholm M, Todd JA. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. Science 2009 Apr 17;324(5925):387-9.
82. Martin JE, Broen JC, Carmona FD, Teruel M, Simeon CP, Vonk MC, *et al.* Identification of CSK as a systemic sclerosis genetic risk factor through Genome Wide Association Study follow-up. Hum Mol Genet 2012 Jun 15;21(12):2825-35.
83. Manjarrez-Orduno N, Marasco E, Chung SA, Katz MS, Kiridly JF, Simpfendorfer KR, *et al.* CSK regulatory polymorphism is associated with systemic lupus erythematosus and influences B-cell signaling and activation. Nat Genet 2012 Nov;44(11):1227-30.
84. Hill RJ, Zozulya S, Lu YL, Ward K, Gishizky M, Jallal B. The lymphoid protein tyrosine phosphatase Lyp interacts with the adaptor molecule Grb2 and functions as a negative regulator of T-cell activation. Exp Hematol 2002 Mar;30(3):237-44.
85. Franke A, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, *et al.* Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet 2010 Dec;42(12):1118-25.
86. Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K, Rostamkhani M, *et al.* A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nat Genet 2004 Apr;36(4):337-8.
87. Vang T, Liu WH, Delacroix L, Wu S, Vasile S, Dahl R, *et al.* LYP inhibits T-cell activation when dissociated from CSK. Nat Chem Biol 2012 May;8(5):437-46.
88. Vang T, Congia M, Macis MD, Musumeci L, Orru V, Zavattari P, *et al.* Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant. Nat Genet 2005 Dec;37(12):1317-9.
89. Habib T, Funk A, Rieck M, Brahmandam A, Dai X, Panigrahi AK, *et al.* Altered B cell homeostasis is associated with type I diabetes and carriers of the PTPN22 allelic variant. J Immunol 2012 Jan 1;188(1):487-96.
90. Rieck M, Arechiga A, Onengut-Gumuscu S, Greenbaum C, Concannon P, Buckner JH. Genetic variation in PTPN22 corresponds to altered function of T and B lymphocytes. J Immunol 2007 Oct 1;179(7):4704-10.

91. Zikherman J, Hermiston M, Steiner D, Hasegawa K, Chan A, Weiss A. PTPN22 deficiency cooperates with the CD45 E613R allele to break tolerance on a non-autoimmune background. J Immunol 2009 Apr 1;182(7):4093-106.
92. Zhang J, Zahir N, Jiang Q, Miliotis H, Heyraud S, Meng X, *et al.* The autoimmune disease-associated PTPN22 variant promotes calpain-mediated Lyp/Pep degradation associated with lymphocyte and dendritic cell hyperresponsiveness. Nat Genet 2011 Sep;43(9):902-7.
93. Sayos J, Martinez-Barriocanal A, Kitzig F, Bellon T, Lopez-Botet M. Recruitment of C-terminal Src kinase by the leukocyte inhibitory receptor CD85j. Biochem Biophys Res Commun 2004 Nov 12;324:640-7.
94. Veillette A, Thibaudeau E, Latour S. High expression of inhibitory receptor SHPS-1 and its association with protein-tyrosine phosphatase SHP-1 in macrophages. J Biol Chem 1998;273:22719-28.
95. Cambier JC. Autoimmunity risk alleles: hotspots in B cell regulatory signaling pathways. J Clin Invest 2013 Apr 24;1-4.
96. Dai X, James RG, Habib T, Singh S, Jackson S, Khim S, *et al.* A disease-associated PTPN22 variant promotes systemic autoimmunity in murine models. J Clin Invest 2013 Apr 24.
97. Horton HM, Chu SY, Ortiz EC, Pong E, Cemerski S, Leung IW, *et al.* Antibody-mediated coengagement of FcgammaRIIb and B cell receptor complex suppresses humoral immunity in systemic lupus erythematosus. J Immunol 2011 Apr 1;186(7):4223-33.
98. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, *et al.* The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol 2009 Jan;10(1):48-57.
99. Boles KS, Vermi W, Facchetti F, Fuchs A, Wilson TJ, Diacovo TG, *et al.* A novel molecular interaction for the adhesion of follicular CD4 T cells to follicular DC. Eur J Immunol 2009 Mar;39(3):695-703.
100. Privratsky JR, Newman DK, Newman PJ. PECAM-1: conflicts of interest in inflammation. Life Sci 2010 Jul 17;87(3-4):69-82.
101. Thomas R, Matthias T, Witte T. Leukocyte immunoglobulin-like receptors as new players in autoimmunity. Clin Rev Allergy Immunol 2010 Apr;38(2-3):159-62.
102. Riella LV, Paterson AM, Sharpe AH, Chandraker A. Role of the PD-1 pathway in the immune response. Am J Transplant 2012 Oct;12(10):2575-87.
103. Shang YJ, Cui QF, Li JL, Guo GN, Zhu WY. [Expression of the costimulatory molecule BTLA in synovial tissues from rheumatoid arthritis patients]. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 2012 Jun;28(6):643-6.
104. Sui L, Li N, Liu Q, Zhang W, Wan T, Wang B, *et al.* IgSF13, a novel human inhibitory receptor of the immunoglobulin superfamily, is preferentially expressed in dendritic cells and monocytes. Biochem Biophys Res Commun 2004 Jul 2;319(3):920-8.
105. Anderson AC, Anderson DE, Bregoli L, Hastings WD, Kassam N, Lei C, *et al.* Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells. Science 2007 Nov 16;318(5853):1141-3.
106. Lobo EO, Zhang Z, Shively JE. Pivotal advance: CEACAM1 is a negative coreceptor for the B cell receptor and promotes CD19-mediated adhesion of B cells in a PI3K-dependent manner. J Leukoc Biol 2009 Aug;86(2):205-18.
107. Adams S, van der Laan LJ, Vernon-Wilson E, Renardel de LC, Dopp EA, Dijkstra CD, *et al.* Signal-regulatory protein is selectively expressed by myeloid and neuronal cells. J Immunol 1998 Aug 15;161(4):1853-9.
108. Meyaard L. The inhibitory collagen receptor LAIR-1 (CD305). J Leukoc Biol 2008 Apr;83(4):799-803.
109. Bhat R, Eissmann P, Endt J, Hoffmann S, Watzl C. Fine-tuning of immune responses by SLAM-related receptors. J Leukoc Biol 2006 Mar;79(3):417-24.

110. van BJ, Koning F. The tortoise and the hare: slowly evolving T-cell responses take hastily evolving KIR. Immunology 2010 Nov;131(3):301-9.
111. Dorner T, Shock A, Smith KG. CD22 and autoimmune disease. Int Rev Immunol 2012 Oct;31(5):363-78.
112. Kerscher B, Willment JA, Brown GD. The Dectin-2 family of C-type lectin-like receptors: an update. Int Immunol 2013 May;25(5):271-7.
113. Borrego F, Kabat J, Sanni TB, Coligan JE. NK cell CD94/NKG2A inhibitory receptors are internalized and recycle independently of inhibitory signaling processes. J Immunol 2002 Dec 1;169(11):6102-11.
114. Lane P. Regulation of T and B cell responses by modulating interactions between CD28/CTLA4 and their ligands, CD80 and CD86. Ann N Y Acad Sci 1997 Apr 5;815:392-400.
115. Holmannova D, Kolackova M, Kondelkova K, Kunes P, Krejsek J, Andrys C. CD200/CD200R paired potent inhibitory molecules regulating immune and inflammatory responses; Part I: CD200/CD200R structure, activation, and function. Acta Medica (Hradec Kralove) 2012;55(1):12-7.

3

ENHANCED SECRETION OF LEUKOCYTE-ASSOCIATED IMMUNOGLOBULIN-LIKE RECEPTOR (LAIR)-2 AND SOLUBLE LAIR-1 IN RHEUMATOID ARTHRITIS:

LAIR-2 IS A MORE EFFICIENT ANTAGONIST OF THE LAIR-1-COLLAGEN INHIBITORY INTERACTION THAN IS SOLUBLE LAIR -1

Marloes J. M. Olde Nordkamp[1], Joël A.G. van Roon[2], Max Douwes[1], Talitha de Ruiter[1], Rolf T. Urbanus[3], Linde Meyaard[1]

Arthritis and Rheumatism 2011 Dec; 63(12):3749-57

[1]Department of Immunology, University Medical Centre Utrecht, the Netherlands
[2]Department of Rheumatology and Clinical Immunology, University Medical Centre Utrecht, the Netherlands
[3]Department of Clinical Chemistry and Haematology, University Medical Centre Utrecht, the Netherlands

ABSTRACT

Objective: Human leukocyte-associated immunoglobulin-like receptor 1 (hLAIR-1) is an immune inhibitory receptor for collagen that is expressed on most immune cells. We previously showed that the LAIR-1–collagen interaction could be antagonized by the secreted homolog hLAIR-2, which can be detected in the synovial fluid of rheumatoid arthritis (RA) patients. In addition, the extracellular part of hLAIR-1 is a putative antagonist upon shedding from the cell membrane. The purpose of this study was to determine the relative roles of LAIR-2 and soluble hLAIR-1 (shLAIR-1) in the regulation of the LAIR-1–collagen interaction.

Methods: The ability of recombinant LAIR proteins to abrogate LAIR-1–collagen binding was tested by flow cytometry and adhesion assays. Collagen binding capacity was analyzed by surface plasmon resonance. Plasma, urine, and synovial fluid were screened for the presence of sLAIR-1 and LAIR-2 by enzyme-linked immunosorbent assay.

Results: Recombinant LAIR-2 proteins abrogated the binding of collagen to LAIR-1 more efficiently than did recombinant sLAIR-1. Consistent with these findings, surface plasmon resonance analysis showed that LAIR-2 had a higher affinity for collagen than did LAIR-1. Activated $CD4^+$ T cells were the main producers of LAIR-2, whereas the source of sLAIR-1 remains elusive. Both soluble LAIR-1 and LAIR-2 could be detected in the plasma and urine of healthy control subjects and patients with RA. Urinary levels of both proteins were significantly increased in RA patients, and LAIR-2 levels in urine were significantly correlated with markers of inflammation.

Conclusion: Our data suggest that LAIR-2 is a more potent antagonist of LAIR-1 function in vivo, while both sLAIR-1 and LAIR-2 are potential biomarkers that may be used to monitor urine samples for evidence of systemic inflammation.

INTRODUCTION

Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is an inhibitory immune receptor for collagens and is expressed on most immune cells (1-3). Upon triggering, LAIR-1 inhibits various cellular functions (2;4-8). Since receptor and ligand are widely expressed, the regulation of the interaction must be tightly controlled to allow for appropriate cellular activation. We and others have previously shown that upon maturation or activation, LAIR-1 expression on neutrophils, T and B cells decreases, providing a regulatory mechanism at the level of receptor expression (8-11).

Another way of regulating the immune response is by the use of shed receptors or soluble proteins which block the interaction between receptor and ligand. The soluble homologue LAIR-2 is produced *in vivo* and the binding of LAIR-1 to collagens I and III can be blocked by recombinant LAIR-2-Fc proteins (12). Furthermore, we have recently shown that recombinant LAIR-2-Fc, but not LAIR-1-Fc is able to interfere with collagen induced platelet aggregation and efficiently inhibits the binding of collagen to glycoprotein VI and von Willebrand factor (13). Next to production of LAIR-2, LAIR-1 can be shed from the membrane to further regulate the LAIR-1 – collagen interaction. Little is known about the function of sLAIR-1. LAIR-1 shedding may dampen the strength of the inhibitory signal by two mechanisms, first by decreasing the amount of LAIR-1 expressed on the cell-surface and second by release of the soluble LAIR-1 (sLAIR-1) protein which could function as receptor antagonist (14). Importantly, published studies are limited to recombinant LAIR dimers, whereas the natural form of sLAIR-1 and LAIR-2 is most likely a monomer.

Data on sLAIR-1 and LAIR-2 production *in vivo* are scarce. Ouyang *et.al.* showed detectable levels of sLAIR-1 in the serum of healthy control subjects and increased levels in sera of patients suffering from hemorrhagic fever with renal syndrome (HFRS) (15). We previously showed that synovial fluid (SF) from rheumatoid arthritis (RA) patients has significantly elevated levels of LAIR-2 (12). In the present study, we investigated the secretion of sLAIR-1 and LAIR-2 *in vivo* in healthy control subjects and in RA patients to examine their role as potential biomarkers in RA. Therefore, we correlated the levels of sLAIR-1 and LAIR-2 in plasma and urine samples of RA patients with inflammation.

To study the role of sLAIR-1 and LAIR-2 as modulators of the immune response, we produced both recombinant LAIR-1 and LAIR-2 monomers and dimers, and then we determined their inhibitory effect on the interaction of cells with collagens in relation to their affinity for collagen, as measured by surface plasmon resonance.

MATERIAL AND METHODS

Cells

K562 human erythroleukemia wild-type (WT) cells and K562 expressing human (hLAIR-1) (2) were cultured in RPMI 1640 (Gibco, Invitrogen,) supplemented with 10% fetal calf serum (FCS; Integro) and antibiotics at 37°C, 5% CO_2.

Biological samples

SF samples were obtained from a first cohort of 16 RA patients and 16 osteoarthritis (OA) patients. SF was obtained upon joint aspiration. Samples were subjected to centrifugation to remove cells prior to storage at -80°C.

From a second cohort of 27 RA patients, plasma and urine were collected and measured. Of these patients, 30% (n = 8) were male and 70% (n = 19) were female. The RA patients had a mean disease activity score (DAS) of 2.29 (range 0.78-4.38) and a mean erythrocyte sedimentation rate (ESR) of 16.6 mm/hour (range 3-62 mm/hour). Morning urine samples were obtained from 21 healthy volunteer donors, and stored at -20°C until further use.

Prior to donation of peripheral blood, plasma, SF and urine samples, all healthy donors and patients gave written informed consent. Approval was obtained from the Institutional Review Board at University Medical Center Utrecht.

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from the blood of healthy donors by Ficoll-paque (1.077 specific gravity, GE Healthcare) centrifugation. Isolated cells were washed with phosphate buffered saline (PBS) at room temperature (RT) and used immediately for sorting or stimulation.

Antibodies

The following antibodies were used in enzyme-linked immunosorbent assays (ELISAs): anti-hLAIR-1 DX26 IgG1 monoclonal antibody (mAb) (16); biotinylated α-hLAIR-1 8A8 mAb (produced in our own laboratory), anti-hLAIR-2 1A7 IgG1 mAb (generously provided by Professor B. Jin, Fourth Military Medical University, Xi'an, Shaanxi Province, China) and biotinylated anti-hLAIR-2 IgG mAb (R&D Systems).

For $CD4^+$ and $CD8^+$ T cell phenotype sorting, the following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated CD3, PerCP-conjugated CD3, PerCP-conjugated CD4, PerCP-conjugated CD8, allophycocyanin-conjugated CD27, and phycoerythrin-conjugated CD45RO. All antibodies were purchased from BD Biosciences except for FITC-conjugated CD3, which was purchased from Sanquin.

Cell Sorting and Stimulation

After isolation, PBMCs were stained for 30 minutes at 4°C after which they were washed three times with ice cold PBS supplemented with 1% bovine serum albumin (BSA). Cells were diluted in RPMI 1640 supplemented with 1% FCS and sorted into a $CD27^+$ $CD45RO^-$ naïve population, $CD27^+$ $CD45RO^+$ memory population and $CD27^-$ $CD45RO^+$ effector population for $CD4^+$ and $CD8^+$ T cells. After sorting the cells were washed with RPMI 1640 1% FCS and cultured in RPMI 1640 10% FCS. Cells were stimulated for 4 days with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (1μM). Finally, supernatant was collected and stored at -20°C until further use.

A total of 150 μl whole blood, or 1:10 in RPMI 1640 diluted blood was added to a NUNC U bottom plate. Then, 200 μg/ml poli(I-C), 100ng/ml lipopolysaccharide (LPS),

1000 *μM* loxoribine, 30 μg/ml CpG-containing oligonucleotide (CpG ODN), or 20 ng/ml interferon-γ (IFNγ) was added, and incubated for 24 hours. A total of 1 μg/ml Pam_3Cys was incubated for 5 hours. Monocytes were isolated by $CD14^+$ magnetic-activated cell sorter (Miltenyi Biotec) analysis and stimulated for 4 days with 10 μg/ml curdlan, 1 μg/ml Pam_3Cys or 1 ng of LPS. Neutrophils were isolated from the blood of healthy donors by Ficoll-Histopaque centrifugation. Isolated cells were washed with PBS at room temperature and transferred to 1640 RPMI containing 10% FCS. Cell were primed for either 0, 10, 30 or 90 minutes with 10 units of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage stimulating factor (GM-CSF), or 500 units of tumor necrosis factor α (TNFα) and subsequently stimulated with 1 *μM* FMLP. Cells were spun down, and supernatant was removed and stored at -20 until further use.

Sandwich ELISA

96-well flat bottom MAXI-sorp plates (Nunc) were coated overnight with either anti-hLAIR-1 DX26 IgG1 mAb (14 μg/ml in 50 μl PBS) or α-hLAIR-2 1A7 IgG1 mAb (12 μg/ml in 50 μl PBS) at 4°C. Three washes were performed with 150 μl PBS. After washing, the plates were incubated for 2 hours at room temperature with PBS supplemented with 3% BSA to block non-specific interactions.

Meanwhile, biologic samples (urine, plasma or synovial fluid) were centrifuged for 5 minutes at 8,765*g*. After blocking, the plates were washed 3 times and supernatant from cells or biologic fluids were screened for the presence of sLAIR-1 or LAIR-2. Recombinant human sLAIR-1 or LAIR-2 (R&D systems) were used as the reference protein and was serially diluted in PBS with 3% BSA. All was incubated for 2 hours at room temperature.

Plates were washed 3 times, and the wells were incubated with biotinylated anti-hLAIR-1 8A8 mAb (5 μg/ml in 50 μl PBS) or biotinylated anti-hLAIR-2 mAb (R&D systems) (0.5 μg/ml in 50 μl PBS) for two hours at room temperature. After 3 washes, the wells were incubated for 1 hour at room temperature with StrepABComplex/HRP (DAKO). After 3 washes, ABTS reagent (100 μl/well; Roche Diagnostics) was added and colour was allowed to develop for 15 minutes at 37°C. Measurements were performed with an ELISA plate reader (BIO-TEK) at 405 nanometer. The detection limit for sLAIR-1 is 1,950 pg/ml and for LAIR-2 230 pg/ml. For statistical analysis, samples below detection limit were given the value of the detection limit. A Mann-Whitney U-test was performed to calculate statistical significance between groups. Spearman's test was used to assess correlations between sLAIR-1 and LAIR-2 and between the ESR and LAIR-2 using Graphpad Prism software, version 5.03.

Recombinant protein production

LAIR-1, LAIR-2 and as a control Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) (17) complementary DNA was cloned into a retroviral pGEMT vector using LAIR-1 extra cellular domain specific forward (5'-GGATCCCAGGAGGAAGATCTGCCCAGACCC-3') and reverse (5'-GCGGCCGCCTCAGCTTTCAGGCTTGGGAAGC-3'), LAIR-2 specific forward

(5'-GGATCCCAGGAGGGGCCCTTCCCAGAC-3') and reverse (5'-GCGGCCGCTGGTGCAT CAAATCCGGAGGCTTC-3') and SIRL-1 extra cellular domain specific forward (5'-GATCCTA CGAAGATGAGAAAAAGAATGAGAAACCGCCCAAG-3') and reverse (5'-GCGGCCGCGTCTG TTTTCATTGAGGGAGCTTCCAAGTTCATCG-3') primers, thereby introducing a *Bam* H1 and *Nod* 1 restriction site. pGEMT vectors were delivered to U-protein express, who provided us with eukaryotic expressed purified 6xHIS C-terminal tagged monomer proteins and (TEV)-hFC C-terminal tagged dimer proteins.

Fluorescent collagen binding

FITC-labelled collagen I or III (final concentration 50 mg/ml) was allowed to incubate for 5 minutes with 10 μg of LAIR-1 or LAIR-2 monomer or dimer protein dissolved in PBS. EDTA was added to a final concentration of 50 m*M* in a total volume of 20 μl. K562 WT or K562 hLAIR-1 expressing cells were washed 3 times with PBS supplemented with 1% BSA and counted. A total of 100,000 cells were added and incubated for 30 minutes at 4°C.

Cells were washed 3 times with PBS with 1% BSA and analysed by flow cytometry on a LSR II fluorescence-activated cell sorter (FACS) using FACS DIVA software (BD Biosciences).

Adhesion Assay

A 96-well flat-bottomed MAXI-sorp plate was coated overnight at 4°C with purified type I collagen (2 μg/ml; Sigma) or BSA (5 μg/ml) in 100 μl PBS, supplemented with 2 m*M* acetic acid. Then, 5 X 10^6 K562 WT or K562 hLAIR-1 expressing cells were fluorescently labeled with Calcein AM (Molecular Probes) in PBS for 30 minutes at 37°C. After three washes with PBS, the wells were incubated with 40, 30, 20 or 0 μg LAIR-1, LAIR-2 or control monomer or dimer recombinant protein. Cells were then assayed for their capacity to adhere to the collagen in the 96-well plate, as described previously (2). The retained fluorescence was determined for each well as a percentage of the input fluorescence; data of wash 4 are shown.

Surface plasmon resonance experiments

Surface plasmon resonance (BIAcore) binding studies were performed with the use of a BIAcore T100 system. Approximately 3000 response units (RU) for the monomer recombinant LAIR proteins or 300 RU for the dimer recombinant LAIR proteins of acid soluble human collagen type I, III or IV (Sigma) were immobilized on a series S CM5 sensor chip by using the amine coupling kit according to the manufacturer's instructions. Analysis was performed in buffer (125 m*M* NaCl, 2.5 m*M* $CaCl_2$, 0.005% [volume/volume] Tween20 and 25 m*M* HEPES pH 7.4) at 25°C at a flow rate of 5 μl/min. Injections with increasing concentrations of recombinant proteins (0, 31.25, 62.5, 125, 250, 500, 1,000 and 2,000 nM for the dimer proteins and 0, 125, 250, 500, 1,000, 2,000, 3,000, 4,000, 5,000, and 6,000 nM for the monomer proteins) were allowed to bind for 10 minutes, after which regeneration by either 0.1*M* H_3PO_4 for the monomer LAIR-2-his protein and the dimer LAIR-1-Fc protein, or by 4*M* $MgCl_2$ followed by 0.2*M* H_3PO_4 for the dimer LAIR-2-Fc protein occurred. Baseline stability was checked after every experiment.

Numbers of maximal binding sites (B_{max}) were converted to number of recombinant proteins interacting with a single collagen trimer by using the theoretical mass of monomer LAIR-2-his, dimer LAIR-1-Fc and dimer LAIR-2-Fc

(15.4 kd, 86 kd and 83.9 kd, respectively, as supplied by the manufacturer) versus types I, III and IV collagen (416.7, 415.7 and 438.7 kDa respectively). Proteins were injected until binding equilibrium was reached. Binding data were analyzed with BIAcore T100 evaluation software (version 2.01). Affinity constants were determined with steady state analysis.

RESULTS

LAIR-2 is a more efficient antagonist of the LAIR-collagen interaction than is sLAIR-1

Theoretically, both shLAIR-1 and hLAIR-2 are able to block the interaction between cell-expressed hLAIR-1 and collagen, thereby functioning as a receptor antagonist (14). We recently found that recombinant LAIR-2-Fc is an efficient inhibitor of the interaction between platelets and collagen, whereas LAIR-1-Fc is not. Recombinant Fc proteins are dimers, whereas sLAIR-1 and LAIR-2 likely circulate in vivo as monomers.

Addition of dimeric LAIR-1-Fc inhibited up to 75% of the binding of fluorescence-labeled collagen to LAIR-1-expressing cells, whereas dimeric LAIR-2-Fc blocked up to 90% (Figures 1A and B). Control recombinant proteins did not interfere with the interaction between the LAIR-1-expressing cells and collagen. To further establish the difference between the blocking capacity of the sLAIR-1-Fc and LAIR-2-Fc we performed an adhesion assay. As shown previously, LAIR-1-expressing K562 cells bound firmly to type I collagen immobilized in microtiter plates, and this could be inhibited by preincubation with different concentrations of the recombinant proteins (2;12). Both LAIR-dimers inhibited adhesion to a similar extent (Figure 1C).

To be able to investigate the difference in binding between monomeric and dimeric LAIR proteins, we constructed recombinant monomers of LAIR-1 (LAIR-1-His) and LAIR-2 (LAIR-2-His) and tested them in the same assays. Addition of monomeric LAIR-1-His reduced the binding of fluorescence-labeled collagen to K562 cells with 20%. In contrast, LAIR-2-His was much more potent in blocking the LAIR-1 – collagen interaction, since a similar concentration of LAIR-2-His abrogated collagen binding with 70% (Figure 1B).

Similarly, LAIR-2-His was more efficient than LAIR-1-His at blocking the adhesion of LAIR-1 – expressing cells to collagen (Figure 1C). These data indicate that LAIR-2 is a better competitor for the collagen-LAIR-1 interaction than is sLAIR-1.

LAIR-2 has a higher affinity for collagen than does LAIR-1

We investigated the differences in binding to types I, III and IV collagen between recombinant LAIR-1 and LAIR-2 monomers and dimers with surface plasmon resonance experiments. Analysis of monomeric LAIR-1-His with insufficient amounts of monomeric LAIR-1 indicated an affinity > 20 μM and was therefore not determined (data not shown). Monomeric LAIR-2-His showed specific binding to collagen, though with low affinity.

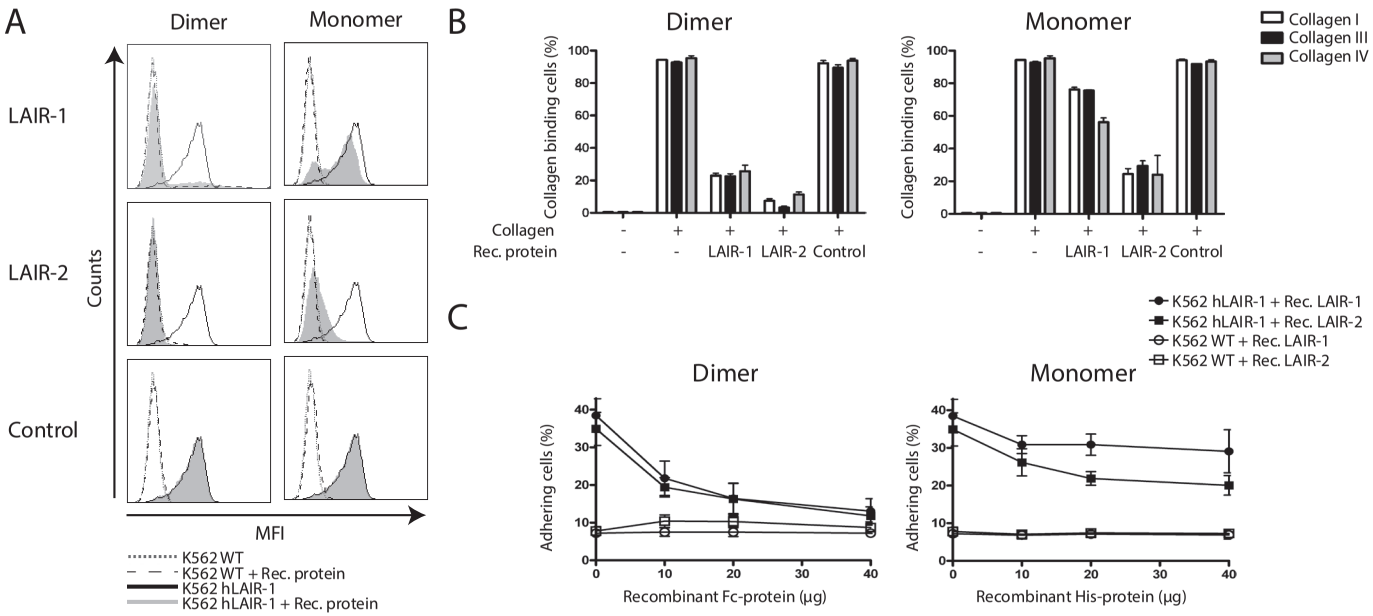


Figure 1: Leukocyte-associated immunoglobulin-like receptor-2 (LAIR-2) is a more efficient competitor for the LAIR-1 – collagen interaction than is soluble LAIR-1 (sLAIR-1). K562 wild-type (WT) or K562 human LAIR-1 (hLAIR-1) expressing cells were incubated with fluorescence-labeled collagen in the presence or absence of recombinant (Rec) LAIR proteins and analyzed by flow cytometry. All experiments were performed at least 3 times. **(A)** Histograms from a representative experiment. K562 WT cells or K562 hLAIR-1-expressing cells were incubated with type I, type III and type IV collagen in the presence or absence of recombinant dimer LAIR-Fc or monomer LAIR-His. **(B)** Percentages of cells binding type I, type III and type IV collagen. Values are the mean ± SEM of 3 experiments. **(C)** Inhibition of collagen binding. Plates (96-well) were coated with type I collagen and incubated with increasing concentrations of recombinant (Rec.) LAIR proteins. Fluorescence-labeled K562 WT or K562 LAIR-1-expressing cells were added to the plates and left to adhere for ~3 hours. The retained fluorescence was determined for each well as a percentage of the input fluorescence.Values are the mean ± SEM percentage of adherent cells relative to the input from wash 4.

The mean ± SEM affinity constants (K_D) were 2,039 ± 227.2 n*M* for type I collagen, 2,047 ± 229.1 n*M* for type III collagen and 1,916 ± 216.2 n*M* for type IV collagen (Figure 2A and Table 1). One single trimer of type I or III collagen was able to bind ~19 molecules of monomeric LAIR-2-His, while a single trimer of type IV collagen could bind 18 LAIR-2-His molecules. LAIR-1-Fc dimers had affinity constants of 425.5 ± 57.79 n*M* for type I collagen, 907.6 ± 103.9 nM for type III collagen and 603.0 ± 39.8 n*M* for type IV collagen (Figure 2B and Table 1). Approximately 10 molecules of LAIR-1-Fc could bind to a single type I or type III collagen trimer, while ~11 molecules could bind to a single trimer of type IV collagen. Dimeric LAIR-2-Fc had such a high affinity for type III and type IV collagen that regeneration failed; therefore, we could only determine the affinity for type I collagen. Type I collagen had 2 different binding sites for LAIR-2-Fc; one high-affinity binding site (K_D = 31.83 ± 3.29 nM) and one low-affinity binding site (K_D = 912.9 ± 127.1 nM) (Figure 2C and Table 1). A single trimer of type I collagen could bind ~7 LAIR-2-Fc molecules to the high-affinity binding site, while ~10 molecules could bind to the low-affinity binding site.

Together, these data show that LAIR-2 has a higher affinity for collagen than does LAIR-1, both as a monomer and as a dimer.

LAIR-2 is produced by activated CD4$^+$ T cells

We set out to determine which cells produce sLAIR-1 and LAIR-2 in vitro. First, we stimulated PBMCs from 20 healthy donors with PMA and ionomycin for 4 days and screened for the presence of sLAIR-1 and LAIR-2 using a sandwich ELISA. LAIR-2 was produced by activated PBMCs in 16 out of 20 donors (Figure 3A). Next, we wanted to

Table 1: Calculated K_D and Bmax values*

	LAIR-1		LAIR-2	
	Monomer	**Dimer**	**Monomer**	**Dimer**
Type I collagen				
K_D	ND	425.5 ± 57.79	2,039 ± 227.2	31.83 ± 3.29
B_{max}	ND	10.48 ± 0.57	19.51 ± 0.87	7.35 ± 0.39
K_D2	ND	ND	ND	912.9 ± 127.1
$B_{max}2$	ND	ND	ND	10.52 ± 0.26
Type III collagen				
K_D	ND	907.6 ± 103.9	2,047 ± 229.1	ND
B_{max}	ND	10.84 ± 0.63	19.57 ± 0.88	ND
Type IV collagen				
K_D	ND	603.0 ± 39.8	1,916 ± 216.2	ND
B_{max}	ND	11.74 ± 0.35	18.02 ± 0.8	ND

* Shown are the calculated affinities (K_D; in n*M*) and number of maximal binding sites (B_{max}; in moles of recombinant protein per mole of collagen) as calculated from the binding at equilibrium (R_{eq}) of the surface plasmon resonance experiments. Values are the mean ± SEM. ND = not determined.

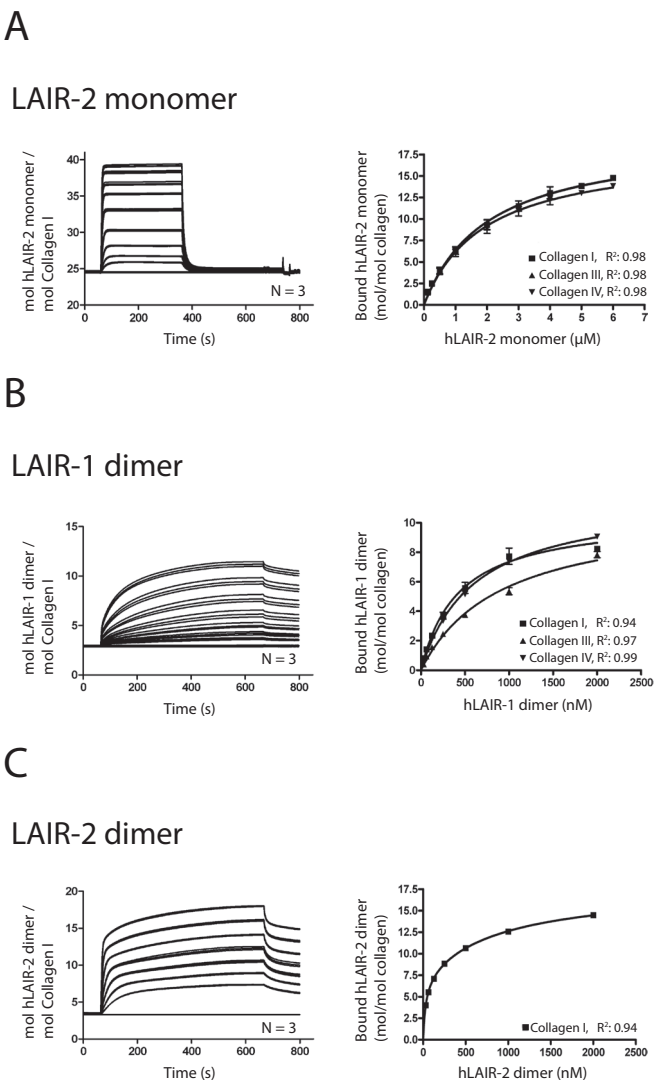


Figure 2: LAIR-2 has a higher affinity for collagens than does sLAIR-1. Binding of **(A)** recombinant monomeric LAIR-2-His, **(B)** dimeric sLAIR-1-Fc and **(C)** dimeric LAIR-2-Fc to immobilized type I, type III, and type IV collagen was measured by surface plasmon resonance analysis. Recombinant proteins were injected at a flow rate of 5 µl/min at a temperature of 25° C through a BIAcore flow cell containing ~300 response units (RU; for LAIR-1-Fc and LAIR-2-Fc) or ~3000 RU (for LAIR-2-His) of immobilized collagen. Triplicate binding curves of recombinant monomeric LAIR-2-His at a concentration of 0, 125, 250, 500, 1,000, 2,000, 3,000, 4,000, 5,000, and 6,000 n*M* and dimeric sLAIR-1-Fc and dimeric LAIR-2-Fc at a concentration of 0, 31.25, 62.5, 125, 250, 500, 1,000 and 2,000 n*M* are shown at the left. Individual symbols representing resonance at equilibrium along with the corresponding concentration of the recombinant protein are shown at the right. Values are the mean ± SEM. See figure 1 for definitions.

know which T cell subset was producing LAIR-2. FACS-sorted $CD4^+$ T cells produced more LAIR-2 than did $CD8^+$ T cells (Figure 3B). Among $CD4^+$ T cells, naive $CD27^+$ $CD45RO^-$ $CD4^+$ T cells produced the highest amount of LAIR-2, followed by central memory $CD27^+$, $CD45RO^+$ and effector $CD27^-$, $CD45RO^+$ cells (Figure 3C).

Soluble LAIR-1 was detected in only a limited number of samples. Increased production of sLAIR-1 by PBMCs, sorted $CD4^+$ and $CD8^+$ T cells and subsets upon in vitro stimulation was not observed (Figure 3A and data not shown). To further investigate the cellular source of sLAIR-1, we stimulated PBMCs with LPS or antiCD3/antiCD28. Furthermore, we isolated monocytes and stimulated them with PMA and several Toll-like receptor agonists (LPS, Pam_3Cys or curdlan). In addition, isolated neutrophils were stimulated with the proinflammatory cytokines G-CSF, GM-CSF, fMLP and TNFα and whole blood cultures were stimulated with poly(I-C), LPS, LPS plus IFNγ, CpG-ODN, loxoribine or Pam_3Cys. Again, none of these conditions led to sLAIR-1 production above background level (data not shown). Thus, while we identified activated T cells as a source of LAIR-2, we were not able to determine which leukocytes shed and/or produce sLAIR-1.

Soluble LAIR-1 and LAIR-2 levels are increased in the urine of patients with chronic inflammation

We previously showed that the amount of LAIR-2 in synovial fluid of 16 RA patients was significantly higher than that in synovial fluid from 16 OA patients (12). In the present study, we found that synovial fluid contains up to 100-fold more sLAIR-1 than LAIR-2, which also is significantly increased in RA patients (n = 16) compared to OA patient controls (n = 16) (figure 4A). To further assess the production of sLAIR-1 or LAIR-2 in vivo, we compared levels of sLAIR-1 and LAIR-2 in plasma and urine samples between healthy controls (21 urine samples; 19 plasma samples) and RA patients (27 urine and plasma samples). LAIR-1 was detectable in the plasma of most healthy controls and RA patients, while LAIR-2 was below the detection limit in most plasma samples from RA and healthy controls (Figure 4B). The plasma concentration of sLAIR-1 was about 10 times higher than that of LAIR-2. No differences were observed between healthy controls and RA patients. However, strikingly increased urine levels of both sLAIR-1 and LAIR-2 were observed in RA patients as compared to healthy controls (Figure 4C). In addition, concentrations of sLAIR-1 and LAIR-2 in the urine of RA patients were significantly correlated (Figure 4D). Furthermore, a significant correlation between LAIR-2 concentrations in urine and ESR, a marker of inflammation, was observed in RA patients (Figure 4D). No significant correlation between levels of sLAIR-1 and ESR were observed ($r = 0.1695$, $P = 0.3979$).

DISCUSSION

Proper functioning of the immune response depends on a delicate balance between activating and inhibitory signals. The inhibitory immune receptor LAIR-1 is expressed on most cells of the immune system, and its ligand collagen is ubiquitously expressed in the tissues. Therefore, triggering of the inhibitory immune receptor LAIR-1 must be carefully

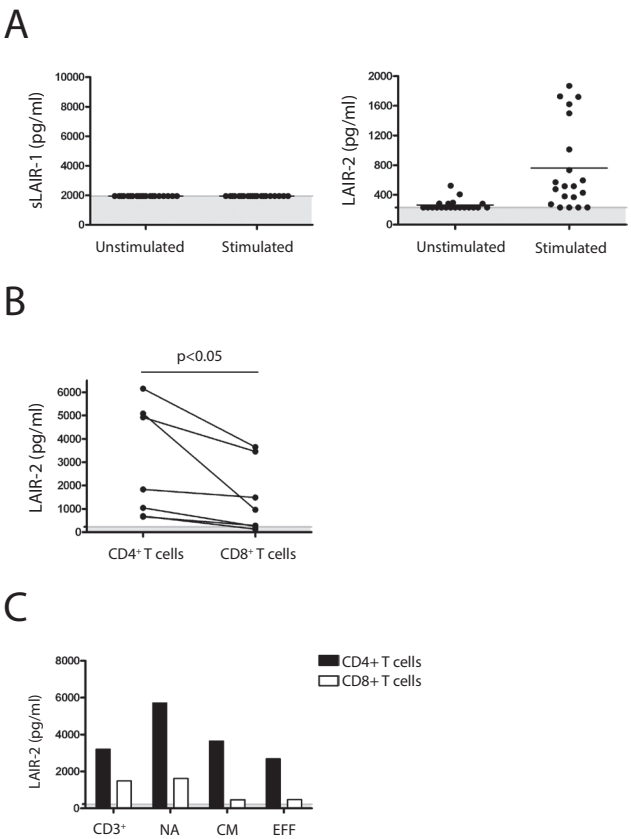


Figure 3: Leukocyte-associated immunoglobulin-like receptor-2 (LAIR-2) is produced by activated T cells. Peripheral blood mononuclear cells (PBMCs) isolated from 20 healthy donors were left unstimulated or were stimulated for 4 days with phorbol myristate acetate and ionomycin, and the supernatant was harvested and tested in a sandwich enzyme-linked immunosorbent assay for the presence of soluble LAIR-1 (sLAIR-1) and LAIR-2. **(A)** High levels of LAIR-2 were detected in the supernatant from stimulated PBMCs whereas sLAIR-1 was not detected. Each data point represents a single subject; horizontal lines show the mean. **(B)** $CD4^+$ and $CD8^+$ T cells sorted from the PBMCs of 6 healthy donors were screened for the production of LAIR-2. Lines connect the data points for individual donors. *P* value was determined by Wilcoxon's signed rank test. **(C)** LAIR-2 levels in $CD3^+$, naive (NA), central memory (CM), and effector (EFF) T cells were determined in stimulated $CD4^+$ and $CD8^+$ T cell subsets sorted from the PBMCs of healthy donors. Data are from a representative donor; 6 donors were tested. Shaded areas in **A-C** indicate the limits of detection.

regulated. In this study, we have investigated the role of two soluble proteins which might contribute to this regulation (14). We show that both sLAIR-1 and LAIR-2 can be detected in body fluids. Upon inflammation, such as in RA, increased concentrations of sLAIR-1 and LAIR-2 can be detected in the urine and in synovial fluid of inflamed joints. Indeed, T cells produce increased amounts of LAIR-2 upon activation in vitro.

Soluble LAIR-1 and LAIR-2 can both potentially function as competitors for collagen binding with cell-expressed LAIR-1. We have now shown that LAIR-2 is more effective than sLAIR-1 in abrogating the LAIR-1 - collagen interaction. Given the low affinity for collagen of the monomer LAIR-1, it is likely that sLAIR-1 will hardly affect the collagen – LAIR-1 interaction in vivo. However, shedding of the molecule will lower the amount of receptor exposed on the cell membrane, thereby lowering the strength of the inhibitory signal given to the cell upon triggering of the receptor. We have previously shown that immature cells express higher amounts of LAIR-1 than do activated cells or cells differentiated towards a more mature phenotype (8-10). Thus, shedding of the receptor may act to regulate the strength of the inhibitory signal delivered to the cell, even if the shed receptor itself does not function as a competitor for LAIR-1 binding to collagen.

In contrast to sLAIR-1, monomer LAIR-2 is an efficient inhibitor of the LAIR-1 – collagen interaction and, thus, may prevent the binding of collagen to LAIR-1 expressed on cells. Endogenous LAIR-2 has been shown to bind the extra cellular matrix of chorionic villi ex vivo (18), thus supporting this notion. LAIR-2 is produced by T cells, which also express LAIR-1. Therefore, LAIR-2 might act locally as an additional regulator of LAIR-1 function on T cells.

For monomer LAIR-1-His, the affinity for collagen was so low, that we were not able to detect this with surface plasmon resonance using the stated concentrations of recombinant protein. Jiang *et al.* (1) used a more sensitive assay to detect low-affinity interactions. They used beads that express multivalent ligands to determine the affinity of the LAIR-1 - collagen interaction by surface plasmon resonance. They reported a K_d for the LAIR-1 – collagen binding of ~20 μM. Although those authors did not determine the K_d for LAIR-2, their K_d for LAIR-1 is a 10-fold lower affinity than we detected for LAIR-2-His in our experiments. This confirms the notion that monomer LAIR-2 binds collagen with a higher affinity than does monomer LAIR-1.

We previously determined that the K_D of LAIR-1-Fc and LAIR-2-Fc were in the same range (2;12). However, our current results showed that LAIR-2-Fc binds collagen with higher affinity than does LAIR-1-Fc. In the current set of experiments, the dissociation of recombinant LAIR dimers was slower than in former experiments, which led us to regenerate the chip after every injection of recombinant proteins. This regeneration step was not necessary in former experiments. These differences in binding kinetics could be indicative of differences in batches of collagen used.

We previously showed that LAIR-2 is capable of binding more immobilized synthetic trimeric peptides encompassing the entire triple-helical domain of human type II and type III collagen than LAIR-1 (19). This might explain the higher affinity measured in the surface plasmon resonance experiments and its ability to abrogate the LAIR-1 – collagen interaction better than sLAIR-1. Importantly, we showed that recombinant LAIR-2-Fc but not LAIR-1-Fc was able to inhibit collagen induced platelet aggregation, which fits with our findings that LAIR-2 has a higher affinity for collagens than does sLAIR-1 (13).

Our finding that sLAIR-1 and LAIR-2 can be detected in increased amounts in the synovial fluid and urine of RA patients suggests that shedding of sLAIR-1 and increased

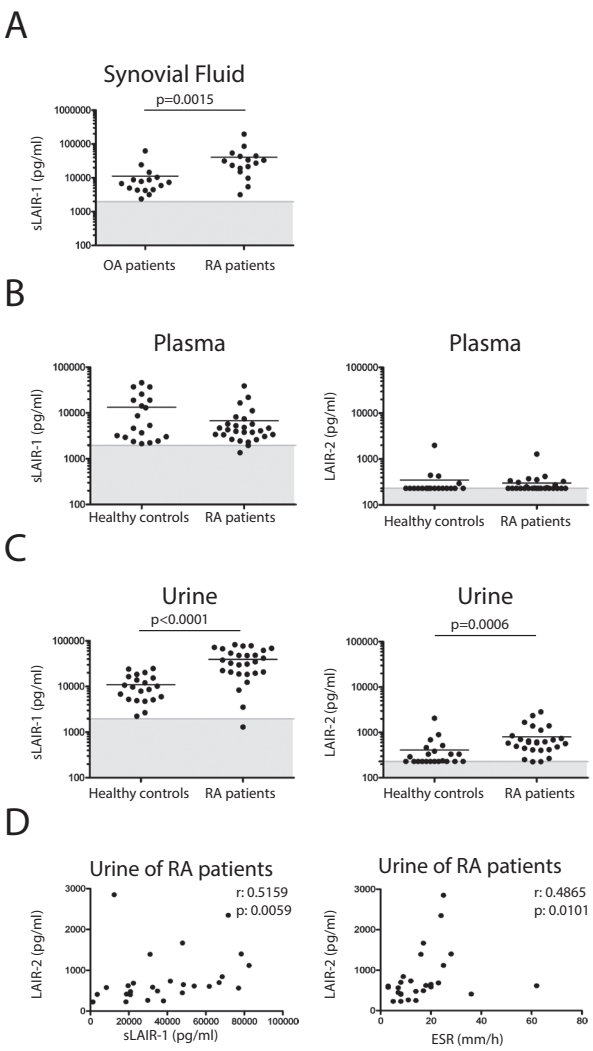


Figure 4: Soluble leukocyte-associated immunoglobulin-like receptor-1 (sLAIR-1) and LAIR-2 are secreted in increased amounts in the urine of patients with rheumatoid arthritis (RA). A-C Concentrations of sLAIR-1 in synovial fluid **(A)**, and sLAIR-1 and LAIR-2 in plasma **(B)** and sLAIR-1 and LAIR-2 in urine **(C)** from osteoarthritis (OA) patients, RA patients, and healthy control subjects were determined by sandwich enzyme-linked immunosorbent assay as described in Material and Methods. All values below the limit of detection were plotted as being at the detection limit. There was a significant difference between the levels of sLAIR-1 in synovial fluid from OA (n = 16) versus RA (n = 16) patients, no significant difference between the levels of sLAIR-1 and LAIR-2 in plasma from healthy controls (n = 19) versus RA patients (n = 27), and a significant difference between the levels of sLAIR-1 and LAIR-2 in urine from healthy controls (n = 21) versus RA patients (n = 27), as determined by Mann-Whitney U test. **(D)** Urinary concentrations of LAIR-2 correlated significantly with the urinary sLAIR-1 concentration and with the erythrocyte sedimentation rate (ESR) in RA patients, as determined by Spearman's correlation test. Shaded areas in **A-C** indicate the limits of detection.

production of LAIR-2 play a role in the regulation of LAIR-1-induced inhibition in these patients. The correlation of the amount of LAIR-2 in the urine of RA patients with a marker of inflammation supports this notion. The amount of urinary LAIR-2 in RA patients correlated significantly with ESR. The increased urinary levels of sLAIR-1 and LAIR-2 in these patients suggest that these proteins are indicative of disease, even though other clinical and laboratory markers of inflammation are minimally up-regulated. These increased levels in RA patients may be indicative of ongoing synovial activation and tissue-destructive processes, which have previously been suggested to contribute to progressing joint destruction in patients with low disease activity (20;21). In this respect these markers could serve as sensitive biomarkers for inflammation and tissue destruction. Future studies are needed to address whether sLAIR-1 and LAIR-2 are also useful markers for the detection of early disease. Moreover, the role of LAIR-2 in the pathogenesis of RA needs to be investigated. The increased level of this protein might contribute to the pathogenesis of RA by releasing the inhibitory LAIR-1 – collagen interaction. Whether enhanced LAIR-2 secretion is the cause or consequence of immune activation is unclear. However, once elevated, a self sustaining loop of immune activation resulting in even higher LAIR-2 production may arise. Modulating this loop could reinstate the inhibition of the LAIR-1 – collagen interaction and dampen the immune system, thereby lowering the rate of ongoing joint destruction.

Ouyang *et al.* (15) showed detectable levels of sLAIR-1 in the serum of healthy control subjects and increased levels in the sera of patients with hemorrhagic fever with renal syndrome. We previously found increased levels of LAIR-2 in pregnant women (12). Other investigators have reported decreased expression of LAIR-2 mRNA in chorionic villous samples of pregnancies destined for preeclampsia (22). From our study findings, we conclude that LAIR-2 is a possible antagonist of LAIR-1 function in vivo. Both sLAIR-1 and LAIR-2 are potential biomarkers for monitoring inflammation in patients with inflammatory disease or altered immune activation or regulation.

AKNOWLEDGEMENTS

We would like to thank P. J. Lenting, J.W. Akkerman and K. Gerritsen for discussions and C.E. Hack and Ph. G. de Groot for critical reading of the manuscript. We are very grateful to Professor B. Jin for generously providing the LAIR-2 antibody and G. Spierenburg for performing the cell sorting.

REFERENCE LIST

1. Jiang L, Barclay AN. New assay to detect low-affinity interactions and characterization of leukocyte receptors for collagen including leukocyte-associated Ig-like receptor-1 (LAIR-1). Eur J Immunol 2009; 39(4):1167-75.
2. Lebbink RJ, De Ruiter T, Adelmeijer J, Brenkman AB, van Helvoort JM, Koch M *et al.* Collagens are functional, high-affinity ligands for the inhibitory immune receptor LAIR-1. J Exp Med 2006; 203:1419-25.
3. Tang X, Narayanan S, Peruzzi G, Apara A, Natarajan K, Margulies DH *et al.* A single residue, arginine 65, is critical for the functional interaction of leukocyte-associated inhibitory receptor-1 with collagens. J Immunol 2009; 182(9):5446-52.
4. Omiya R, Tsushima F, Narazaki H, Sakoda Y, Kuramasu A, Kim Y *et al.* Leucocyte-associated immunoglobulin-like receptor-1 is an inhibitory regulator of contact hypersensitivity 1. Immunology 2009; 128(4):543-55.
5. Meyaard L, Hurenkamp J, Clevers H, Lanier LL, Phillips JH. Leukocyte-associated Ig-like receptor-1 functions as an inhibitory receptor on cytotoxic T cells. J Immunol 1999; 162:5800-4.
6. Poggi A, Tomasello E, Ferrero E, Zocchi MR, Moretta L. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. Eur J Immunol 1998; 28:2086-91.
7. Saverino D, Fabbi M, Merlo A, Ravera G, Grossi CE, Ciccone E. Surface density expression of the leukocyte-associated Ig-like receptor-1 is directly related to inhibition of human T cell functions. Hum Immunol 2002; 63:534-46.
8. Van der Vuurst de Vries A, Clevers H, Logtenberg T, Meyaard L. Leukocyte Associated Ig-like Receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. Eur J Immunol 1999; 29:3160-7.
9. Jansen CA, Cruijsen C, De Ruiter T, Nanlohy N, Willems N, Janssens-Corpela P *et al.* Regulated expression of the inhibitory receptor LAIR-1 on human peripheral T cells during T cell activation and differentiation. Eur J Immmunol 2007; 37:914-24.
10. Verbrugge A, de RT, Geest C, Coffer PJ, Meyaard L. Differential expression of leukocyte-associated Ig-like receptor-1 during neutrophil differentiation and activation. J Leukoc Biol 2006; 79(4):828-36.
11. Maasho K, Masilamani M, Valas R, Basu S, Coligan JE, Borrego F. 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 2005; 42:1521-30.
12. Lebbink RJ, van den Berg MCW, De Ruiter T, Raynal N, van Roon JAG, Lenting PJ *et al.* The soluble LAIR-2 antagonizes the collagen/LAIR-1 inhibitory immune interaction. J Immunol 2008; 180:1662-9.
13. Lenting PJ, Westerlaken GHA, Denis CV, Akkerman J.W., Meyaard L. Efficient inhibition of collagen-induced platelet activation and adhesion by LAIR-2, a soluble Ig-like receptor family member. Plos One 2010; 5:e12174.
14. Meyaard L. The inhibitory collagen receptor LAIR-1 (CD305). J Leukoc Biol 2008; 83(4):799-803.
15. Ouyang W, Xue J, Liu J, Jia W, Li Z, Xie X *et al.* Establishment of an ELISA system for determining soluble LAIR-1 levels in sera of patients with HFRS and kidney transplant. J Immunol Methods 2004; 292(1-2):109-17.
16. Meyaard L, Adema GJ, Chang C, Woollatt E, Sutherland GR, Lanier LL *et al.* LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. Immunity 1997; 7:283-90.
17. Steevels TAM, Lebbink RJ, Westerlaken GHA, Coffer PJ, Meyaard L. Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) is a novel functional inhibitory immune receptor expressed on human phagocytes. J Immunol 2010; 184:4741-8.
18. Founds SA, Fallert-Junecko B, Reinhart TA, Conley YP, Parks WT. LAIR2 localizes specifically to sites of extravillous trophoblast invasion. Placenta 2010; 31(10):880-5.

19. Brondijk TH, de RT, Ballering J, Wienk H, Lebbink RJ, van IH *et al.* Crystal structure and collagen-binding site of immune inhibitory receptor LAIR-1: unexpected implications for collagen binding by platelet receptor GPVI. Blood 2010; 115:1364-73.
20. Brown AK, Conaghan PG, Karim Z, Quinn MA, Ikeda K, Peterfy CG *et al.* An explanation for the apparent dissociation between clinical remission and continued structural deterioration in rheumatoid arthritis. Arthritis Rheum 2008; 58(10):2958-67.
21. Aletaha D, Funovits J, Breedveld FC, Sharp J, Segurado O, Smolen JS. Rheumatoid arthritis joint progression in sustained remission is determined by disease activity levels preceding the period of radiographic assessment. Arthritis Rheum 2009; 60(5):1242-9.
22. Founds SA, Conley YP, Lyons-Weiler JF, Jeyabalan A, Hogge WA, Conrad KP. Altered global gene expression in first trimester placentas of women destined to develop preeclampsia. Placenta 2009; 30(1):15-24.

4

sLAIR-1 IN AMNIOTIC FLUID IS OF FETAL ORIGIN AND POSITIVELY ASSOCIATES WITH LUNG COMPLIANCE

Michiel L Houben[1], Marloes JM Olde Nordkamp[2], Peter Nikkels[3], Cornelis K van der Ent[1], Linde Meyaard[2], Louis J Bont[1,2]

Submitted

[1)] Dept of Pediatrics, [2)] Dept of Immunology, Laboratory for Translational Immunology, [3)] Dept of Pathology, University Medical Centre Utrecht, Utrecht, the Netherlands

ABSTRACT

The soluble form of the inhibitory immune receptor Leukocyte-Associated Ig-like Receptor-1 (sLAIR-1) is present in plasma, urine and synovial fluid and correlates to inflammation. We and others previously showed inflammatory protein expression in normal amniotic fluid at term. We hypothesized that sLAIR-1 is present in amniotic fluid during term parturition and is related to fetal lung function development.

sLAIR-1 was detectable in all amniotic fluid samples (n = 355) collected during term spontaneous deliveries. First, potential intra-uterine origins of amniotic fluid sLAIR-1 were explored. Although LAIR-1 was expressed on the surface of amniotic fluid neutrophils, LAIR-1 was not secreted upon ex vivo neutrophil stimulation with LPS, or PMA and ionomycin. Cord blood concentrations of sLAIR-1 were fourfold lower than and not related to amniotic fluid concentrations and placentas showed no or only sporadic LAIR-1 positive cells. Similarly, in post-mortem lung tissue of term neonates that died of non-pulmonary disorders LAIR-1 positive cells were absent or only sporadically present. In fetal urine samples, however, sLAIR-1 levels were even higher than in amniotic fluid and correlated with amniotic fluid sLAIR-1 concentrations.

Second, the potential relevance of amniotic fluid sLAIR-1 was studied. sLAIR-1 concentrations had low correlation to amniotic fluid cytokines. We measured neonatal lung function in a convenient subset of 152 infants, using the single occlusion technique, at a median age of 34 days (IQR 30-39). The amniotic fluid concentration of sLAIR-1 was independently correlated to airway compliance ($\rho = 0.29$, $P = .001$).

Taken together, we show for the first time the consistent presence of sLAIR-1 in amniotic fluid, which originates from fetal urine. Concentrations of sLAIR-1 in amniotic fluid during term deliveries are independent from levels of other soluble immune mediators. The positive association between concentrations of amniotic fluid sLAIR-1 and neonatal lung compliance suggests that amniotic fluid sLAIR-1 may be useful as a novel independent marker of neonatal lung maturation.

INTRODUCTION

The immune system plays a pivotal role in the defense of our body by eliminating pathogens on a daily basis. Activation is ensured by pathogen recognition receptors which recognize microbial products such as lipopolysaccharide, flagellin or zymosan (1). To ensure that there is no damage to self by an excess immune response, the immune system needs to be tightly regulated. The expression of inhibitory immune receptors can provide the necessary fine tuning of an immune response (2). Soluble variants of inhibitory immune receptors may interfere with the function of the membrane bound variant of the receptor.

Leukocyte-associated Ig-like receptor-1 (LAIR-1) was discovered in 1997 as an inhibitory immune receptor that is expressed on multiple peripheral blood leukocytes, including T, B, and NK cells, eosinophils, monocytes and dendritic cells (3;4). Collagens are ligands for LAIR-1 and cross-linking results in an increase of the threshold for activating signals on several immune cells (5). Interestingly, its family member LAIR-2 is expressed as a soluble receptor which also binds collagen molecules, and may function as a natural competitor of membrane-bound LAIR-1, serving as a regulator of LAIR-1 (6). sLAIR-1 can also be detected in serum and urine, probably by shedding from the surface of LAIR-1 expressing cells (7). The affinity of sLAIR-1 for collagen most likely is too low to function as a regulator of LAIR-1 by competing for collagen binding sites (8). Ouyang and colleagues have demonstrated sLAIR-1 in serum of healthy individuals and at increased concentrations in patients with renal disorders (7). We recently demonstrated that sLAIR-1 levels in urine of rheumatoid arthritis patients are significantly increased as compared to healthy controls. Furthermore, synovial fluid of rheumatoid arthritis patients contained significantly more sLAIR-1 than synovial fluid of osteoarthritis patients, leading to the conclusion that sLAIR-1 is a marker of inflammation (8).

In this study, we investigated the levels of sLAIR-1 in amniotic fluid. Intra-uterine inflammation, in absence of a fetal inflammatory response syndrome, promotes fetal lung maturation (9-12). In a cohort of 761 preterm infants, chorioamnionitis protected against the development of chronic lung disease (CLD) (13). Moreover, in preterm lambs, experimental intra-amniotic injection of bacterial endotoxin induced an inflammatory response in membranes and in amniotic fluid, resulting in increased fetal lung compliance and lung volume (9;11;12;14). The mechanisms underlying the beneficial effects of intra-uterine inflammation on fetal lung maturation have not yet been identified.

We hypothesized that sLAIR-1 is present in utero during term spontaneous onset of labor delivery and reflects a general state of immune activation (15;16). Subsequently, we studied whether intra-amniotic sLAIR-1 is a marker of normal fetal lung function in a large healthy birth cohort.

METHODS

Study population and baseline characteristics

This study was performed as part of the Netherlands Amniotic Fluid study, described previously (17;18). In short, 372 healthy term newborns were included in a birth cohort. See supplementary table 1 for baseline and clinical characteristics. The study protocol was approved by the institutional review boards of the University Medical Center Utrecht and the Diakonessen Hospital (both in Utrecht, The Netherlands) and written informed consent was provided by the parents of all participating children. This provision was waived by the board of the University Medical Center Utrecht in the three (retrospective) cases of pathology of lung tissue of fatal neonatal cases.

Collection of amniotic fluid, urine, cord blood plasma, placentas and newborn lung tissue

Amniotic fluid was sampled during labor, purified, and stored (-80°C), as described previously (17). Amniotic fluid cell immunophenotyping and stimulation were performed on fresh amniotic fluid samples. First newborn urine (i.e. fetal urine) was collected from boys, using a urine collection bag, and stored at -80°C. Cord blood was collected directly after birth and anticoagulated using sodium heparin. Plasma was prepared by centrifugation (5 min, 500 *g*), and stored at -80°C. Placentas were stored at +4°C and processed within 72 hours, as described previously (17). Amniotic fluid lecithin-sphingomyelin (L/S) ratios were determined by thin layer chromatography according to Gluck and Kulovich (19;20). Representative sections of both lungs of fatal neonatal cases were stored according to standard operating procedures. Three cases were selected retrospectively from a coded database of perinatal autopsies, kept by the pediatric pathologist. The selection was based on delivery at term and on the absence of any signs of pulmonary disorders from the history, physical examination, and macroscopy and microscopy at autopsy.

sLAIR-1 ELISA

The concentration of sLAIR-1 in amniotic fluid, in cord blood plasma, in fetal urine, and in supernatant of stimulated amniotic fluid cells was measured by sandwich ELISA (in-house manufactured; limit of detection 1.95 ng/mL) (8). The intra-assay and the inter-assay correlations were high (Spearman's $\rho = 0.98$, $P = .005$, $n = 5$; Pearson's $\rho = 0.81$, $P < .001$, $n = 21$).

Amniotic fluid cytokine and chemokine measurements

In a subsample of 42 amniotic fluid samples, the concentrations of interleukin (IL)-1β, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, IL-18, IL-23, TNF-α, MCP-1, MIF, sICAM, MIP-1α, eotaxin, IP-10, and MIG were measured using ELISA (IL-6, IL-8, and TNF-α; CLB, Sanquin, Amsterdam, The Netherlands) (17) or Luminex (21). Correlations between these cytokines and amniotic fluid sLAIR-1 were calculated.

Immunophenotyping of amniotic fluid neutrophils

Amniotic fluid samples were stained with PE-labelled α-hLAIR-1, APC labelled α-CD11b, Pacific Blue labelled α-CD16 and APC-Cy7 labelled α-CD14 antibodies. IgG1 isotype controls were carried out using PE labelled mouse IgG1κ, to rule out non-specific binding (all antibodies were purchased from BD Pharmingen). Neutrophils were selected from the live cell gate of the forward-sideward scatter plot as $CD11b^+$ / $CD16^+$ / $CD14^-$ cells. Macrophages were selected accordingly as $CD14^+$ / $CD16^-$ cells. Flow cytometry was performed using a LSRII flow cytometer (BD Biosciences, San Diego, CA). Data were analyzed using FlowJo version 7.6 software (Tree Star, Ashland, USA).

Stimulation of amniotic fluid cells

Fresh amniotic fluid was filtered twice through a 70 μm filter. Cells were isolated by centrifugation, and cultured in RPMI 1640 (Gibco, Invitrogen, The Netherlands) supplemented with 10% fetal calf serum (Integro, Dieren, the Netherlands) and antibiotics at 37°C, 5% CO_2. Cells were stimulated with LPS (1 ng/mL and 10 ng/mL), and PMA (50 ng/mL) and ionomycin (1 μM) for 24 hours. The supernatant was harvested and stored at -20°C until further use in a sandwich ELISA for the presence of sLAIR-1.

Placenta and newborn lung immunohistology

Placenta and newborn lung tissue was analyzed for the presence of membrane-bound LAIR-1. Two sections of the umbilical cord, at the fetal and placental side, a membrane roll, one sample from the umbilical cord insertion, and three slides of normal placental parenchyma, including both decidua and chorionic plate and representative sections of newborn lung specimens were collected.

The samples were embedded in paraffin wax by standard histological procedures. Histological sections were cut at 3-4 μm and were mounted on coated slides. For all immunohistochemical staining, the same antigen-retrieval method was followed. Before staining with antibodies, slides were placed in boiling citrate buffer (pH 6.0) for 20 min. Monoclonal mouse antibodies against CD68/ED1 (Novo Castra, Newcastle, UK) were used to determine the presence of macrophages. For the placenta slides, staining was visualized using vectastain ABC (Vector Laboratories) and diaminobenzidine. For the lung slides, staining was visualized using the Bond-max (Leica) automatic stainer using polymer refine red detection. Haematoxylin was used as counterstaining.

To differentiate between cells of trophoblastic (fetal) and decidual (maternal) origin, the samples were stained for the presence of keratin (positive in trophoblast). Histological chorioamnionitis was diagnosed based on the presence of polymorphonuclear cells (neutrophilic granulocytes) in the chorionic plate or the extraplacental membranes (17). Pharyngeal tonsillary tissue from regular pediatric tonsillectomy specimens was used as positive control tissue for LAIR-1 staining. No negative controls were tested.

Infant lung function

Infant lung function was measured before the age of two months (median age 34 days, IQR 30-39), during natural sleep and without the use of any sedation, as described previously (22-24). Lung function was assessed from measurement of passive respiratory mechanics (compliance and resistance of the total respiratory system) using the single occlusion technique (SOT). The results of compliance and resistance were standardized by correction for length, weight and age during lung function measurement.

Clinical definitions

Antepartum exposure to tobacco smoke was defined as maternal smoking of at least one cigarette per day during the second semester of pregnancy. Parental atopy was defined as the presence of any atopic diagnosis (asthma, eczema or hay fever) made by a physician in one or both parents. Parental asthma was defined accordingly (25).

Statistical analysis

Baseline and lung function characteristics were compared between groups using Student's T test, Mann-Whitney U test or X^2 test, as appropriate. After logarithmic transformation, the concentration of amniotic fluid sLAIR-1 was normally distributed. To assess the association between the amniotic fluid concentration of sLAIR-1 and the standardized airway compliance and resistance, Pearson's correlation coefficient was calculated. Linear regression analysis was carried out to adjust for baseline characteristics. Spearman's correlation was calculated for the association with L/S ratios, and the correlation of cytokine and chemokine measurements.

RESULTS

sLAIR-1 is detectable in all amniotic fluid samples of term deliveries

The mean concentration of amniotic fluid sLAIR-1 was 4.82 ng/mL (95% CI 2.5-9.4), comparable to the levels of sLAIR-1 found in synovial fluid of ostheoarthritis patients, plasma in rheumatoid arthritis patients, and urine in healthy controls (8). sLAIR-1 was detectable in all 355 amniotic fluid samples. There were no differences detected for gender, gestational age (Figure 1A-B), parental asthma, or exposure to maternal tobacco smoking during pregnancy (data not shown).

Amniotic fluid neutrophils express LAIR-1 but do not secrete sLAIR-1 upon stimulation

We sought to identify the intra-uterine origin of amniotic fluid sLAIR-1. LAIR-1 was expressed on the cell surface of neutrophils in amniotic fluid, as assessed by flow cytometry (Figure 2A). However, upon ex vivo culture and stimulation of isolated amniotic fluid cells with LPS or PMA / ionomycin, no secretion of sLAIR-1 in supernatant was detected (Figure 2B). Previous studies have revealed the presence of macrophages in amniotic fluid at term, next to neutrophil abundancy (17). Flow cytometry did not

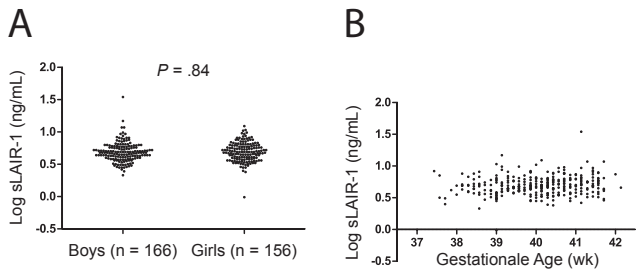


Figure 1: Distribution of amniotic fluid sLAIR-1 during term deliveries. Amniotic fluid was collected transvaginally during term physiologic deliveries (n = 345) as described previously (17). sLAIR-1 was measured by sandwich ELISA (limit of detection 1.95 ng/mL). **(A)** Amniotic sLAIR-1 concentration of newborn boys and girls. **(B)** Amniotic sLAIR-1 concentration plotted against gestational age.

consistently demonstrate the expression of LAIR-1 on the cell surface of amniotic fluid macrophages (data not shown).

Amniotic fluid sLAIR-1 is not related to cord blood sLAIR-1

Next, we measured the concentration of sLAIR-1 in paired cord blood and amniotic fluid samples, to assess for spill-over of sLAIR-1 from cord blood to amniotic fluid. sLAIR-1 was detected in 50% of cord blood plasma samples versus 100% of amniotic fluid samples (Figure 2C). There was no correlation between the concentrations of sLAIR-1 in paired samples of cord blood plasma (with detectable sLAIR-1) and amniotic fluid (Figure 2D).

No correlation between amniotic fluid sLAIR-1 and L/S ratio

Lecithin and sphingomyelin are surfactant glycoproteins and their ratio is used to predict preterm lung maturation. Amniotic fluid L/S ratios were determined in 21 amniotic fluid samples to assess the relation to surfactant synthesis at term. The mean L/S ratio was 9.2 (SD 4.1, range 3.1 to 18.2). Amniotic fluid L/S ratios were not correlated to the amniotic fluid concentrations of sLAIR-1 (Spearman's $\rho = 0.02$, $P = .94$, $n = 21$). In addition, L/S ratios were not associated with amniotic fluid pro-inflammatory cytokine concentrations, airway compliance or resistance, or gestational age or birth weight (data not shown).

No LAIR-1 positive cells present in placenta tissue during spontaneous onset of labor at term

The placentas of three children were analyzed. The two placentas without or with mild signs of histological chorioamnionitis were LAIR-1 negative (Supplementary Figure 1A-B). The placenta with signs of severe chorioamnionitis showed infrequent positive LAIR-1 staining of cells in the chorionic plate, stromal cells in Wharton's jelly of the umbilical cord, and the maternal side of the chorionic membranes (Figure 2E, Supplementary

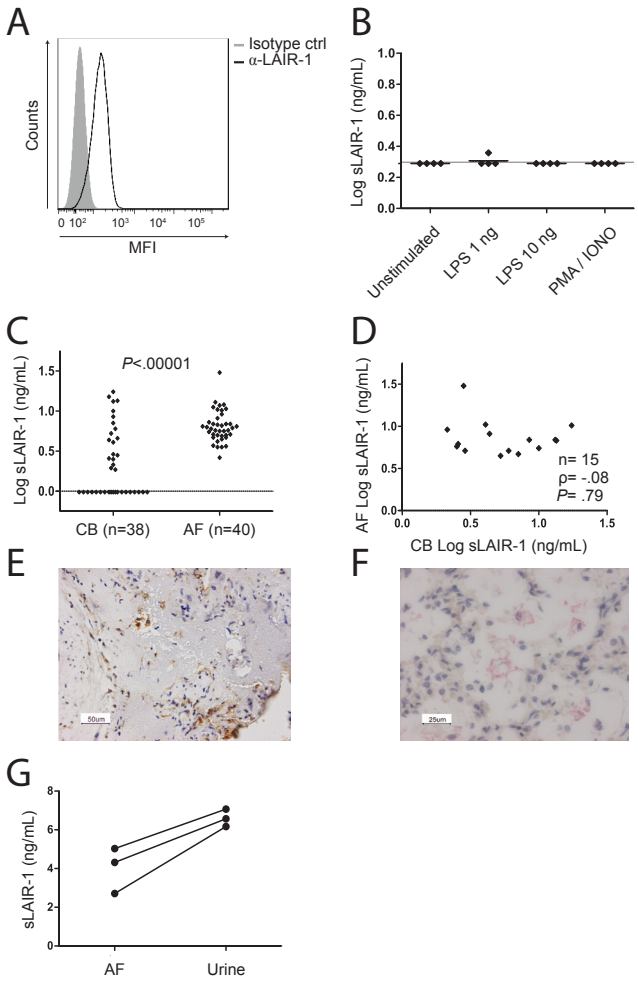


Figure 2: Fetal urine is the source of amniotic fluid sLAIR-1. The origin of amniotic fluid sLAIR-1 was investigated in multiple compartments. **(A)** LAIR-1 flow cytometry on fresh amniotic fluid samples. Neutrophils were selected from the live cell gate of the forward-sideward scatter plot as $CD11b^{+}$ / $CD16^{+}$ / $CD14^{-}$ cells. LAIR-1 expression was measured (representative of a series of $n = 5$). **(B)** Amniotic fluid cells were stimulated with LPS or PMA / ionomycin ($n = 4$). After 24 hours supernatant was harvested and the concentration of sLAIR-1 was measured in a sandwich ELISA. All values below the limit of detection were plotted as the limit of detection. Notably, flow cytometry did not consistently demonstrate the expression of LAIR-1 on the cell surface of amniotic fluid macrophages (data not shown). **(C)** sLAIR-1 ELISA in samples of cord blood (CB) and amniotic fluid (AF). Comparison of the concentration of sLAIR-1 in CB and AF samples. Student's T test for unpaired analysis. **(D)** Correlation between detectable sLAIR-1 in paired cord blood and amniotic fluid samples (Pearson's correlation coefficient). **(E)** Immunohistology of a placenta with signs of severe chorioamnionitis. LAIR-1 positive cells are infrequently present in the chorionic plate. **(F)** Immunohistology of lung tissue of a child that died perinatally of an urea cycle defect. Lung microscopy without signs of inflammation and negative LAIR-1 staining, except for sporadic interstitial cells and for alveolary macrophages. **(G)** sLAIR-1 ELISA in paired samples of amniotic fluid (AF) and fetal urine.

Figure 1C). Within the chorionic membranes, LAIR-1 was only detectable in regions that stained negative for keratin, suggesting a maternal origin of the LAIR-1 positive cells. LAIR-1 was predominantly located on the membrane of cells, most consistently in the keratin negative regions (Supplementary Figure 1D). Cells that stained LAIR-1 positive were mononuclear on microscopy and stained CD68 negative (data not shown). Tonsillary tissue (positive control) stained positive for LAIR-1 (data not shown).

No LAIR-1 positive cells present in lung tissue of fatal perinatal cases

We analyzed lung tissue of three children that died perinatally without evidence of any pulmonary disorder. The first case was a girl born after a gestational age of 37 weeks, who died from perinatal asphyxia. Lung microscopy showed sporadic infiltrates of neutrophils. Staining for LAIR-1 was negative (data not shown). The second case was a boy born after a gestational age of 37 weeks, who died from multiple congenital anomalies, including ventricular septal defect, schizencephaly, hydrocephalus and corpus callosum agenesis. Lung microscopy showed no abnormalities, except for weak LAIR-1 staining in the interstitium (Supplementary Figure 2A). The third case was a boy born after a gestational age of 38 weeks, who developed hypothermia, convulsions and severe hyperammonemia on the second day of life. He died under the diagnosis of an urea cycle disorder. Lung microscopy showed no signs of inflammation and LAIR-1 staining was negative, except for alveolar macrophages (Figure 2F, Supplementary Figure 2B). In summary, LAIR-1 was demonstrated to be virtually absent in term fetal lung parenchyma and epithelial tissue. In some cases alveolar macrophages stained positive.

Urine is the fetal source of sLAIR in amniotic fluid

Finally, we studied whether sLAIR is introduced in the amniotic cavity by fetal urine, because amniotic fluid is mainly produced by fetal urine. Indeed, we found high concentrations of sLAIR-1 in fetal urine samples which exceeded levels of paired amniotic fluids samples (Figure 2G). We therefore conclude that the sLAIR-1 in amniotic fluid is of fetal origin.

Infant lung function is associated with the concentration of amniotic fluid sLAIR-1

We hypothesized that the abundant level of sLAIR-1 in amniotic fluid could relate to a positive effect on the infant lung development. Lung function testing of all newborns was carried out, using the single occlusion technique. Successful lung function measurement was performed in 152 infants. The major reasons for failure of lung function measurement were: technical (insufficient quality and / or number of occlusions, 42%) and no sleep or too short period of sleep (52%). Children with successful lung function measurements and those who failed had similar baseline characteristics (Supplementary Table 1). Compliance and resistance of the respiratory system were normally distributed after logarithmic transformation (Supplementary Figure 3).

There was a moderately strong, positive association between the amniotic fluid concentration of sLAIR-1 and infant lung compliance ($\rho = 0.29$, $P = .001$, $n = 129$) (Figure 3).

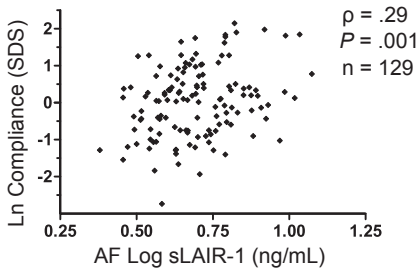


Figure 3: Correlation between amniotic fluid sLAIR-1 and infant lung compliance. Amniotic fluid sLAIR-1 was measured by sandwich ELISA (limit of detection 1.95 ng/mL). Infant lung compliance was assessed using the single occlusion technique during physiologic sleep. Compliance measurements were standardized, correcting for length, weight, and age during lung function measurement. Pearson's correlation was calculated.

Adjustment for sex yielded identical results (ρ = 0.28, *P* = .002, *n* = 129; girls ρ = 0.23, *P* = .07, *n* = 63; boys ρ = 0.33, *P* = .006, *n* = 66). Adjustment for antepartum maternal smoking yielded similar results (correction: ρ = 0.28, *P* = .001, *n* = 129; no smoking ρ = 0.28, *P* = .002, *n* = 113). There was no association between the amniotic fluid concentration of sLAIR-1 and infant resistance of the respiratory system (ρ = -0.08, *P* = .37, *n* = 131).

Amniotic sLAIR-1 is a distinct marker for fetal lung development

To study whether this relation is specific for amniotic fluid sLAIR-1, we measured a series of established (mainly) pro-inflammatory cytokines and chemokines in a subsample of the cohort. We found high correlations between amniotic fluid concentrations of IL-1β, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, IL-18, IL-23, TNF-α, MCP-1, MIF, sICAM, MIP-1α, eotaxin, IP-10, and MIG, (Supplemental Table 2), resembling the "acute inflammation gene expression signature" described by Haddad and colleagues (15). Interestingly, the pattern of amniotic fluid sLAIR-1 concentration was distinct and poorly associated with the before mentioned cytokines. The effect of sLAIR-1 on infant lung function was not found for other pro-inflammatory cytokines nor for placenta histopathology (data not shown). Thus amniotic fluid sLAIR-1 is a distinct marker for fetal lung development.

DISCUSSION

In a birth cohort of healthy term newborns, we demonstrated the consistent presence of sLAIR-1 in amniotic fluid. Concentrations were independent from amniotic fluid cytokine levels. Clinical evaluation at age one month of these newborns showed an association between intra-amniotic sLAIR-1 and newborn lung function. We found a positive correlation (ρ = 0.29) between the amniotic fluid concentration of sLAIR-1 and compliance of the total respiratory system. High levels of sLAIR-1 in paired fetal urine and amniotic fluid samples showed that fetal urine is the most likely source of amniotic fluid sLAIR-1.

The number of studies on sLAIR-1 is limited. Previous studies show that sLAIR-1 reflects a state of immune activation, and might even be used as a biomarker for disease activity (7;8;26). It was hypothesized that LAIR-1 is shed from the cell membrane of activated lymphocytes, possibly blocking the interaction with its ligand, thereby allowing for persistent lymphocyte activation (4;7). However, we recently showed that the affinity of sLAIR-1 is too low to function as a competitor for ligand binding sites (8). Still, when shed of the cell membrane, LAIR-1 expression on the cell surface is decreased, and thereby the inhibitory signal is diminished. We think that amniotic fluid sLAIR-1 reflects perinatal in utero immune activation (15;16). Immune activation could contribute to increased lung maturation at term and amniotic fluid sLAIR-1 could be either a direct signal for lung maturation, or, more likely, a reflection of the fetal inflammatory state.

Experimental studies in animals and observational studies in humans support the suggestion of a (complex) relation between fetal lung maturation, intra-amniotic inflammation and sLAIR-1. Intra-amniotic induction of a pro-inflammatory response doubled lung compliance and lung volume in prematurely delivered lambs (11;14). Similarly, intra-amniotic injection of IL-1β, TNF-α, IL-6, and IL-8 in pregnant rhesus monkeys induced accumulation of neutrophils in the fetal lungs (27). Studies on the relation between intra-amniotic inflammation and lung function in term newborns are lacking. Haddad and colleagues showed that an "acute inflammation gene expression signature" is generally present during physiologic term deliveries, and it was speculated that tissue homeostasis could be promoted by intra-uterine inflammation (15). Previously, we have demonstrated that extensive intra-uterine inflammation is present during term delivery with spontaneous onset of labor (17). Remarkably, we found that amniotic fluid sLAIR-1 levels are independent of the levels of other pro-inflammatory cytokines, and that only sLAIR-1 correlates to newborn lung function. Conceivably, sLAIR-1 is a unique representative of tissue inflammation or cell activation with limited overlap with pro-inflammatory cytokines, such as TNF-α or IL-1-β.

A limitation of our study is the observational nature. The complex relation between intra-amniotic inflammation, amniotic fluid sLAIR-1 and infant lung function remains to be unraveled. The major strength of our study is that we had the opportunity to study sLAIR-1 in a large cohort of healthy term deliveries and that several intra-uterine samples were available for extensive assessment.

Our study potentially has clinical implications. The association between intra-uterine sLAIR-1 and infant lung function may extend our insight on the origin of childhood respiratory disorders (28-32). Furthermore, it increases our insight in the mechanisms underlying fetal and newborn lung development (11;14;33). In future, amniotic fluid proteins such as sLAIR-1 possibly may serve as biomarkers for early detection of the susceptibility to viral lower respiratory tract infections or recurrent wheeze (34-36). Eventually, such a biomarker can also be applied to target new preventive or treatment strategies to children at high risk of respiratory syncytial virus (RSV) or asthma development (37;38).

In conclusion, infant lung compliance is positively correlated with the amniotic fluid concentration of sLAIR-1 in healthy term infants. Fetal urine is the source of amniotic fluid sLAIR-1. The high level of amniotic fluid sLAIR-1 during term parturition probably reflects a general state of perinatal immune activation and may be a biomarker of lung maturation in term fetuses. These novel findings may improve our understanding of the origins of childhood respiratory disorders in general, and of the physiologic maturation of fetal and infant lung function specifically.

ACKNOWLEDGEMENTS

We gratefully acknowledge Joyce Tersmette, lung function technician, Gerard Visser, gynaecologist, Eltje Bloemen-Carlier, Gertrude Roza and Annelotte Visser, research nurses, Berry Wilbrink, virologist, Jojanneke Dekkers and Arendje Peterusma, laboratory technicians, Hans Kemperman, clinical chemist, and Sanne van Haren Noman and Jona Walk, medical students, for their contribution to the collection of data; and Thomas Dobber, medical photographer, for his assistance with the histological photographs.

REFERENCE LIST

1. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; 124(4):783-801.
2. Ravetch JV, Lanier LL. Immune inhibitory receptors. Science 2000; 290(5489):84-89.
3. Meyaard L, Adema GJ, Chang C *et al.* LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. Immunity 1997; 7(2):283-290.
4. Meyaard L. The inhibitory collagen receptor LAIR-1 (CD305). J Leukoc Biol 2008; 83(4):799-803.
5. Lebbink RJ, deRuiter T., Adelmeijer J *et al.* Collagens are functional, high affinity ligands for the inhibitory immune receptor LAIR-1. J Exp Med 2006; 203(6):1419-1425.
6. Lebbink RJ, van den Berg MC, deRuiter T. *et al.* The soluble leukocyte-associated Ig-like receptor (LAIR)-2 antagonizes the collagen/LAIR-1 inhibitory immune interaction. J Immunol 2008; 180(3):1662-1669.
7. Ouyang W, Xue J, Liu J *et al.* Establishment of an ELISA system for determining soluble LAIR-1 levels in sera of patients with HFRS and kidney transplant. J Immunol Methods 2004; 292(1-2):109-117.
8. Olde Nordkamp MJ, van Roon JA, Douwes M, de RT, Urbanus RT, Meyaard L. Enhanced secretion of leukocyte-associated immunoglobulin-like receptor 2 (LAIR-2) and soluble LAIR-1 in rheumatoid arthritis: LAIR-2 is a more efficient antagonist of the LAIR-1-collagen inhibitory interaction than is soluble LAIR-1. Arthritis Rheum 2011; 63(12):3749-3757.
9. Kallapur SG, Kramer BW, Nitsos I *et al.* Pulmonary and systemic inflammatory responses to intra-amniotic IL-1alpha in fetal sheep. Am J Physiol Lung Cell Mol Physiol 2011; 301(3):L285-L295.
10. Kramer BW, Jobe AH. The clever fetus: responding to inflammation to minimize lung injury. Biol Neonate 2005; 88(3):202-207.
11. Newnham JP, Moss TJ, Kramer BW, Nitsos I, Ikegami M, Jobe AH. The fetal maturational and inflammatory responses to different routes of endotoxin infusion in sheep. Am J Obstet Gynecol 2002; 186(5):1062-1068.
12. Willet KE, Kramer BW, Kallapur SG *et al.* Intra-amniotic injection of IL-1 induces inflammation and maturation in fetal sheep lung. Am J Physiol Lung Cell Mol Physiol 2002; 282(3):L411-L420.
13. Lahra MM, Beeby PJ, Jeffery HE. Intrauterine inflammation, neonatal sepsis, and chronic lung disease: a 13-year hospital cohort study. Pediatrics 2009; 123(5):1314-1319.
14. Kramer BW, Moss TJ, Willet KE *et al.* Dose and time response after intraamniotic endotoxin in preterm lambs. Am J Respir Crit Care Med 2001; 164(6):982-988.
15. Haddad R, Tromp G, Kuivaniemi H *et al.* Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature. Am J Obstet Gynecol 2006; 195(2):394-24.
16. Smith R. Parturition. N Engl J Med 2007; 356(3):271-283.
17. Houben ML, Nikkels PG, van Bleek GM *et al.* The association between intrauterine inflammation and spontaneous vaginal delivery at term: a cross-sectional study. PLoS ONE 2009; 4(8):e6572.
18. Houben ML, Rovers MM, Wilbrink B *et al.* High concentrations of amniotic fluid proinflammatory cytokines in healthy neonates are associated with low risk of respiratory syncytial virus bronchiolitis. Pediatr Infect Dis J 2012; 31(9):931-934.
19. Gluck L, Kulovich MV. Lecithin-sphingomyelin ratios in amniotic fluid in normal and abnormal pregnancy. Am J Obstet Gynecol 1973; 115(4):539-546.
20. Wijnberger LD, de KM, Voorbij HA *et al.* The effect of clinical characteristics on the lecithin/sphingomyelin ratio and lamellar body count: a cross-sectional study. J Matern Fetal Neonatal Med 2003; 14(6):373-382.

21. de Jager W, te VH, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 2003; 10(1):133-139.
22. Katier N, Uiterwaal CS, de Jong BM, Kimpen JL, van der Ent CK. Feasibility and variability of neonatal and infant lung function measurement using the single occlusion technique. Chest 2005; 128(3):1822-1829.
23. Katier N, Uiterwaal CS, de Jong BM, Verheij TJ, van der Ent CK. Passive respiratory mechanics measured during natural sleep in healthy term neonates and infants up to 8 weeks of life. Pediatr Pulmonol 2006; 41(11):1058-1064.
24. Lesouef PN, England SJ, Bryan AC. Passive respiratory mechanics in newborns and children. Am Rev Respir Dis 1984; 129(4):552-556.
25. Lakwijk N, Van Strien RT, Doekes G, Brunekreef B, Gerritsen J. Validation of a screening questionnaire for atopy with serum IgE tests in a population of pregnant Dutch women. Clin Exp Allergy 1998; 28(4):454-458.
26. Lebbink RJ, Meyaard L. Non-MHC ligands for inhibitory immune receptors: novel insights and implications for immune regulation. Mol Immunol 2007; 44(9):2153-2164.
27. Sadowsky DW, Adams KM, Gravett MG, Witkin SS, Novy MJ. Preterm labor is induced by intraamniotic infusions of interleukin-1beta and tumor necrosis factor-alpha but not by interleukin-6 or interleukin-8 in a nonhuman primate model. Am J Obstet Gynecol 2006; 195(6):1578-1589.
28. Haland G, Carlsen KC, Sandvik L *et al.* Reduced lung function at birth and the risk of asthma at 10 years of age. N Engl J Med 2006; 355(16):1682-1689.
29. Martinez FD. The origins of asthma and chronic obstructive pulmonary disease in early life. Proc Am Thorac Soc 2009; 6(3):272-277.
30. Stern DA, Morgan WJ, Wright AL, Guerra S, Martinez FD. Poor airway function in early infancy and lung function by age 22 years: a non-selective longitudinal cohort study. Lancet 2007; 370(9589):758-764.
31. Vonk JM, Boezen HM, Postma DS, Schouten JP, Van Aalderen WM, Boersma ER. Perinatal risk factors for bronchial hyperresponsiveness and atopy after a follow-up of 20 years. J Allergy Clin Immunol 2004; 114(2):270-276.
32. World Health Organization. Global surveillance, prevention and control of chronic respiratory diseases: A comprehensive approach. 1-146. 2007. Ref Type: Report
33. Prendergast M, May C, Broughton S *et al.* Chorioamnionitis, lung function and bronchopulmonary dysplasia in prematurely born infants. Arch Dis Child Fetal Neonatal Ed 2010.
34. Gravett MG, Novy MJ, Rosenfeld RG *et al.* Diagnosis of intra-amniotic infection by proteomic profiling and identification of novel biomarkers. JAMA 2004; 292(4):462-469.
35. Houben ML, Bont L, Wilbrink B *et al.* Clinical prediction rule for RSV bronchiolitis in healthy newborns: prognostic birth cohort study. Pediatrics 2011; 127(1):35-41.
36. Simoes EA, Carbonell-Estrany X, Fullarton JR *et al.* A predictive model for respiratory syncytial virus (RSV) hospitalisation of premature infants born at 33-35 weeks of gestational age, based on data from the Spanish FLIP study. Respir Res 2008; 9(1):78.
37. DeVincenzo J, Lambkin-Williams R, Wilkinson T *et al.* A randomized, double-blind, placebo-controlled study of an RNAi-based therapy directed against respiratory syncytial virus. Proc Natl Acad Sci U S A 2010; 107(19):8800-8805.
38. Olszewska W, Openshaw P. Emerging drugs for respiratory syncytial virus infection. Expert Opin Emerg Drugs 2009; 14(2):207-217.

SUPPLEMENTARY TABLES

Supplementary table 1. Baseline characteristics

	Lung function measurement			
	Total ***n*** **= 372**	**Passed** ***n*** **= 152**	**Failed** ***n*** **= 220**	***P*-value**
Delivery and birth				
Gestational age (wk)	40.0 (1.0)	40.1 (1.1)	39.9 (1.0)	.07
Birth weight (kg)	3.61 (0.47)	3.65 (0.47)	3.58 (0.46)	.17
Male gender	51%	52%	51%	.77
Antepartum maternal smoking	10%	10%	11%	.90
Parental asthma	23%	24%	23%	.83
Parental atopy	56%	54%	57%	.61

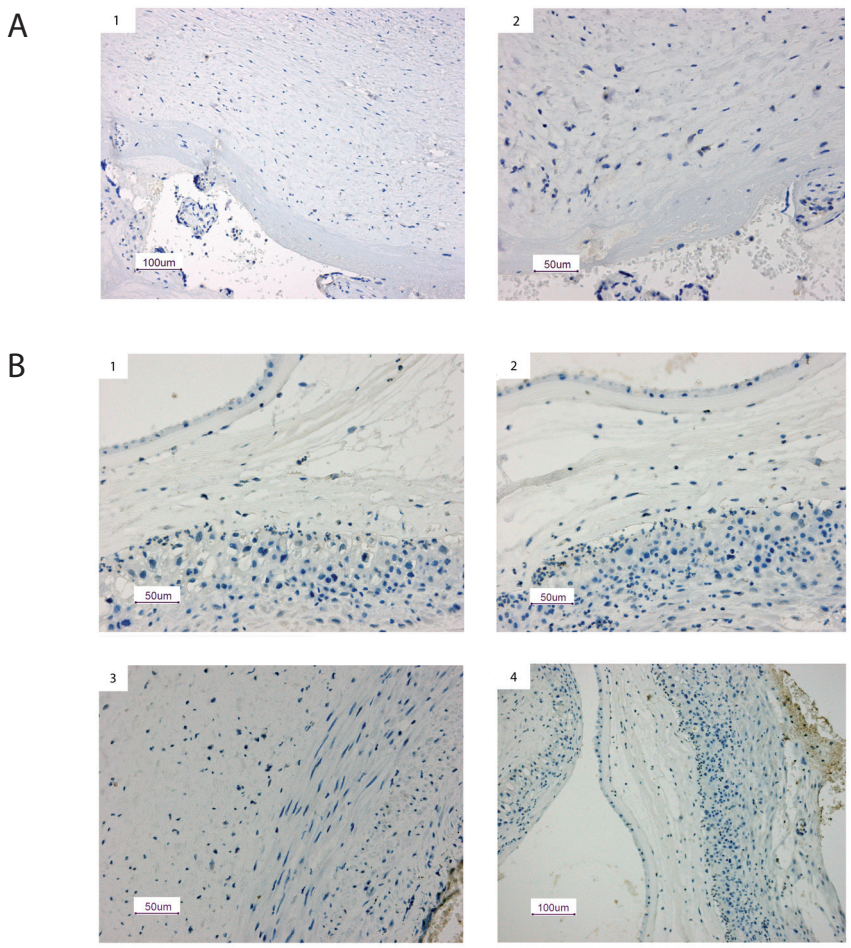
Values represent mean (SD) or percentage. *P*-values for Student's T test or X^2 test. Missing values: parental atopy and / or asthma *n* = 7 (2%), maternal antepartum smoking *n* = 13 (3%). NA denotes not applicable.

Supplementary table 2. Amniotic fluid cytokine and chemokine correlation matrix

	IL6	IL8	TNFα	IL1β	IL5	IL10	IL12p70	IL17	IL18	MCP1	MIF	sICAM	MIP1α	Eotaxin	IP10	MIG	IL23
sLAIR-1	-0,07	-0,04	0,21	0,22	0,21	0,03	0,10	0,06	0,07	-0,03	0,10	0,24	0,02	-0,19	-0,29	-0,10	0,07
	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
IL-6		0,73	0,51	0,55	0,25	0,72	0,16	0,30	0,62	0,40	0,05	0,30	0,62	0,50	0,05	0,22	0,33
		<0,01	<0,01	<0,01	NS	<0,01	NS	NS	<0,01	0,01	NS	NS	<0,01	<0,01	NS	NS	0,04
IL-8			0,64	0,69	0,34	0,58	0,23	0,43	0,52	0,54	0,38	0,32	0,61	0,46	0,05	0,40	0,45
			<0,01	<0,01	0,03	<0,01	NS	0,01	<0,01	<0,01	0,02	0,045	<0,01	<0,01	NS	0,01	<0,01
TNF-α				0,69	0,33	0,63	0,24	0,35	0,37	0,44	0,48	0,43	0,61	0,39	0,01	0,22	0,43
				<0,01	0,04	<0,01	NS	0,03	0,02	<0,01	<0,01	0,01	<0,01	0,01	NS	NS	0,01
IL-1β					0,36	0,60	0,60	0,51	0,60	0,42	0,52	0,42	0,62	0,34	0,02	0,35	0,43
					0,02	<0,01	<0,01	<0,01	<0,01	0,01	<0,01	0,01	<0,01	0,03	NS	0,03	0,01
IL-5						0,65	0,49	0,73	0,16	0,60	0,34	0,52	0,52	0,56	0,29	0,57	0,83
						<0,01	<0,01	<0,01	NS	<0,01	0,03	<0,01	<0,01	<0,01	0,07	<0,01	<0,01
IL-10							0,56	0,74	0,49	0,73	0,30	0,53	0,87	0,78	0,25	0,57	0,81
							<0,01	<0,01	<0,01	<0,01	NS	<0,01	<0,01	<0,01	NS	<0,01	<0,01
IL-12p70								0,76	0,42	0,42	0,42	0,43	0,50	0,49	0,21	0,43	0,63
								<0,01	0,01	0,01	0,01	0,01	<0,01	<0,01	NS	0,01	<0,01
IL-17									0,40	0,66	0,39	0,54	0,68	0,60	0,32	0,63	0,89
									0,01	<0,01	0,01	<0,01	<0,01	<0,01	0,048	<0,01	<0,01
IL-18										0,17	0,02	0,14	0,32	0,24	-0,01	0,19	0,29
										NS	NS	NS	0,045	NS	NS	NS	NS
MCP-1											0,49	0,37	0,78	0,84	0,41	0,65	0,70
											<0,01	0,02	<0,01	<0,01	0,01	<0,01	<0,01
MIF												0,30	0,41	0,33	0,04	0,15	0,41
												NS	0,01	0,04	NS	NS	0,01
sICAM													0,45	0,35	0,06	0,26	0,58
													<0,01	0,02	NS	NS	<0,01
MIP-1α														0,81	0,35	0,56	0,68
														<0,01	0,03	<0,01	<0,01
Eotaxin															0,48	0,56	0,69
															<0,01	<0,01	<0,01
IP10																0,68	0,25
																<0,01	NS
MIG																	0,62
																	<0,01

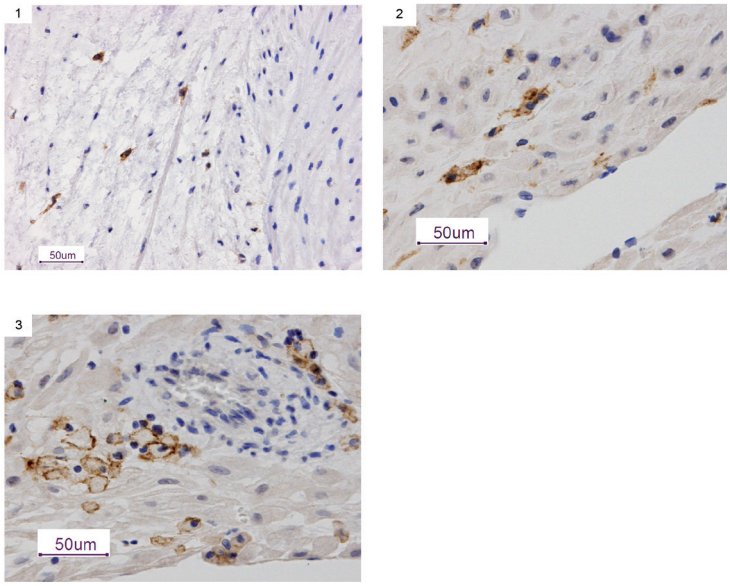
Spearman's ρ (upper) and *P*-value (lower). Sample sizes were 40-42.

SUPPLEMENTARY FIGURES

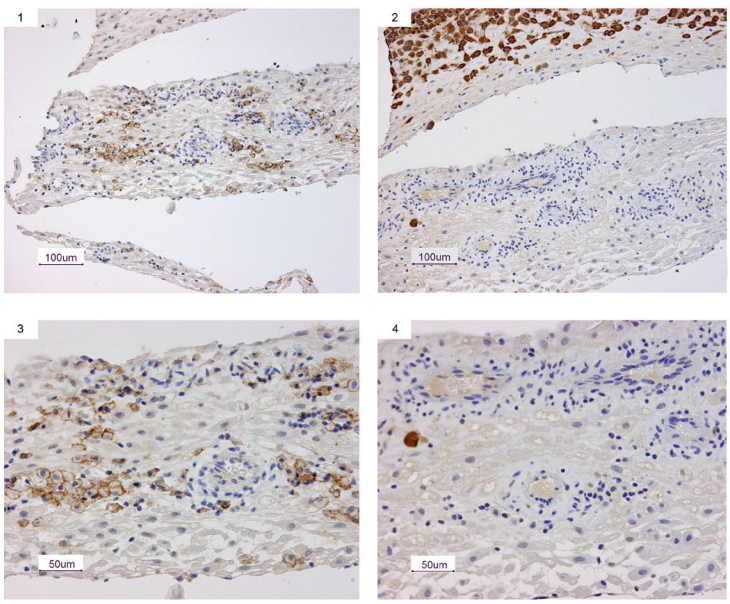


Supplementary figure 1: No LAIR-1 positive cells present in placenta tissue during spontaneous onset of labour at term. Immunohistology of placentas. Standard haematoxylin / eosin staining was applied to samples without immunohistology staining. **(A)** Placenta without histological signs of chorioamnionitis. LAIR-1 positive cells are absent in the chorionic plate (1+2), the umbilical cord, and the chorionic membranes (data not shown). **(B)** Placenta with mild signs of chorioamnionitis. LAIR-1 positive cells are sparsely present in the chorionic membranes (1+2+4), the stromal cells of Wharton's jelly of the umbilical cord (3), and the chorionic plate (data not shown). ▶

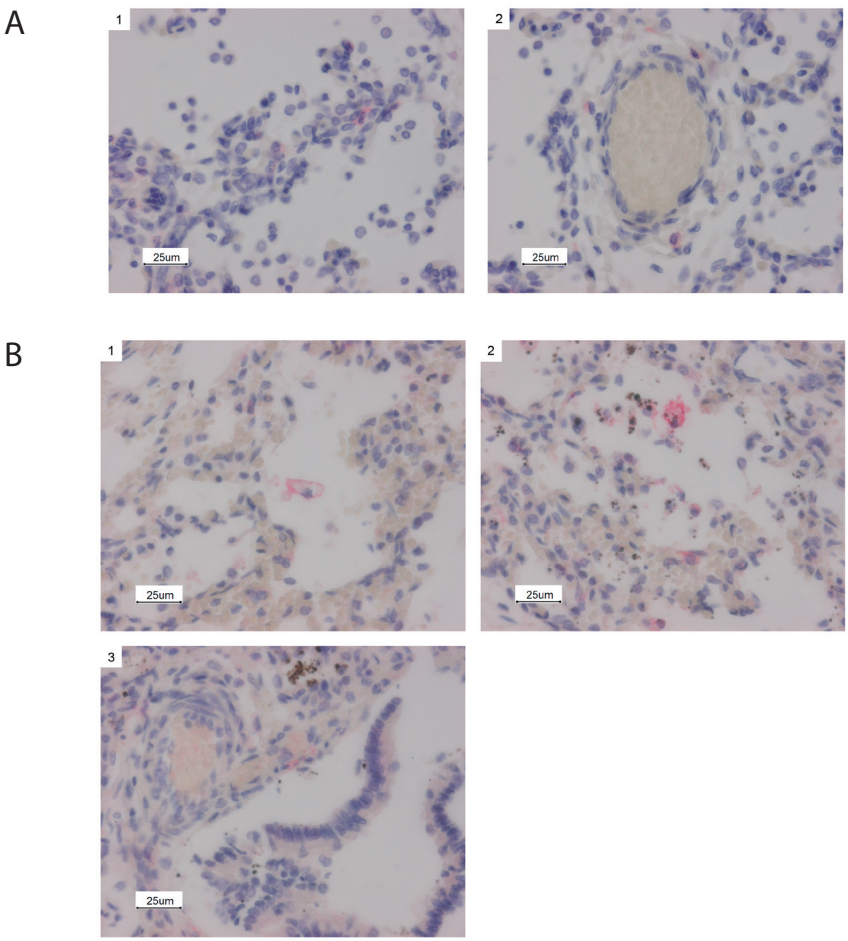
C



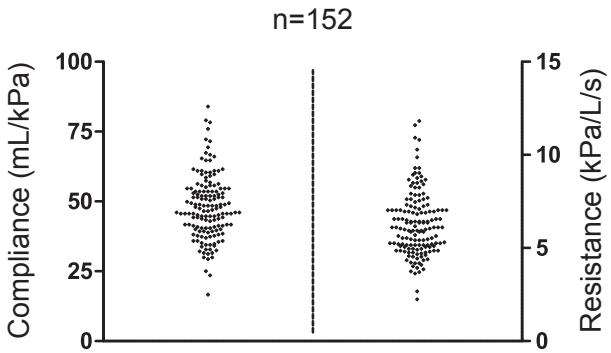
D



Supplementary figure 1 (continued): (C) Placenta with severe signs of chorioamnionitis. LAIR-1 positive cells are infrequently present in the chorionic plate (see figure 2E), the stromal cells of Wharton's jelly of the umbilical cord (1), and the maternal side of the chorionic membranes (2+3). **(D)** Placenta with severe signs of chorioamnionitis. LAIR-1 (left, 1+3) and keratin (right, 2+4) immunohistology. LAIR-1 positive cells are present in keratin negative regions (decidua) of the chorionic membranes. On these mononuclear cells, LAIR-1 staining is predominantly found on the cell membrane. CD68 staining (macrophage marker) of these cells is negative (data not shown). LAIR-1 positive cells are sparsely present in keratin positive regions (trophoblast) of chorionic membranes.



Supplementary figure 2: No LAIR-1 positive epithelial cells present in lung tissue of term fatal neonatal cases. Immunohistology of lung tissue of two children that died perinatally without evidence of any pulmonary disorder. **(A)** Lung microscopy without abnormalities, except for weak positive LAIR-1 staining interstitially (1+2). **(B)** Lung microscopy without signs of inflammation and negative LAIR-1 staining, except for alveolar macrophages (1-3).



Supplementary figure 3: Compliance and resistance of the respiratory system of healthy term infants. Infant compliance (left Y-axis) and resistance (right Y-axis) were assessed using the single occlusion technique during physiologic sleep. Mean (standard deviation) compliance 47.8 (11.3) mL/kPa. Mean (standard deviation) resistance 6.27 (1.7) kPa/L/s.

5

LEUKOCYTE ASSOCIATED IG-LIKE RECEPTOR-1 IS A NOVEL INHIBITORY RECEPTOR FOR SURFACTANT PROTEIN D

Marloes J.M. Olde Nordkamp[1], Martin van Eijk[2], Rolf T. Urbanus[3], Louis Bont[1,4], Henk P. Haagsman[2], Linde Meyaard[1]

Submitted

[1]Laboratory of Translational Immunology, Department of Immunology, University Medical Centre Utrecht, the Netherlands
[2]Department of Infectious Diseases and Immunology, Division Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, the Netherlands
[3]Department of Clinical Chemistry and Hematology, University Medical Centre Utrecht, the Netherlands
[4]Department of Pediatrics, Wilhelmina Children's Hospital, Utrecht, the Netherlands

ABSTRACT

The collagenous C-type lectin, surfactant protein D (SP-D), is a multi-trimeric glycoprotein present at mucosal surfaces and is involved in host defense against infections in mammals. SP-D has immunomodulatory properties, but the underlying mechanisms are incompletely understood. SP-D contains collagen domains. Leukocyte Associated Ig-like Receptor-1 (LAIR-1) is an inhibitory immune receptor at the cell surface of various immune competent cells that binds collagen. We hypothesized that the immunomodulatory functions of SP-D can be mediated via interactions between its collagen domain and LAIR-1.

Binding assays show that SP-D interacts via its collagenous domain with LAIR-1 and the related LAIR-2. This does not affect the opsonin function of SP-D. SP-D induces cross-linking of LAIR-1 in a cellular reporter assay. Functional assays show that SP-D inhibits the production of Fcα-receptor-mediated reactive oxygen via LAIR-1. Our studies indicate that SP-D is a functional ligand of the immune inhibitory receptor LAIR-1. Thus, we have identified a novel pathway for the immunomodulatory functions of SP-D mediated via binding of its collagenous domains to LAIR-1. This may provide a mechanism for the unexplained immunomodulatory function of the collagenous domains of SP-D.

INTRODUCTION

The lung contains a large alveolar surface area that is constantly exposed to pathogens such as viruses and bacteria. Surfactant protein-D (SP-D) belongs to the family of the 'collectins' and functions as a first line (innate) defense component that contributes to protection against pathogens in various mucosal tissues, such as the lung and gastro-intestinal tract (1). SP-D is composed of four domains: a short cysteine-rich N-terminal region, a 177-aa-long collagenous domain, and an α-helical neck domain which links the collagen to a C-type lectin domain or 'carbohydrate recognition domain' (CRD) (Figure 1A) (2). One SP-D structural subunit is composed of three polypeptide chains and these trimers further assemble into cruciform-like dodecamers (2). SP-D functions as an opsonin by binding to various pathogens such as influenza A virus, but it also binds to LPS on the cell wall of Gram-negative bacteria (reviewed in (3)). Besides functioning as an opsonin, SP-D has immunomodulatory properties. In human systems, SP-D inhibits T cell proliferation, as well as interleukin-2 production *in vitro* (4;5). SP-D also functions as a chemo-attractant and can modulate the surface expression of Toll-like receptor-4 (reviewed in (3;6)). In a human epithelial airway model, SP-D diminished suppollen particles induced IL-8 secretion (7).

SP-D$^{-/-}$ mice show a clear defect in immune regulation, since the lungs show a state of constitutive immune activation. Innate immune cells in the lungs of SP-D$^{-/-}$ mice show abnormalities such as the constitutive release of cytokines and an altered morphology. Despite the exaggerated inflammatory response, SP-D$^{-/-}$ mice have impaired host defense (reviewed in (8)). Attempts to correct the phenotype by administrating or overexpressing truncated forms of SP-D showed that the collagenous domain is essentially involved in immune homeostasis (9;10). There are several candidate receptors which may exert the immunomodulatory functions of SP-D. Most candidate receptors have shown to bind SP-D via its CRD and require the presence of calcium ions. Gardai *et al.* showed binding of SP-D to the inhibitory signal regulatory protein α (SIRPα), resulting in a reduced capacity of alveolar macrophages to ingest apoptotic cells (11). Furthermore, SP-D binds to the SIRPα homologue SIRPβ, although the functionality of this binding remains unclear (12). SP-D binds CD14 and rough LPS via the CRD, whereby the binding of SP-D to CD14 inhibits the binding of rough and smooth LPS (13). SP-D has been shown to bind to calreticulin/CD91 on macrophages via its collagenous domain, resulting in increased chemotaxis (14). SP-D function can be modulated through post-translational modification by nitric oxide (NO). The altered structure then initiates a pro-inflammatory response via calreticulin/CD91 and p38 activation (14).

Despite the increasing number of SP-D binding receptors, the mechanism via which SP-D exerts its immunoinhibitory role is unclear. LAIR-1 is an inhibitory immune receptor that is expressed on most immune cells for which collagens are high-affinity ligands (15-17). LAIR is capable of binding multiple collagens and different binding sites on collagen II and III have been mapped (18). Upon collagen-mediated ligation, LAIR-1

inhibits various cellular functions including maturation, proliferation and degranulation of cells (19). In this study we set out to investigate whether the collagen-containing innate immune protein SP-D can exert its immunomodulatory functions via the inhibitory receptor LAIR-1.

MATERIAL AND METHODS

Cells

PLB-985 cells, a human myeloid leukemia cell line derived from HL-60, which were a kind gift from Dr. Timo van den Berg and 2B4 NFAT-GFP T cell reporters were cultured as described (16;20;21). For granulocytic differentiation, cells were exposed to 0.65% (v/v) DMF (Thermo Fisher Scientific, Waltham, USA) for 4-5 days. A stable LAIR-1-knockdown of PLB-985 was obtained by transducing a pSicoR vector containing a shRNA for LAIR-1 (22).

Recombinant proteins and antibodies

Anti-LAIR-1 (clone 8A8, IgG1) (23) and anti-SIRL (24) antibodies, recombinant LAIR proteins (25), recombinant human SP-D (RhSPD) and a truncated neckCRD trimeric fragment of human SP-D (26;27) were produced and characterized as previously described. $F(ab')_2$ fragments were made by using the Mouse IgG1 Fab and $F(ab')_2$ Preparation Kit (Thermo Fisher Scientific).

Collagen 1 was purchased from Sigma-Aldrich (St. Louis, USA). Bovine serum albumin (BSA) (fraction V) from Roche Diagnostics (Basel, Switzerland), peroxidase conjugated goat anti human IgG (FC fragment specific) from Jackson Immunoresearch (Suffolk, UK). Serum derived IgA was purchased from MP biomedicals (Santa Ana, California, USA).

Collagen peptides were a part of collagen toolkits obtained from Richard Farndale (University of Cambridge) (18).

Binding ELISA

Maxisorp plates (Thermo Fisher Scientific) were coated overnight at 4°C with 50µl/well collagen I (in 2m*M* acetic acid in PBS), BSA (in PBS) or recombinant human SP-D (in 10 m*M* carbonate buffer pH 9.6). After 4 washings with PBS, 10 µg of LAIR-1-Fc or 0.63 µg of LAIR-2-Fc in PBS supplemented with 1% BSA and 10m*M* EDTA was added and incubated for 30 min at room temperature (RT) (quantities of recombinant proteins had been established in pilot experiments). After washing, peroxidase conjugated goat anti human IgG (FC fragment specific) (Jackson Immunoresearch, Suffolk, UK) was added for 60 min at RT. After 3 washes, ABTS reagent (100 µl/well; Roche Diagnostics, Basel, Switzerland) was added, and color was allowed to develop. Measurements were performed at 405 nm.

Biacore

Surface plasmon resonance (BIAcore) binding studies were performed with the BIAcore T100 system (GE Healthcare, Buckinghamshire, United Kingdom). Approximately 1100

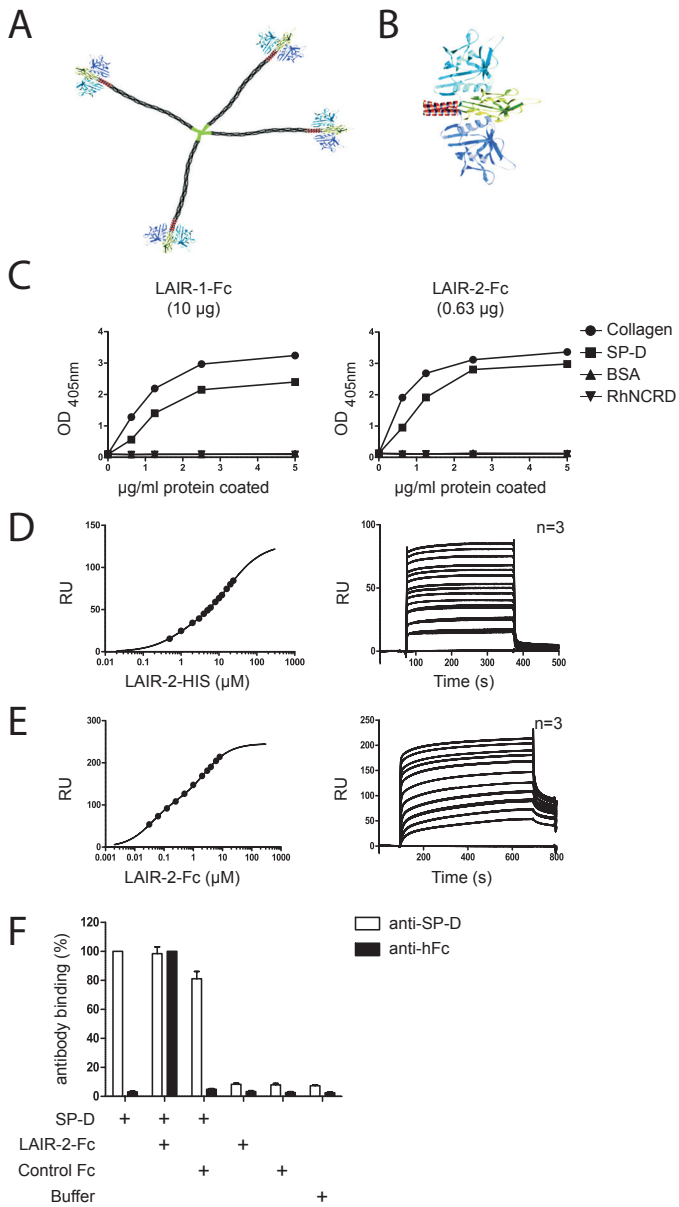


Figure 1: LAIR-1 and LAIR-2 proteins bind SP-D. (A) Schematic representation of an SP-D dodecamer molecule. CRD domains in blue, neck region in red, collagenous domains in black and N terminal region in green. **(B)** Schematic representation of the trimeric RhSP-DNCRD mutant, which lacks the collagenous domain and N terminal region. **(C)** Binding of LAIR-1-Fc (10 µg/ml) and LAIR-2-Fc (0.63 µg/ml) to plate-coated SP-D, RhNCRD, Collagen I or BSA. Detection with peroxidase-conjugated anti-human Fc antibody. Results are of a representative experiment (n = 3). **(D)** Binding of LAIR-2-HIS and **(E)** LAIR-2-Fc to immobilized SPD was measured by surface plasmon resonance analysis. In the left panels, individual symbols representing resonance at equilibrium along with the corresponding ▶

response units (RU) of SP-D in 10 m*M* MES buffer pH 6.1 were immobilized on a series S CM5 sensor chip by the amine coupling kit according to the manufacturer's instructions. After coupling the chip was pulsed for 30 seconds with EDC/NHS, subsequently followed by a 30 seconds pulse with ethanolamine. Analysis was performed in buffer (125 m*M* NaCl, 2.5 m*M* $CaCl_2$, 0.005% (v/v) Tween 20, and 25 m*M* HEPES, pH 7.4) at 25°C and at a flow rate of 20 µl/minute. Injections with increasing concentrations of recombinant proteins (0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20 and 24 µ*M* for LAIR-2-HIS and 0, 0.031, 0.063, 0.125, 0.25, 0.5, 1, 2, 3, 4, 6 and 8 µ*M* for LAIR-2-Fc) were allowed to bind for 5 minutes for LAIR-2-HIS and 10 minutes for LAIR-2-Fc, after which regeneration by either flowing buffer for 10 minutes for the LAIR-2-HIS or 10 m*M* formic acid for LAIR-2-Fc occurred. Baseline stability was checked after every experiment. Proteins were injected until binding equilibrium was reached. Binding data were analyzed with BIAcore T100 evaluation software (version 2.01). Affinity constants were determined by steady state analysis. The affinity is calculated using graphpad prism (version 5.03). The formula used for a two-site specific binding is: $Y = ((B_{max1} * X) / (K_{d1} + X)) + ((B_{max2} * X) /(K_{d2} + X))$ where X represents the ligand concentration in n*M*.

Mannan-immobilized SP-D-binding assay

Costar 9018 high-binding 96-well plates (Corning, Corning, New York, USA) were coated overnight at 4°C with 10 µg/ml of mannan (Sigma M-7504) dissolved in 100 m*M* sodium bicarbonate buffer (pH 9.6). The experiment was carried out at RT with 100 µl/well unless indicated otherwise. After removal of the coating buffer, the wells were washed 3 times with washing buffer (10 mM Tris-HCl, pH 7.4; 0.9% NaCl, 5 mM $CaCl_2$) and blocked with 1% BSA in washing buffer for 1h, followed by the washing procedure. RhSP-D (1 µg/ml) in washing buffer was mixed with either LAIR2-Fc or SIRL-Fc (both 0.5µg/ml) or buffer only, and after pre-incubation for 1h in solution, the mixtures were applied to the mannan-coated plates. After 1h incubation, plates were washed. SP-D detection: incubation for 1h with rabbit-anti human SP-D polyclonal serum (1:1000 (26)), washing and 1h incubation with goat anti-rabbit HRPO (Nordic Immunological Laboratories, Eindhoven, The Netherlands). hFc detection: incubation with rabbit anti-mouse Fab_2 HRPO. After a final wash, 100 µl of stabilized TMB chromogen (Life Technologies Europe BV, Bleiswijk, The Netherlands) was added. After color development, the reaction was

▶ concentration of proteins are shown. Values are the mean ± SEM. Triplicate binding curves of LAIR-2-HIS and LAIR-2-Fc are shown in the right panels. **(F)** Binding of LAIR-bound SP-D to mannan coated plates. SP-D was either pre-incubated with LAIR-2-Fc or a control inhibitory receptor coupled to an Fc (SIRL) or not. The SP-D/LAIR-2-mixture or the SP-D-control-mixture was added to mannan-coated wells. SP-D binding to mannan was detected with rabbit anti-human SP-D serum, followed by goat anti-rabbit HRPO incubation and a standard TMB staining solution. Detection of LAIR-2 or control-Fc protein to SP-D was achieved with an HRPO-conjugated anti-human Fc antibody. Values are the mean ± SD. n = 3

stopped by adding 50 µl of 2*M* H_2SO_4. Absorbance was measured at 450 nm. Background values were obtained for all conditions in absence of mannan-coating or in presence of 5m*M* EDTA during SP-D incubation. Data are expressed as relative intensities as compared to incubation with SP-D only (goat anti-rabbit HRPO signal set to 100%), or to incubation with SP-D followed by LAIR-2-incubation (rabbit anti-mouse Fab_2 HRPO signal set to 100%).

LAIR-1-Reporter assay:
2B4 NFAT-GFP T cell reporters stably expressing LAIR-1-CD3ζ chimeric receptors were used to detect functional LAIR-1-ligand binding as described before (16).

Oxidative Burst assay:
ROS production was measured using Amplex Red as described before (28) with minor adjustments. Statistical analysis was performed using SPSS software.

RESULTS AND DISCUSSION

SP-D binds LAIR proteins with high affinity

We applied several techniques to establish if SP-D binds to LAIR proteins and to deduce which domain of SP-D would be involved in this interaction (Figure 1). Recombinant LAIR-1 as well as LAIR-2 bind to immobilized full-length SP-D (Figure 1C). The presence of 10 mM EDTA ruled out Ca^{++}-dependent interactions of the CRDs of SP-D (Figure 1C). The amount of LAIR-1 needed to reach the same optical densities was sixteen times higher than that for LAIR-2, indicating a lower affinity of SP-D for LAIR-1 than for LAIR-2, in line with our previously study showing a higher affinity of LAIR-2 for collagens (25). Binding of LAIR proteins to SP-D was slightly lower than binding to collagen I. To further characterize the binding mechanism of LAIR to SP-D, we also tested a SP-D-mutant that lacks the collagenous domain (RhNCRD) (Figure 1B) for LAIR binding. RhNCRD is a neck CRD trimer and binds sugars (data not shown). We observed no LAIR-binding to RhNCRD (Figure 1C). From these data we can conclude that the binding sites for the LAIR-1 and LAIR-2 proteins are located in the collagenous domain of SP-D.

Table 1: Calculated K_D values of the SP-D-LAIR binding

		LAIR-2	
		Monomer	**Dimer**
SP-D	K_D	0.83 ± 0.09	0.04 ± 0.002
	$K_D 2$	20.68 ± 2.79	2.57 ± 0.17

Calculated affinities (K_D in µM) from the binding at equilibrium (R_{eq}) of the surface plasmon resonance experiments. Values are the mean ± standard error of the mean (SEM).

Equilibrium binding data obtained with surface plasmon resonance studies showed that LAIR-2-HIS monomers bind to SP-D with moderate affinity (Figure 1D and Table 1). We previously showed that the affinity for collagen I, III and IV is around 2 μ*M*. Therefore, LAIR-2 binds to SP-D with a somewhat higher affinity than to collagen. The affinity of dimeric LAIR-2-Fc (0.037 ± 0.002 μ*M*) is in the same range as the affinity of LAIR-2-Fc for collagen I, (0.032 ±0.003 μ*M*) (25) (Figure 1E and Table 1). LAIR-1 protein also binds immobilized SP-D (Figure 1C), but we were unable to determine the affinity of LAIR-1 proteins for SP-D since no binding to SP-D in the surface plasmon resonance assay was observed. Previously, we have shown that LAIR-2 has more and different binding sites on collagen than LAIR-1 (18). The lack of LAIR-1 binding in the surface plasmon resonance assay might be due to differences in the conformation of SP-D after binding to the CM5 chip compared to plastic, which blocks the LAIR-1 binding sites. In conclusion, SP-D binds both recombinant LAIR-1 and LAIR-2 in a Ca^{++}-independent manner and dimeric LAIR-2-Fc does so with high affinity.

SP-D binding to LAIR does not interfere with the pathogen binding site of SP-D

To investigate the effect of binding of LAIR to SP-D on the opsonin function of SP-D, SP-D was pre-incubated with either LAIR-2-Fc or SIRL-1-Fc as a control inhibitory receptor which does not bind collagen (24;25) and added to solid-phase mannan (Figure 1F). Binding of SP-D to mannan was detected with an anti-SP-D antibody, while LAIR-2 binding to SP-D was detected with an antibody against the hFc-fragment fused to LAIR-2. Mannan-binding of untreated SP-D was set at 100%. Pre-incubation of SP-D with LAIR-2-Fc did not reduce the mannan binding activity of SP-D, while we could detect the binding of LAIR-2 to mannan-bound SP-D. SIRL-1-Fc showed no binding to either SP-D or mannan. From these data we conclude that mannan and LAIR can bind to SP-D simultaneously and that binding of LAIR to SP-D may not interfere with the ability of SP-D to opsonize/bind microorganisms via its CRDs.

SP-D functionally engages LAIR-1

We used cells expressing chimeric proteins consisting of the extracellular part of LAIR-1 fused to the intracellular part of the CD3ζ and an NFAT-GFP reporter to investigate if binding of cell-expressed LAIR-1 to the collagen domain of SP-D could also functionally ligate the receptor (Figure 2A). We previously used this assay to demonstrate functional binding of collagens and collagen-derived peptides to LAIR-1 (18). As a control we used cells only containing the NFAT-GFP reporter construct (wild type cells) or cells expressing a chimera of SIRL-1 and the reporter construct (24). Cells were incubated overnight with solid phase collagen I, SP-D or BSA (as a control) and GFP expression was assessed by flow cytometry (Figure 2A). Collagen I induced GFP expression in up to 70% of the LAIR-reporter cells and SP-D was almost as effective, inducing up to 50% GFP-expressing cells (Figure 2B). Based upon these findings, we conclude that SP-D can act as a functional ligand for LAIR-1.

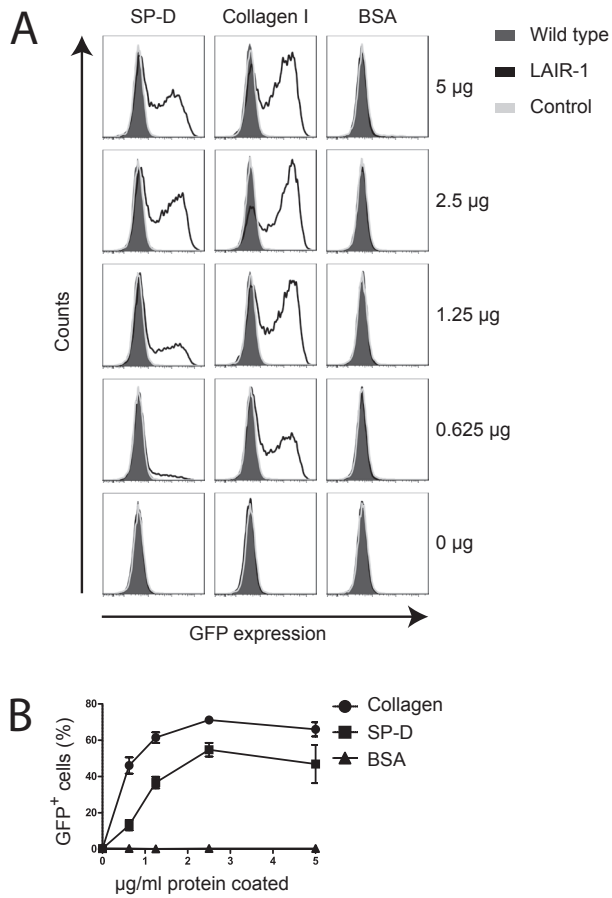


Figure 2: SP-D functionally ligates LAIR-1. (A) NFAT-GFP reporter cells stably transfected with either a LAIR-1-CD3ζ, a control protein-CD3ζ chimera or wild type cells, were incubated in 96-well MAXIsorp plates coated with SP-D, collagen I or BSA in various concentrations. Overlays of flow cytometry pictures of 1 representative experiment out of 3 are shown. GFP-expression (MFI) is shown on the X-axis; the amount of coated protein/ml used is indicated adjacent to each row of panels. **(B)** Calculated percentages of GFP-positive cells. Values shown are the means ± SD of three experiments.

SP- D inhibits reactive oxygen species production through LAIR-1

Reactive oxygen species (ROS) production by neutrophils and monocytes is an important part of the defense mechanism against pathogens by the innate immune system, although it can also cause severe tissue damage when released into the extracellular milieu (29). The effect of LAIR-1 ligation on ROS production has not been studied previously. We activated PLB 985 cells, which were matured with DMF towards a granulocytic lineage, via the Fcα Receptor (FcαR) to induce an oxidative burst. First we tested whether ligation of LAIR-1 via collagen regulates this oxidative burst. Collagen II and III have multiple

binding sites for LAIR-1. We previously identified trimeric collagen peptides that do (III-38) or do not (II-20) induce inhibitory signaling via LAIR-1 (18). Simultaneous ligation of FcαR and LAIR-1 via collagen peptide III-38 reduced the oxidative burst by 50%, while collagen peptide II-20 did not significantly affect the oxidative burst (Figure 3A and B). Blockade of the LAIR-1-collagen interaction with specific $F(ab')_2$ fragments significantly reversed the inhibition of the oxidative burst, demonstrating that the inhibition is LAIR-1 mediated (Figure 3A and B). $F(ab')_2$ fragments alone, in combination with IgA (data not shown) or in combination with IgA and collagen peptide II20 did not significantly affect the ROS production (Figure 3A and B). Thus, LAIR-1 ligation through collagen inhibits the FcαR–induced oxidative burst. Next, we tested SP-D for its ability to inhibit ROS production in this set-up. As a control, we used the RhNCRD peptide that does not bind LAIR-1 (Figure 1B). SP-D significantly inhibited the FcαR-induced oxidative burst in a concentration dependent manner up to 60% while RhNCRD did not (Figure 3C and D). LAIR-1 specific $F(ab')_2$ fragments prevented SP-D mediated inhibition of ROS production, while addition of $F(ab')_2$ fragments to RhNCRD did not (Figure 3D), indicating that SP-D inhibits the ROS production via LAIR-1.

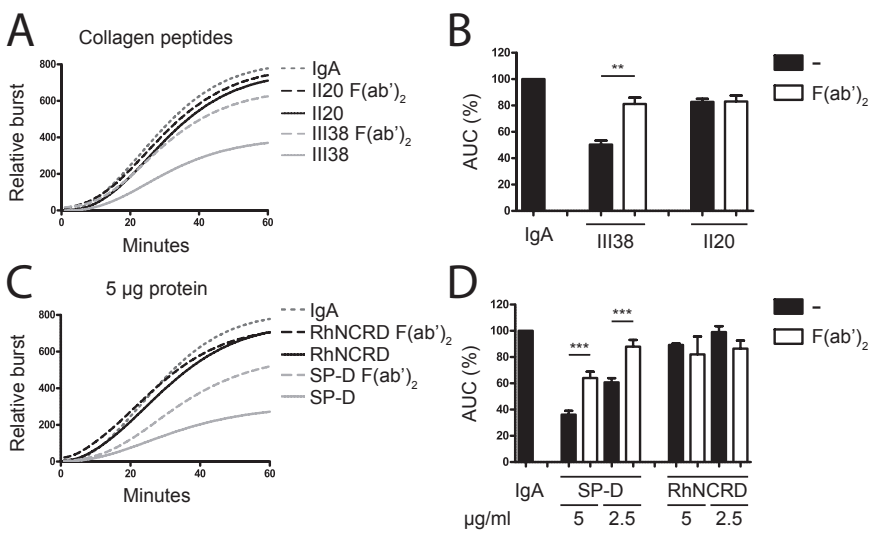


Figure 3: SP-D inhibits reactive oxygen species production by engaging LAIR-1. PLB985 cells were matured towards a granulocytic phenotype. Measurements were corrected for spontaneous ROS production. Cells were either pre-incubated with 10 µg/ml (final concentration) anti-LAIR $F(ab')_2$ fragments or not. **(A)** Cells were stimulated with 20 µg/ml IgA either in the presence of 5 µg/ml collagen peptides or not. A representative curve of the cumulative ROS production is shown. (B) Area under the curve (AUC), n = 3. **(C)** Cells were stimulated with 20 µg/ml IgA either in the presence of 5 µg/ml or 2.5 µg/ml SP-D or RhNCRD or not. A representative curve of the cumulative ROS production is shown. **(D)** Area under the curve, n = 3, mean ± SD. Significance was tested by an unpaired two tailed student T test. ** = $p \leq 0.01$, *** = $p \leq 0.001$.

To further confirm that the SP-D mediated inhibition of the oxidative burst is LAIR-1 mediated, we constructed a LAIR-1 knockdown cell line by using shRNA specific for LAIR-1 (22), which resulted in a decreased LAIR-1 surface expression (Figure 4A). We compared the knockdown cell line to the wild type cell line in the same experimental set-up as described above. Although SP-D, like collagen peptide III-38, is still able to inhibit the FcαR-induced oxidative burst, the inhibition in the LAIR-1 knockdown cell line was significantly lower than in the wild type cell line (Figure 4B and C). This confirmed that the inhibitory action of SP-D is mediated, at least in part, by LAIR-1 (Figure 4C).

SP-D has several immunomodulatory functions. However, the mechanisms via which SP-D exerts these functions are incompletely understood. In this study, we assessed the possibility that these functions are partially mediated by the inhibitory immune receptor LAIR-1. We show collagen domain-specific, Ca^{++}-independent binding of immobilized SP-D to LAIR proteins. Moreover, binding of SP-D to cells that express LAIR-1 leads to functional ligation of this receptor. In the lung, SP-D functions as a first line defense molecule by opsonizing pathogens (30). Since LAIR-binding to SP-D does not hinder its opsonin function, we speculate that SP-D can function as an opsonin without mounting a fulminant immune response.

Alveolar macrophages of SP-$D^{-/-}$ mice show a 10-fold higher production of hydrogen peroxide as well as an increase in lipid peroxide in lung homogenates as compared to

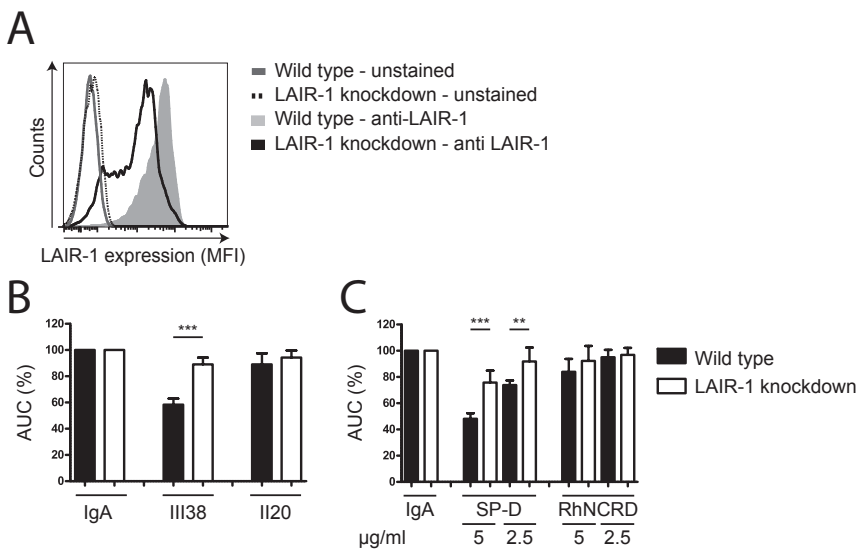


Figure 4: LAIR-1 knockdown significantly reduces the inhibitory effect of SP-D. (A) LAIR-1 expression by PLB-985 wild type and LAIR-1 knockdown cells as determined by flow cytometry. **(B)** Cells were stimulated with 20 µg/ml IgA either in the presence of 5 µg/ml collagen peptides or not. Area under the curve (AUC) is shown, n = 4. **(C)** Cells were stimulated with 20 µg/ml IgA either in the presence of 5 µg/ml or 2.5 µg/ml SP-D or RhNCRD or not. AUC is shown, n = 4, mean ± SD. Significance was tested by an unpaired two tailed student T test. ** = $p \leq 0.01$, *** = $p \leq 0.001$.

wild type mice (31). In view of our data, this may reflect a lack of immune regulation in the lung via LAIR-1 and/or other inhibitory receptors. In line with this, attempts to rescue the phenotype of the SP-D$^{-/-}$ mice by intranasal delivery or over expression of recombinant SP-D fragments revealed that a fragment without the collagenous domain does not display therapeutic effects. Thus, the collagenous domain is involved in the immunomodulatory function of SP-D (reviewed in (8)), for which we now show that LAIR-1 is a receptor.

SP-D has been shown to function as a chemo-attractant for neutrophils (32). Previously, we showed that neutrophils express LAIR-1 upon activation (33). Now, we show that SP-D can also functionally reduce the production of reactive oxygen species via LAIR-1 signaling in a neutrophilic cell line. This may be important to limit damage to the lungs during pathogen clearance. SP-D was first found in lung lavage. Therefore, most studies were devoted to investigate the role of SP-D in pulmonary infections and inflammation. However, SP-D is expressed in many tissues and serves a more general role in the protection of mucosal epithelia. It will be interesting to study its putative anti-inflammatory function in the gastro-intestinal tract and other tissues (1;34).

Our data reveal a novel immunomodulatory axis, through which SP-D exerts its functions in both steady state and inflammatory conditions. Previously, Gardai *et al.* showed CRD dependent binding of SP-D to SIRPα, which induces SHIP-1 activity and suppresses the capacity of alveolar macrophages to ingest apoptotic cells (11). They propose a model in which SP-D binds SIRPα via its CRD domains in the resting lung and, after pathogen binding, activates the immune system by binding calreticulin/CD91 with its collagenous domain (35). Our data show that the collagenous domains of SP-D can also inhibit immune activation via LAIR-1. Thus, SP-D dampens the inflammatory response mediated by immune cells not only through its CRD domain via SIRPα, but also via LAIR-1 through its collagenous domain.

We found a novel functional receptor for SP-D that can dampen immune cell function in mucosal areas which are continuously exposed to pathogens and other triggers. Binding of SP-D via its collagenous domains to LAIR-1 may provide a mechanism for the unexplained immunomodulatory function of the collagenous domains of SP-D. We reveal a novel level of immune regulation that could contribute to lung homeostasis and prevents excessive inflammation and ROS production and thus damage to the fragile lung epithelium.

ACKNOWLEDGMENTS

We thank Timo van den Berg for kindly providing us with the PLB-985 cells, Robert Jan Lebbink for providing the LAIR-1 shRNA constructs and Amelia Lacna for technical assistance. We thank Richard Farndale (University of Cambridge) for providing us with the collagen Toolkit peptides.

This work was supported by the Dutch Arthritis Foundation (grant 06-1-403) and by the Technology Foundation STW (grant 10388).

REFERENCE LIST

1. Herias M.V., Hogenkamp A., van Asten A.J., Tersteeg M.H., Eijk, M.van, and Haagsman H.P.; 2007. Expression sites of the collectin SP-D suggest its importance in first line host defence: power of combining in situ hybridisation, RT-PCR and immunohistochemistry. *Mol. Immunol.* 44:3324-3332.
2. Haagsman H.P., Hogenkamp A., Eijk, M.van, and Veldhuizen E.J.; 2008. Surfactant collectins and innate immunity. *Neonatology.* 93:288-294.
3. Wright J.R. 2005. Immunoregulatory functions of surfactant proteins. *Nat. Rev. Immunol.* 5:58-68.
4. Borron P.J., Crouch E.C., Lewis J.F., Wright J.R., Possmayer F., and Fraher L.J.; 1998. Recombinant rat surfactant-associated protein D inhibits human T lymphocyte proliferation and IL-2 production. *J. Immunol.* 161:4599-4603.
5. Borron P.J., Mostaghel E.A., Doyle C., Walsh E.S., Heyzer-Williams M.G., and Wright J.R.; 2002. Pulmonary surfactant proteins A and D directly suppress $CD3^+/CD4^+$ cell function: evidence for two shared mechanisms. *J. Immunol.* 169:5844-5850.
6. Kishore U., Greenhough T.J., Waters P., Shrive A.K., Ghai R., Kamran M.F., Bernal A.L., Reid K.B., Madan T., and Chakraborty T.; 2006. Surfactant proteins SP-A and SP-D: structure, function and receptors. *Mol. Immunol.* 43:1293-1315.
7. Schleh C., Rothen-Rutishauser B.M., Blank F., Lauenstein H.D., Nassimi M., Krug N., Braun A., Erpenbeck V.J., Gehr P., and Hohlfeld J.M.; 2012. Surfactant Protein D modulates allergen particle uptake and inflammatory response in a human epithelial airway model. *Respir. Res.* 13:8.
8. Orgeig S., Hiemstra P.S., Veldhuizen E.J., Casals C., Clark H.W., Haczku A., Knudsen L., and Possmayer F.; 2010. Recent advances in alveolar biology: evolution and function of alveolar proteins. *Respir. Physiol Neurobiol.* 173 Suppl:S43-S54.
9. Knudsen L., Wucherpfennig K., Mackay R.M., Townsend P., Muhlfeld C., Richter J., Hawgood S., Reid K., Clark H., and Ochs M.; 2009. A recombinant fragment of human surfactant protein D lacking the short collagen-like stalk fails to correct morphological alterations in lungs of SP-D deficient mice. *Anat. Rec. (Hoboken.)* 292:183-189.
10. Kingma P.S., Zhang L., Ikegami M., Hartshorn K., McCormack F.X., and Whitsett J.A.; 2006. Correction of pulmonary abnormalities in Sftpd-/- mice requires the collagenous domain of surfactant protein D. *J. Biol. Chem.* 281:24496-24505.
11. Gardai S.J., Xiao Y.Q., Dickinson M., Nick J.A., Voelker D.R., Greene K.E., and Henson P.M.; 2003. By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation. *Cell* 115:13-23.
12. Fournier B., Andargachew R., Robin A.Z., Laur O., Voelker D.R., Lee W.Y., Weber D., and Parkos C.A.; 2012. Surfactant protein D (Sp-D) binds to membrane-proximal domain (D3) of signal regulatory protein alpha (SIRPalpha), a site distant from binding domain of CD47, while also binding to analogous region on signal regulatory protein beta (SIRPbeta). *J. Biol. Chem.* 287:19386-19398.
13. Sano H., Chiba H., Iwaki D., Sohma H., Voelker D.R., and Kuroki Y.; 2000. Surfactant proteins A and D bind CD14 by different mechanisms. *J. Biol. Chem.* 275:22442-22451.
14. Guo C.J., tochina-Vasserman E.N., Abramova E., Foley J.P., Zaman A., Crouch E., Beers M.F., Savani R.C., and Gow A.J.; 2008. S-nitrosylation of surfactant protein-D controls inflammatory function. *PLoS. Biol.* 6:e266.
15. Jiang L. and Barclay A.N.; 2009. New assay to detect low-affinity interactions and characterization of leukocyte receptors for collagen including leukocyte-associated Ig-like receptor-1 (LAIR-1). *Eur. J. Immunol.* 39:1167-1175.
16. Lebbink R.J., De Ruiter T., Adelmeijer J., Brenkman A.B., van Helvoort J.M., Koch M., Farndale R.W., Lisman T., Sonnenberg A., Lenting P.J., and Meyaard L.; 2006. Collagens are functional, high-affinity ligands for the inhibitory immune receptor LAIR-1. *J. Exp. Med.* 203:1419-1425.

17. Tang X., Narayanan S., Peruzzi G., Apara A., Natarajan K., Margulies D.H., Coligan J.E., and Borrego F.; 2009. A single residue, arginine 65, is critical for the functional interaction of leukocyte-associated inhibitory receptor-1 with collagens. *J. Immunol.* 182:5446-5452.
18. Lebbink R.J., Raynal N., De Ruiter T., Bihan D., Farndale R.W., and Meyaard L.; 2009. Identification of multiple potent binding sites for human leukocyte associated Ig-like receptor LAIR on collagens II and III. *Matrix Biol* 28:202-210.
19. Meyaard L. 2008. The inhibitory collagen receptor LAIR-1 (CD305). *J. Leukoc. Biol.* 83:799-803.
20. Zhen L., King A.A., Xiao Y., Chanock S.J., Orkin S.H., and Dinauer M.C.; 1993. Gene targeting of X chromosome-linked chronic granulomatous disease locus in a human myeloid leukemia cell line and rescue by expression of recombinant gp91phox. *Proc. Natl. Acad. Sci. U. S. A* 90:9832-9836.
21. Voehringer D., Rosen D.B., Lanier L.L., and Locksley R.M.; 2004. CD200 receptor family members represent novel DAP12-associated activating receptors on basophils and mast cells. *J. Biol. Chem.* 279:54117-54123.
22. Bassik M.C., Lebbink R.J., Churchman L.S., Ingolia N.T., Patena W., LeProust E.M., Schuldiner M., Weissman J.S., and McManus M.T.; 2009. Rapid creation and quantitative monitoring of high coverage shRNA libraries. *Nat. Methods* 6:443-445.
23. Meyaard L., Adema G.J., Chang C., Woollatt E., Sutherland G.R., Lanier L.L., and Phillips J.H.; 1997. LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. *Immunity.* 7:283-290.
24. Steevels T.A.M., Lebbink R.J., Westerlaken G.H.A., Coffer P.J., and Meyaard L.; 2010. Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) is a novel functional inhibitory immune receptor expressed on human phagocytes. *J Immunol* 184:4741-4748.
25. Olde Nordkamp M.J.M., van Roon J.A., Douwes M., De Ruiter T., Urbanus R.T., and Meyaard L.; 2011. Enhanced secretion of Leukocyte-Associated Immunoglobulin-like Receptor (LAIR)-2 and soluble LAIR-1 in rheumatoid arthritis: LAIR-2 is a more efficient antagonist of the LAIR-1-collagen inhibitory interaction than soluble LAIR -1. *Arthritis Rheum.* 63:3749-3757.
26. van Eijk M., Bruinsma L., Hartshorn K.L., White M.R., Rynkiewicz M.J., Seaton B.A., Hemrika W., Romijn R.A., van Balkom B.W., and Haagsman H.P.; 2011. Introduction of N-linked glycans in the lectin domain of surfactant protein D: impact on interactions with influenza A viruses. *J. Biol. Chem.* 286:20137-20151.
27. van Eijk M., Rynkiewicz M.J., White M.R., Hartshorn K.L., Zou X., Schulten K., Luo D., Crouch E.C., Cafarella T.R., Head J.F., Haagsman H.P., and Seaton B.A. 2012. A unique sugar-binding site mediates the distinct anti-influenza activity of pig surfactant protein D. *J. Biol. Chem.* 287:26666-26677.
28. Steevels T.A., van Avondt K., Westerlaken G.H., Walk J., Bont L., Coffer P.J., and Meyaard L.; 2013. Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) negatively regulates the oxidative burst in human phagocytes. *Eur. J Immunol* In Press
29. Weissmann G., Smolen J.E., and Korchak H.M.; 1980. Release of inflammatory mediators from stimulated neutrophils. *N. Engl. J. Med.* 303:27-34.
30. Pikaar J.C., Voorhout W.F., van Golde L.M., Verhoef J., van Strijp J.A., and van Iwaarden J.F.; 1995. Opsonic activities of surfactant proteins A and D in phagocytosis of gram-negative bacteria by alveolar macrophages. *J. Infect. Dis.* 172:481-489.
31. Yoshida M. and Whitsett J.A.; 2006. Alveolar macrophages and emphysema in surfactant protein-D-deficient mice. *Respirology.* 11 Suppl:S37-S40.
32. Crouch, E.C., Persson A., Griffin G.L., Chang D., and Senior R.M.; 1995. Interactions of pulmonary surfactant protein D (SP-D) with human blood leukocytes. *Am. J. Respir. Cell Mol. Biol.* 12:410-415.
33. Verbrugge, A., De Ruiter T., Geest C., Coffer P.J., and Meyaard L.; 2006. Differential expression of Leukocyte Associated Ig-like Receptor-1 during neutrophil differentiation and activation. *J Leukoc Biol* 79:828-836.

34. Stahlman, M.T., Gray M.E., Hull W.M., and Whitsett J.A.; 2002. Immunolocalization of surfactant protein-D (SP-D) in human fetal, newborn, and adult tissues. *J. Histochem. Cytochem.* 50:651-660.

35. Janssen, W.J., McPhillips K.A., Dickinson M.G., Linderman D.J., Morimoto K., Xiao Y.Q., Oldham K.M., Vandivier R.W., Henson P.M., and Gardai S.J.; 2008. Surfactant proteins A and D suppress alveolar macrophage phagocytosis via interaction with SIRP alpha. *Am. J. Respir. Crit Care Med.* 178:158-167.

6

INHIBITION OF THE CLASSICAL AND LECTIN PATHWAY OF THE COMPLEMENT SYSTEM BY RECOMBINANT LAIR-2

Marloes J.M. Olde Nordkamp[1], Peter Boross[1], Cafer Yildiz[1], J.H. Marco Jansen[1], Jeanette H.W. Leusen[1], Rolf T. Urbanus[2], C. Erik Hack[1, 3], Linde Meyaard[1]

Submitted

[1] Laboratory of Translational Immunology, department of Immunology, [2] Department of Clinical Chemistry and Haematology, and [3] Department of Dermatology, Rheumatology and Clinical Immunology, University Medical Centre Utrecht, the Netherlands

ABSTRACT

Activation of complement may cause severe tissue damage in antibody-mediated allograft rejection and other antibody mediated clinical conditions; therefore novel potent complement inhibitors are needed. Previously, we described binding of the inhibitory receptor LAIR-1 and its soluble family member LAIR-2 to collagen. Here, we investigated binding of LAIR-1 and LAIR-2 to the complement proteins C1q and MBL, which both have collagen-like domains, and evaluated the effect of this binding on complement function. We demonstrate specific binding of recombinant LAIR proteins to both C1q and MBL. Surface plasmon resonance experiments showed that LAIR-2-Fc protein bound C1q and MBL with the highest affinity. We therefore hypothesized that LAIR-2-Fc is a potent complement inhibitor. Indeed, LAIR-2-Fc inhibited C4 fixation to IgG or mannan, reduced activation of C4 by aggregated IgG in plasma and inhibited iC3b deposition on cells. Finally, LAIR-2-Fc inhibited complement mediated lysis of cells sensitized with anti-HLA antibodies in an *ex vivo* model for antibody-mediated transplant rejection. Thus, LAIR-2-Fc is an effective novel complement inhibitor that may be used for the treatment and prevention of antibody-mediated allograft rejection and antibody-mediated clinical conditions.

INTRODUCTION

The complement system is a double edged sword: on the one hand it is a vital part of the immune system contributing to the defence against microbes, whereas on the other hand it can contribute to severe pathology. For example, in antibody-mediated rejection (AMR) of transplanted organs by anti-HLA or anti-blood group antibodies complement is involved (1;2). Deposition of C4d in kidney allografts is often used as a diagnostic hallmark for AMR (3). Also, reperfusion of transplanted organs triggers complement activation which contributes to the ischemia-reperfusion injury (1). This can lead to major complications such as damage to the graft and even stimulate cell mediated rejection (4). Most vital organs such as the heart, lung and kidneys are susceptible to complement mediated injury (4). Yet, application of complement inhibitors to protect grafts from these processes has not been approved, possibly because potent complement inhibitors that effectively prevent deleterious complement activation in the grafts and improve transplant survival, are currently not available in the clinic.

The complement system is activated via three pathways: the classical, the lectin and the alternative pathway. Activation of complement by IgG or IgM antibodies and in ischemia-reperfusion conditions mainly involves the classical and lectin pathways. The classical pathway is triggered by the binding of the C1 complex to IgG or IgM antibodies complexed with antigens. Binding of C1 is mediated by its sub-component C1q, which is a multimeric protein containing 6 subunits each consisting of a trimer of 3 different polypeptide chains. Each chain contains a C-terminal globular head region bound to an N-terminal collagen-like region (5). The lectin pathway is initiated by binding of mannose binding lectin (MBL) to carbohydrate structures on pathogens, which subsequently activates the MBL-associated serine proteases (MASPs). The MASPs in turn activate C4 and C2. Similar to C1q, MBL is an oligomeric protein made up of up to 6 subunits which each consists of a homo-trimer of the same polypeptide chain. This chain also contains a C-terminal globular domain, coupled to an N-terminal collagen-like region. The alternative pathway can be activated by various triggers and amplifies complement activation at the level of C3 initiated by any pathway (4).

Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is an inhibitory immune receptor expressed on most immune cells and LAIR-2 is a homologous protein which is released by cells. Ligation of LAIR-1 generates a threshold for activation of various cellular functions (6). Collagens serve as ligands for LAIR-1 (7;8), and we recently found that in particular dimeric recombinant LAIR proteins have a high affinity for collagen (9). Recently, Son *et al.* reported C1q as ligand for LAIR-1 on dendritic cells (10). In this report we assessed binding of LAIR-proteins to C1q as well as to MBL and explored the potential of LAIR proteins as complement inhibitors. We demonstrate that LAIR-1 and LAIR-2 bind to the collagen-like regions of C1q and of MBL. Moreover, we show that upon increasing the affinity of LAIR to C1q and MBL by dimerization of the protein, LAIR-2 can efficiently inhibit complement activation via classical and lectin pathways. Thus, dimerized LAIR-2,

produced as LAIR-2-Fc protein, is a promising new complement inhibitor which may be applied in organ transplantation to prevent AMR and ischemia-reperfusion injury.

MATERIAL AND METHODS

Biological samples

Approval for the study was obtained from the Institutional Review Board at the University Medical Center Utrecht. PBMCs were isolated by Ficoll-Paque centrifugation using standard procedures. Normal aged serum was prepared by incubating fresh serum for one week at 37°C in the presence of 0.02% (w/v) sodium azide.

Cell-lines, recombinant proteins and antibodies

2B4 NFAT-GFP T cell reporters, K562 stably transduced with hLAIR-1 and EL-4 CD20 have been described (8;11). Recombinant LAIR and signal inhibitory receptor on leukocytes-1 (SIRL-1) proteins were expressed in HEK293 cells as described (9). Fc- and His-tagged proteins of LAIR-1, LAIR-2 and SIRL-1 were made. Recombinant human MBL was purchased from R&D systems, human C1q and Collagen I from Sigma-Aldrich, bovine serum albumin (BSA) (fraction V) from Roche Diagnostics, HRP conjugated goat anti-human IgG (Fc-fragment specific) from Jackson Immunoresearch, anti-MBL from Bioporto, horseradish peroxidase (HRP)-conjugated secondary anti-mouse antibodies from DAKO, goat anti-mouse IgG Alexa fluor 488 conjugated from Life Technologies, monoclonal antibody (mAb) anti-C4-1 from Sanquin, and herceptin and rituximab from Roche Diagnostics.

Goat anti-human C4 polyclonal antibody was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin according the manufacturer's protocol. Aggregated IgG was prepared by heating a therapeutic human IgG preparation (Gammaquin, Sanquin) at a concentration of 80 mg/ml in phosphate buffered saline (PBS) for 20 min at 63°C (12).

Binding ELISA

Maxisorp plates (Thermo Fisher Scientific) were coated overnight at 4°C with 50 µl/well of collagen I (5 µg/ml in 2m*M* acetic acid in PBS), BSA (5 µg/ml in PBS), C1q or MBL (each at 5 µg/ml in 10 m*M* carbonate buffer, pH 9.6). Wells were incubated with 10 µg of LAIR-1-Fc, 625 ng of LAIR-2-Fc or 10 µg of SIRL-1-Fc in 50 µl PBS supplemented with 10 mg/ml BSA and 10 m*M* ethylenediaminetetraacetic acid (EDTA) for 30 min at room temperature (RT) (quantities of recombinant proteins had been established in pilot experiments). HRP-conjugated goat anti-human IgG was added and incubated for 60 min at RT. Plates were washed and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) reagent (100 µl/well; Roche Diagnostics) was added. Substrate conversion was measured at 405 nm.

Immune precipitation

Protein A/G plus-Sepharose beads (Santa Cruz Biotechnology) were incubated with 13 µg LAIR-1-Fc or LAIR-2-Fc (10 µl of packed Sepharose per sample) for 4 hours at 4°C. Beads were washed twice with lysis buffer where after 10 µg of MBL was added in 500 µl

wash buffer (13), incubated for 3 hours at 4°C, washed and 30 µl SDS-sample buffer was added. Beads were centrifuged and samples were subjected to SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore) and detected using anti-MBL antibodies and HRP-conjugated secondary antibodies. Enhanced chemiluminescent (ECL) substrate (Thermo Fisher Scientific) was used to visualize bound antibodies.

Flow Cytometry

K562 wild type or hLAIR-1 expressing cells were incubated with 100 ng MBL in the presence of 10%, v/v, human pooled serum in PBS with 10 m*M* EDTA for 30 min at RT. Cells were washed with PBS containing 10 mg/ml BSA and 0.1%, w/v, sodium azide and incubated with anti-MBL antibody for 30 min at RT. Binding was detected using goat anti-mouse IgG Alexa fluor 488 conjugated antibody.

2B4 T cell hybridoma cells stably transduced with an NFAT-GFP reporter and hLAIR-1-CD3ζ were used to study functional activity of LAIR-1 ligands as described previously (8).

Complement deposition from mouse serum onto a target cell was measured as described before (11). Cells were analyzed on an LSR II FACS using FACSDiva software (BD Biosciences).

BIAcore

Surface plasmon resonance (BIAcore) (GE Healthcare) binding studies were performed with the use of a BIAcore T100 system. Approximately 1100 response units (RU) of C1q in 10 m*M* MES buffer pH 6.1 and 900 of MBL in 10 m*M* sodium acetate pH 4.5 buffer were coated for the for the LAIR-2-HIS proteins and 1100 RU of C1q and 1500 of MBL for the LAIR-2-IgG proteins were immobilized on a series S CM5 sensor chip by using the amine coupling kit according to the manufacturer's instructions. After coupling the chip was pulsed for 30 seconds with EDC/NHS was followed by a 30 seconds pulse with ethanolamine. Analysis was performed in buffer (125m*M* NaCl, 2.5m*M* $CaCl_2$, 0.005% (w/v) Tween 20, and 25m*M* HEPES, pH 7.4) at 25°C and at a flow rate of 20 µl/minute. Injections with increasing concentrations of recombinant proteins (0, 500, 1000, 2000, 3000, 4000, 5000, 6000, 8000, 10000, 12000, 16000, 20000 and 24000 n*M* for LAIR-2-HIS and 0, 31.25, 62.5, 125, 250, 500, 1000, 2000, 3000, 4000, 6000 and 8000 n*M* for LAIR-2-IgG) were allowed to bind for 5 minutes for LAIR-2-HIS and 10 minutes for LAIR-2-IgG, after which regeneration by either flowing buffer for 10 minutes for the LAIR-2-HIS or 10 m*M* formic acid for LAIR-2-IgG occurred. Baseline stability was checked after every experiment. Proteins were injected until binding equilibrium was reached. Binding data were analyzed with BIAcore T100 evaluation software (version 2.01). Affinity constants were determined by steady state analysis.

Complement activity assays

The Wieslab complement system screen (Euro Diagnostica) was used to determine the effects of LAIR proteins on the activity of the complement system. The assays were performed following the manufacturer's protocol with minor modifications. 20 µl of fresh

human serum were mixed with 13 µg of fusion protein in 20 µl PBS and incubated for 30 min at 37°C. The absorbance was read at 405 nm. Background values were subtracted.

Serum samples mixed with LAIR-proteins were also tested for CH50 titers in a routine diagnostic laboratory (Sanquin).

Fluid phase activation of C4

Activated C4 in plasma or serum samples was measured with an Enzyme-linked immunosorbent assay (ELISA) in which mAb anti-C4-1 against a neo-epitope exposed on C4b, C4bi and C4c was used as a catching antibody (14). Results were related to those obtained with dilutions of aged serum and expressed as arbitrary units (AU) per ml. Samples tested for generation of fluid phase C4b/c were prepared by mixing 30 µl normal fresh serum with 30 µl protein to be tested (LAIR-1-Fc at 1.72 mg/ml, SIRL-Fc at 2.76 mg/ml, LAIR-2-Fc at 1.3 mg/ml, and non-aggregated human IgG (Gammaquin, Sanquin) at 1.3 mg/ml as control). As a positive control 30 µl normal fresh serum was mixed with 30 µl veronal buffered saline (VB) (Lonza).

Complement-dependent cytotoxicity crossmatch test

A cross match assay for anti-HLA antibodies was modified to assess the effects of the LAIR proteins on the complement-dependent cytotoxicity. Heat inactivated (30 min at 56°C) patient serum (1 µl) containing anti-HLA antibodies was incubated in Terasaki plates (Greiner BIO-One) with PBMCs (1 µl of 2-5x10^6 cells/ml) of a selected HLA-typed donor for 1 hour at RT. Meanwhile samples to be tested were prepared by mixing 5 µl fresh serum, 15 µl VB and 5 µl VB containing LAIR-proteins. A positive control was made by adding 5 µl normal fresh serum to 20 µl VB without protein, a negative control was made by mixing 5 µl normal fresh serum, 15 µl VB and 5 µl 100 m*M* EDTA. Subsequently, all samples were incubated for 20 min at RT. Next, 10 µl of each sample were added to the wells in duplicate and incubated for two hours at RT. Then, 5 µl of eosin 5% (VWR) was added. After 3 min 5 µl of 25% formaldehyde (Sigma-Aldrich) supplemented with 5 mg $CaCO_3$ (Sigma – Aldrich) was added. Finally 8 µl paraffin oil (Fagron, Barsbüttel, Germany) was added. Lysis was scored by light microscopy.

RESULTS

LAIR-1 and LAIR-2 bind MBL and C1q

LAIR-1 binds collagens (7;8). C1q and MBL both contain collagen-like regions. Son *et al.* recently showed binding of C1q to LAIR-1 and LAIR-2 (10). We investigated binding of LAIR-1 and -2 to both complement proteins. LAIR-Fc-fusion proteins, which are dimeric, bound to both solid-phase C1q and MBL (Figure 1A), whereas SIRL-1, an Ig-like inhibitory receptor that does not bind to collagen (13), did not bind (data not shown). The globular head domains of MBL bind to activators in a divalent cation-dependent manner. Addition of EDTA had no effect on LAIR binding to MBL, nor C1q, suggesting that the binding site of MBL for LAIR proteins is different from that for activators. This

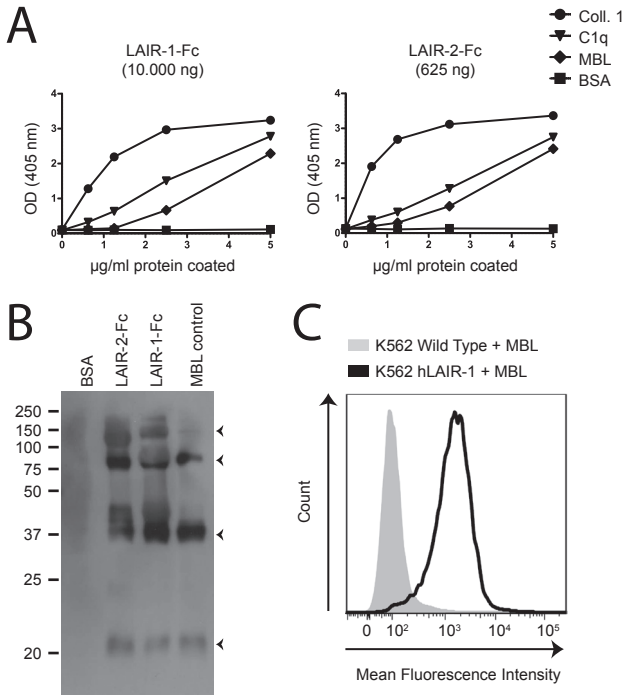


Figure 1: Recombinant LAIR proteins bind complement MBL. (A) Binding of LAIR-1-Fc (10,000 ng) and LAIR-2-Fc (625 ng) to plate coated MBL and C1q. Detection with HRP-conjugated anti-human IgG antibody. Results of a representative experiment (n = 3) are shown. **(B)** LAIR-1-Fc, LAIR-2-Fc and BSA (as a control) were coupled to Sepharose beads that were incubated with MBL. Precipitates were subjected to electrophoresis. MBL was detected with western blotting (n = 2). Arrowheads indicate specific bands **(C)** K562 cells with or without stable expression of LAIR-1 were incubated with MBL and analyzed by flow cytometry. A representative experiment is shown (n = 3).

Table 1: Calculated K_D values

		LAIR-2	
		Monomer	**Dimer**
MBL	K_D	1889 ± 52	76 ± 4
	K_D 2		1719 ± 141
C1q	K_D	9812 ± 251	186 ± 22
	K_D 2		2608 ± 168

Calculated K_D values
Calculated affinities (K_D in n*M*) from the binding at equilibrium (R_{eq}) of the surface plasmon resonance experiments. Values are the mean ± standard error of the mean (SEM).

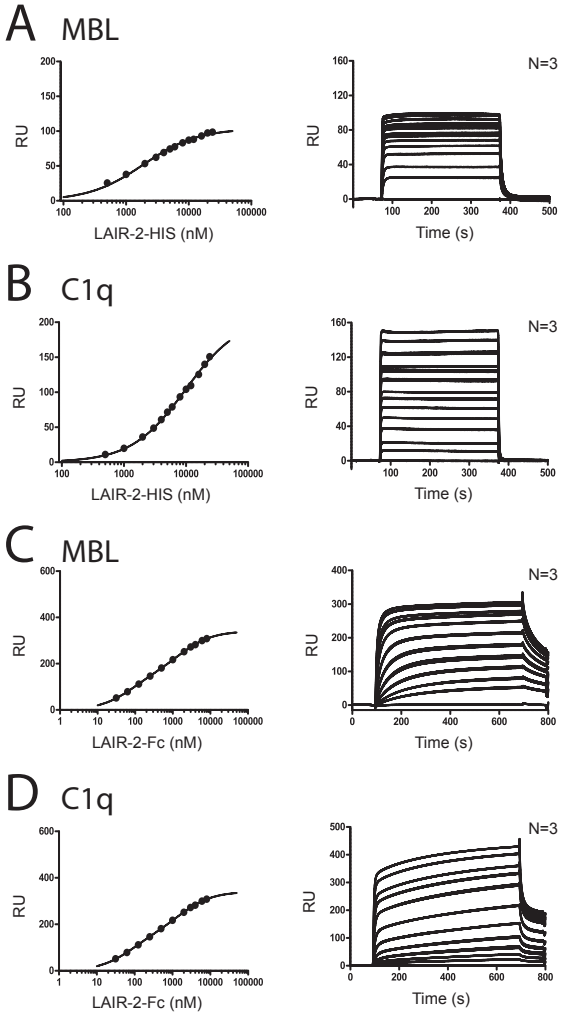


Figure 2: LAIR-2-Fc binds MBL and C1q with high affinity. Binding of LAIR-2-HIS to immobilized **(A)** MBL or **(B)** C1q and binding of LAIR-2-Fc to immobilized **(C)** MBL or **(D)** C1q was measured by surface plasmon resonance analysis. Recombinant proteins were injected at a flow rate of 20 µl/minute at a temperature of 25°C through a BIAcore flowcell containing 1100 RU C1q for the HIS and Fc fusion proteins, 900 RU MBL for the HIS proteins or 1500 RU MBL for the Fc proteins. In the left panels, individual symbols representing resonance at equilibrium along with the corresponding concentration of the protein are shown. Triplicate binding curves of LAIR-2-HIS at a concentration of 0, 500, 1000, 2000, 3000, 4000, 5000, 6000, 8000, 10000, 12000, 16000, 20000 and 24000 n*M* and LAIR-2-Fc at a concentration of 0, 31.25, 62.5, 125, 250, 500, 1000, 2000, 3000, 4000, 6000 and 8000 n*M* are shown in the right panels. Values are the mean ± SEM.

is consistent with the notion that the binding sites for the LAIR proteins are located in the collagen-like domains of MBL and C1q. Our data confirm the recently reported C1q binding to LAIR-1, though in our hands LAIR-2 binds to human C1q with a higher affinity than LAIR-1. Furthermore, we extend this concept to MBL (10): Immune precipitation experiments (Figure 1B) as well as flow cytometry on LAIR-1 transfectants (Figure 1C) further confirmed binding of MBL to LAIR.

Sixteen times more LAIR-1-Fc was needed than LAIR-2-Fc to obtain comparable optical densities (ODs) in the binding experiments (Figure 1A). This indicates that LAIR-2 has a higher affinity for C1q and MBL than LAIR-1. Equilibrium binding data obtained with surface plasmon resonance analysis revealed that monomeric LAIR-2-HIS specifically bound to MBL (Figure 2A) and C1q (Figure 2B), albeit with low affinity (Table 1). To increase the affinity we generated dimers of either LAIR protein by producing either protein as a Fc-fusion protein (9). Indeed LAIR-2-Fc fusion proteins bound MBL and C1q with a higher affinity (Figure 2C and Table 1), though the affinity for C1q is lower than for MBL (Figure 2D and Table 1).

These data are in line with our previous findings that LAIR-2 has a higher affinity for collagen than LAIR-1 and dimeric recombinant LAIR proteins have a higher affinity than monomeric forms (9).

Together, these data show that LAIR-1 and LAIR-2 are capable of binding the collagen-domain of C1q and MBL. LAIR-2-Fc binds MBL with a similar affinity as collagen, and C1q with a somewhat lower affinity.

LAIR-2-Fc inhibits complement activation

We next questioned whether binding of LAIR proteins to C1q and MBL may interfere with complement function of the classical and lectin pathways, respectively. We tested the effect of LAIR proteins added to fresh serum on complement activity as assessed with the Wieslab complement system screen (15). This screen uses deposition of C5b-9 as readout and measures the activity of the three complement pathways separately. To assess the effect on this deposition, serum was supplemented with recombinant LAIR proteins and tested. Untreated serum was set at 100%. The addition of a control recombinant protein had no effect on the activity of complement while the addition of LAIR-2-Fc significantly reduced C5b-9 deposition generated via the classical and lectin pathway but not that via the alternative pathway (Figure 3A), consistent with the absence of a protein with a collagen-like region in the alternative pathway. Thus, binding of LAIR-2-Fc to MBL and C1q interferes with activation of the lectin and classical pathways of the complement system, respectively. LAIR-2-Fc inhibited C4b/c production in fluid phase, while none of the control proteins did, confirming the specificity of the complement inhibition by LAIR-2 (Figure 3B). Finally, the data obtained with the Wieslab complement screening assays were confirmed by evaluating the effects of LAIR protein on the CH50 titer of normal human serum. The addition of 600 µg/ml LAIR-2 rendered the CH50 titers of normal serum undetectable, while the addition of PBS or human

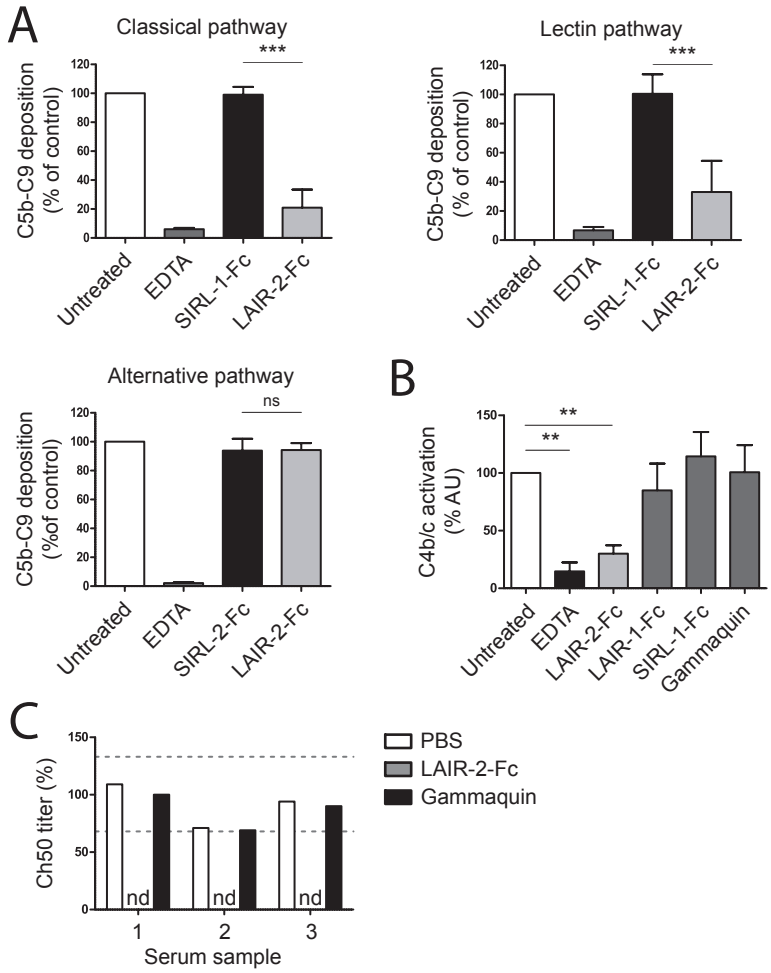


Figure 3: LAIR-2-Fc inhibits complement deposition. (A) Complement C5b-C9 deposition was measured in a pathway specific Wieslab complement assay. Sera of 4 individual donors were either left untreated, incubated with EDTA to completely abrogate complement deposition, or incubated with a control-Fc (SIRL-1) or LAIR-2-Fc. Complement deposition of the untreated condition was set at a 100%, mean ± SD is shown. Significance was tested with an anova test, using a bonferroni correction. (*** = $p \leq 0.001$). Results for the classical, lectin and alternative pathway are shown. **(B)** C4b/c detection in arbitrary units (AU) after fluid phase complement activation. Sera were either left untreated, incubated with EDTA or incubated with LAIR-1-Fc, a control-Fc (SIRL-1), Gammaquin or LAIR-2-Fc. AU of the untreated condition were set at a 100%. Mean ± SD is shown, n = 3. Significance was tested with an anova test, using a bonferroni correction. (** = $p \leq 0.005$, *** = $p \leq 0.001$). **(C)** CH50 titer. The area between the dotted lines depicts the range of normal complement: 68–133%. nd = not detectable.

IgG at the same concentration (Gammaquin) had no effect (Figure 3C). Together these data show that LAIR-2-Fc specifically inhibits the classical and lectin pathways of the complement system.

LAIR-2-Fc inhibits complement mediated cytotoxicity in an ex vivo model for AMR
To test whether LAIR-2-Fc also inhibits cellular complement deposition, we sensitized a CD20 expressing cell line with a CD20 antibody. After incubation with fresh mouse serum, we detected mouse iC3b/C3b and C3c deposition on the cells by flow cytometry. Incubation of the mouse serum with LAIR-2-Fc significantly diminished iC3b/C3b and C3c deposition on the sensitized cells whereas a control protein did not (Figure 4A). Thus, LAIR-2-Fc inhibits human as well as mouse complement.

We then tested the effect of LAIR-2-Fc in a human *ex vivo* model for antibody mediated transplant rejection. Human PBMCs were sensitized with anti-HLA antibodies and lysed by subsequent addition of fresh human serum as a source of complement. LAIR-2-Fc as well as control proteins were added to this serum and the effect on cell lysis was measured. The addition of LAIR-2-Fc significantly reduced the lysis of the cells sensitized with anti-HLA antibodies, while the addition of control proteins or control IgG had no effect (Figure 4B).

DISCUSSION

In this study, we show binding of recombinant LAIR proteins to the complement activators C1q and MBL. Consistent with our previous data on the interaction with collagen (9), the affinity of the dimeric forms of LAIR for C1q and MBL is higher than that of the monomeric proteins, and the affinity of LAIR-2 for these complement proteins is higher than that of LAIR-1. Our observation that LAIR-2-Fc but not LAIR-1-Fc inhibits complement is also in line with our finding that LAIR-2-Fc but not LAIR-1-Fc is an efficient platelet aggregation inhibitor (16). This may reflect differences in affinity but it also could be that LAIR-1 binds to different sites on C1q and MBL than LAIR-2. Our finding that LAIR-1 binds to MBL and C1q fixed to ELISA plates (Figure 1A), but showed no binding during surface plasmon resonance, may reflect that binding of MBL and C1q to the CM5 chip specifically blocked the LAIR-1 binding sites.

Son *et al.* recently showed that LAIR-1 has a higher affinity for C1q than LAIR-2 (10), which is not consistent with our data. Possibly this contrasting result reflects that Son *et al.* evaluated monomeric LAIR proteins whereas we included dimeric proteins, which have a higher affinity.

An interesting implication of MBL binding to LAIR-1 is the possibility that it acts as a functional ligand for LAIR-1. Indeed, we were able to demonstrate functional ligation of LAIR-1 by MBL in a reporter assay, revealing MBL as a potential circulating ligand for LAIR-1 (Supplemental Figure 1). Together with the findings of Son *et al.* that C1q can function as a functional ligand for LAIR-1, this extents LAIR-mediated control of immune cell function by collagens in the tissues to ligands in the circulation (19).

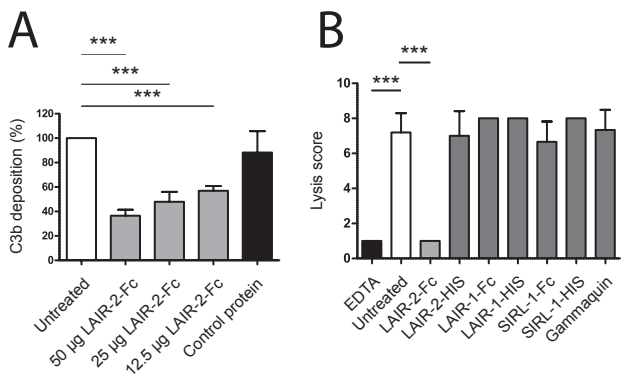


Figure 4: LAIR-2-Fc inhibits complement mediated cell lysis *in vitro.* (A) Mouse iC3b/C3b/C3c deposition on EL-4 CD20 cells was measured by FACS. Untreated control was set at a 100%, at least n = 3 for all conditions, except for the control protein n = 2. Significance was tested with an anova test, using a bonferroni correction. **(B)** Cell lysis in an *ex vivo* transplant model. Cells were either left untreated, incubated with EDTA or incubated with LAIR-2-Fc, LAIR-2-HIS, LAIR-1-Fc, LAIR-1-HIS, a control-Fc protein, a control-HIS protein or Gammaquin. Lysis was scored by light microscopy. Minimal lysis was scored as 1, maximal lysis as 8. All conditions n = 3, except for the HIS proteins n = 2. Mean ± SD is shown. Significance was tested with an anova test, using a bonferroni correction. (*** = $p \leq 0.001$)

Our in vitro data indicate that monomeric sLAIR-1 and LAIR-2 are unlikely to have a physiological role on the complement system considering their relatively low affinities for MBL and C1q. However, dimerization of LAIR-2 led to a substantial increase of the affinity for these two complement proteins, and indeed LAIR-2-Fc reduces complement deposition on complement activators and inhibits lysis of cells sensitized with anti-HLA antibodies in a human *ex vivo* antibody mediated rejection model. Complement can induce significant tissue injury when activated during hyper-acute rejection of transplanted organs, when complement split products such as C4d and C3d are deposited on the vascular endothelium (17). Our data suggest that administration of LAIR-2-Fc fusion protein may constitute a therapeutic option for patients suffering from such complications. The C5 inhibitor eculizumab is currently being tested for its ability to inhibit complement mediated graft rejection in the clinic and shows promising results (18). However, this inhibitor, in contrast to a LAIR-2 based inhibitor, does not inhibit C4 and C3 activation. Observations in patients with paroxysmal nocturnal hemoglobinuria indicate that cells still can be killed in a complement-dependent fashion when the system is blocked at the level of C5, probably through interaction with phagocytes via receptors for fixed C3 (19).

We previously reported that LAIR-2-Fc is able to inhibit collagen-induced platelet aggregation (16). This unique combination of inhibition of complement and thrombosis makes LAIR-2 fusion protein an interesting therapeutic option to prevent damage following reperfusion of transplanted organs as well as to prevent antibody mediated rejection.

In conclusion, antibody mediated rejection and ischemia reperfusion injury are two major problems in de field of organ transplantation. Both processes are dependent on activation of the classical and lectin pathways. We here present LAIR-2-Fc as a potent inhibitor of these two pathways of the complement system. Administration of LAIR-2-Fc may therefore be an interesting novel therapeutic strategy to prevent or treat antibody-mediated rejection as well as ischemia-reperfusion injury of for instance kidney allografts.

ACKNOWLEDGEMENTS

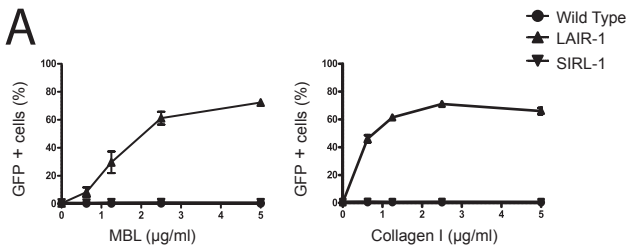
We thank Martin de Smet and Amelia Lacna for technical assistance, and Louis Bont for the help with statistical analysis. This study was funded by the Dutch Arthritis Foundation (grant 06-1-403).

REFERENCE LIST

1. Diepenhorst GM, van Gulik TM, Hack CE: Complement-mediated ischemia-reperfusion injury: lessons learned from animal and clinical studies Ann.Surg. 2009; 249: 889-899.
2. Ricklin D, Hajishengallis G, Yang K, Lambris JD: Complement: a key system for immune surveillance and homeostasis Nat.Immunol. 2010; 11: 785-797.
3. Bohmig GA, Exner M, Habicht A, Schillinger M, Lang U, Kletzmayr J, Saemann MD, Horl WH, Watschinger B, Regele H: Capillary C4d deposition in kidney allografts: a specific marker of alloantibody-dependent graft injury J.Am.Soc.Nephrol. 2002; 13: 1091-1099.
4. Sacks SH, Zhou W: The role of complement in the early immune response to transplantation Nat.Rev.Immunol. 2012; 12: 431-442.
5. Sellar GC, Blake DJ, Reid KB: Characterization and organization of the genes encoding the A-, B- and C-chains of human complement subcomponent C1q. The complete derived amino acid sequence of human C1q Biochem.J. 1991; 274 (Pt 2): 481-490.
6. Meyaard L: The inhibitory collagen receptor LAIR-1 (CD305) J.Leukoc.Biol. 2008; 83: 799-803.
7. Tang X, Narayanan S, Peruzzi G, Apara A, Natarajan K, Margulies DH, Coligan JE, Borrego F: A single residue, arginine 65, is critical for the functional interaction of leukocyte-associated inhibitory receptor-1 with collagens J.Immunol. 2009; 182: 5446-5452.
8. Lebbink RJ, De Ruiter T, Adelmeijer J, Brenkman AB, van Helvoort JM, Koch M, Farndale RW, Lisman T, Sonnenberg A, Lenting PJ, Meyaard L: Collagens are functional, high-affinity ligands for the inhibitory immune receptor LAIR-1 J.Exp.Med. 2006; 203: 1419-1425.
9. Olde Nordkamp MJM, van Roon JA, Douwes M, De Ruiter T, Urbanus RT, Meyaard L: Enhanced secretion of Leukocyte-Associated Immunoglobulin-like Receptor (LAIR)-2 and soluble LAIR-1 in rheumatoid arthritis: LAIR-2 is a more efficient antagonist of the LAIR-1-collagen inhibitory interaction than soluble LAIR -1. Arthritis Rheum. 2011; 63: 3749-3757.
10. Son M, Santiago-Schwarz F, Al-Abed Y, Diamond B: C1q limits dendritic cell differentiation and activation by engaging LAIR-1 Proc.Natl.Acad.Sci.U.S.A 2012.
11. Boross P, Jansen JH, de HS, Beurskens FJ, van der Poel CE, Bevaart L, Nederend M, Golay J, van de Winkel JG, Parren PW, Leusen JH: The in vivo mechanism of action of CD20 monoclonal antibodies depends on local tumor burden Haematologica 2011; 96: 1822-1830.
12. Hack CE, Belmer AJ: The IgG detected in the C1q solid-phase immune-complex assay is not always of immune-complex nature Clin.Immunol.Immunopathol. 1986; 38: 120-128.
13. Steevels TAM, Lebbink RJ, Westerlaken GHA, Coffer PJ, Meyaard L: Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) is a novel functional inhibitory immune receptor expressed on human phagocytes. J Immunol 2010; 184: 4741-4748.
14. Wolbink GJ, Bollen J, Baars JW, ten Berge RJ, Swaak AJ, Paardekooper J, Hack CE: Application of a monoclonal antibody against a neoepitope on activated C4 in an ELISA for the quantification of complement activation via the classical pathway J.Immunol.Methods 1993; 163: 67-76.
15. Fredrikson GN, Truedsson L, Sjoholm AG: New procedure for the detection of complement deficiency by ELISA. Analysis of activation pathways and circumvention of rheumatoid factor influence J.Immunol.Methods 1993; 166: 263-270.
16. Lenting PJ, Westerlaken GHA, Denis CV, Akkerman J.W., Meyaard L: Efficient inhibition of collagen-induced platelet activation and adhesion by LAIR-2, a soluble Ig-like receptor family member Plos One 2010; 5: e12174.
17. Wasowska BA: Mechanisms involved in antibody- and complement-mediated allograft rejection Immunol.Res. 2010; 47: 25-44.
18. Stegall MD, Chedid MF, Cornell LD: The role of complement in antibody-mediated rejection in kidney transplantation Nat.Rev.Nephrol. 2012; 8: 670-678.

19. Hill A, Rother RP, Arnold L, Kelly R, Cullen MJ, Richards SJ, Hillmen P: Eculizumab prevents intravascular hemolysis in patients with paroxysmal nocturnal hemoglobinuria and unmasks low-level extravascular hemolysis occurring through C3 opsonization Haematologica 2010; 95: 567-573.

SUPPLEMENTARY MATERIAL



Supplementary Figure 1: MBL is a functional ligand for LAIR-1. NFAT-GFP reporter cells stably transfected with either a LAIR-1-CD3ζ chimera, a control protein (SIRL-1) CD3ζ chimera or wild type cells were incubated with MBL and collagen. GFP expression was analyzed by FACS. N = 3, mean ± SD of % positive cells is shown.

7

GENERAL DISCUSSION

Inhibitory immune receptors are important for the balance of the immune system. They are differentially expressed by various immune cells and regulate the immune system by counter acting activation signals (1). At present day, over 60 inhibitory receptors have been characterized, while genome wide analysis indicates that there might be as much as 300 (2). The functioning of these receptors is regulated by variance in expression on different cell subsets in different stages of immune activation (3-5). Even within one cell type, the expression of an inhibitory immune receptor can vary in different stages of the immune response. It is known, for instance, that blood neutrophils do not express leukocyte associated immunoglobulin-like receptor-1 (LAIR-1). However, upon stimulation with GM-CSF, LAIR-1 expression on neutrophils is induced. The availability of the ligand also regulates the immune system. In blood, for instance, is no readily available collagen to induce LAIR-1 signaling. Furthermore, soluble receptors might block ligand binding sites, thereby further regulating the immune response. In spite of their abundance, these receptors are not redundant. As discussed in Chapter 2 of this thesis, malfunction of a single inhibitory receptor can lead to predisposition for several autoimmune diseases. However, genetic screenings such as GWAS and META-analysis show a significant association of only a few of these receptors with autoimmunity. Still, mouse models, as well as expression studies and functional analysis of these receptors on patient cells show overwhelming evidence for the involvement of these receptors in autoimmunity. As we state in Chapter 2, genetic variation in common signaling pathways of inhibitory receptors does occur. *PTPN22* and *CSK* are associated with multiple autoimmune disorders (6-9). While the signaling molecules encoded by these genes play an important role in the regulation of the T cell receptor, we and others have found that CSK associates with several inhibitory receptors (10-12). We therefore hypothesize that the variation in *PTPN22* and *CSK* also influence the signaling of these receptors.

LAIR-1

This thesis focuses on the role of LAIR-1 and LAIR-2 in immune regulation. LAIR-1 is an inhibitory receptor of the Ig-superfamily which expresses an extracellular Ig-like domain and two intracellular ITIMs (13). It is expressed on a wide variety of immune cells, among which natural killer (NK) cells, T cells, B cells, eosinophils, monocytes and dendritic cells (3;13). LAIR-1 can be shed off the cell surface, thereby forming soluble LAIR-1 (sLAIR-1) (14). Furthermore, a homologue without a transmembrane and intracellular signaling domain is detected in the circulation; LAIR-2 (15). In 2006 it was discovered that the ligands of LAIR-1 are collagens (16).

LAIR-1 REGULATES THE OXIDATIVE BURST

Cross linking of LAIR-1 can lead to inhibition of cytotoxicity on T cells, inhibition of target cell lysis on NK cells and decreased B cell receptor induced calcium mobilization (14). Furthermore, dendritic cell maturation can be inhibited by LAIR-1 crosslinking,

as well as inhibition of the Fc-epsilon receptor (17-19). In Chapter 5 we show for the first time that ligation of LAIR-1 can lead to inhibition of the oxidative burst. One of the most important anti-microbial defense mechanisms is the oxidative burst generated by phagocytes such as monocytes, macrophages or neutrophils. The nicotinamide adenine dinucleotide phosphate (NADPH) complex is responsible for converting oxygen into superoxide, using NADPH as an electron donor (20;21). Superoxide can be further converted into hydrogen peroxide and hypochlorous acid which are more aggressive reactive oxygen species (ROS) (20). High concentrations of ROS are toxic to invading microorganisms but can also cause severe damage to the host. Therefore, it is crucial to control the oxidative burst to limit the amount of collateral damage. In Chapter 5 we show that ligation of LAIR-1 by collagen or SP-D on a neutrophilic cell line is able to limit the oxidative burst generated by triggering of the Fcα receptor. Other inhibitory receptors regulate the oxidative burst as well. SIRPα, for instance, inhibits the oxidative burst by down regulating $gp91^{phox}$, the key catalytic component of the NADPH oxidase complex (22). SIRL-1 inhibits the phosphorylation of ERK, thereby intervening in an upstream event of the signaling cascade leading to the inhibition of the oxidative burst (23). By what mechanism LAIR-1 limits the oxidative burst needs to be studied in order to further understand the immune regulation mediated by LAIR-1. Most likely, LAIR-1 ligation will inhibit the oxidative burst in a similar way as SIRL-1. SHP-1, one of the downstream mediators of LAIR-1, is known to regulate the oxidative burst and to be essential in the MAPK ERK pathway (24). However, LAIR-1 is not dependant on SHP-1, SHP-2 and SHIP for signaling, since it can also recruit CSK. Further studies are needed to clarify the exact mechanism.

MULTIPLE LAIR LIGANDS

Up until now, collagens were the only known ligands for LAIR-1. In this thesis, we show in Chapters 5 and 6 that the collagenous domain containing surfactant protein-D (SP-D) and the collagenous domain containing mannose binding lectin (MBL) can also function as ligands for LAIR-1. Furthermore, Son *et al.* recently showed that C1q can inhibit dendritic cell differentiation by crosslinking LAIR-1 (18). This puts LAIR-1 mediated immune regulation in a whole new perspective. The hypothesis was that upon an infection or injury, tissue is damaged and collagen is exposed (Figure 1A). Inflammatory mediators are released by the infected or injured cells, and immune cells are recruited. Upon arrival to the infection or injury, LAIR-1 expressing immune cells come into contact with exposed collagen, which raises the threshold for immune activation and fine-tunes the immune response to limit collateral damage. When the activating signal is of such strength that it overrules the inhibitory signal given by LAIR-1, the immune cell will exert its effector functions. The exposed collagen in injured or inflamed tissue causes signaling of LAIR-1 and may help the immune system to return to a more quiescent state. The activation status of the immune system and the individual cells depends on

this delicate balance of activating and inhibitory signals, thereby creating a specific inflammatory nice which is tailor made for the specific injury or infection.

In this thesis we show that this hypothesis needs to be revised, as exposed collagen is not the only way to mediate inhibition via LAIR-1. In Chapter 5 we show that SP-D binds LAIR-1 via its collagen domain, and is able to mediate ligation of the receptor. SP-D is a member of the collectin family, containing a collagen like domain witch is coupled to a carbohydrate recognition domain by a coiled neck region (25). It is mainly expressed in mucosal surfaces, where it acts as a first line defense against pathogens (26). When taking the lung as an exemplary mucosal surface for LAIR-1 mediated immune regulation, alveolar and airway macrophages are the main residential myeloid cells expressing LAIR-1. In normal, steady-state conditions, these cells can mediate both opsonic and non-opsonic phagocytosis of inhaled pathogens (27). These residential myeloid LAIR-1 expressing cells would come in to contact with SP-D, produced and excreted by type II alveolar cells (28). The immune activation state of the myeloid cells may be lowered because of the constitutive contact with SP-D, which would lead to continuous inhibition via LAIR-1 (Figure 1B). Indeed, SP-D$^{-/-}$ mice show a clear defect in immune regulation. Their lungs show a state of constitutive immune activation, with

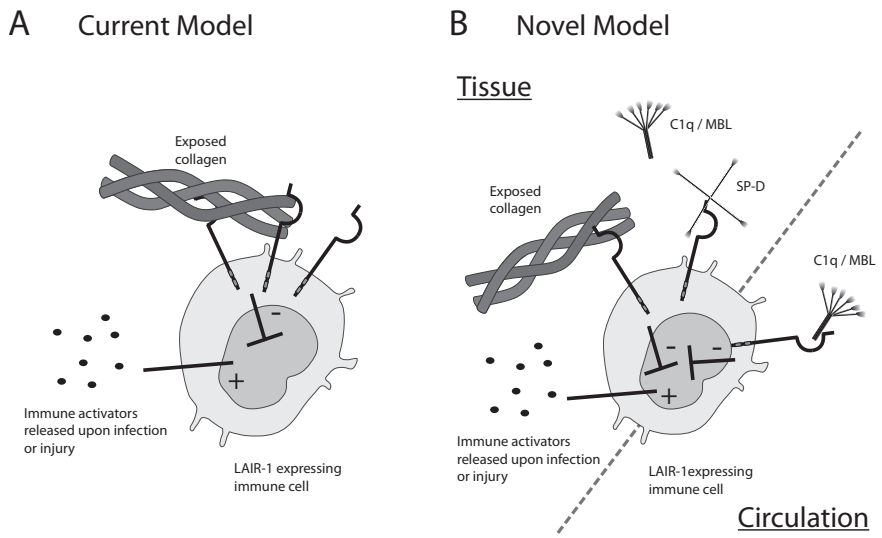


Figure 1: Inhibition via new LAIR-1 ligands. (A) The current model of immune regulation: LAIR-1 comes into contact with collagen that is exposed upon injury or infection. Immune regulation is ensured through a balance of inhibitory and activating signals. **(B)** The novel model of immune regulation shows that LAIR-1 can also be ligated by the novel ligands MBL, C1q and SP-D to regulate immune activation. C1q and MBL are present outside tissues; constitutive ligation of LAIR-1 by the constant availability of C1q and MBL may determine the immune systems threshold for activation. Figure is not drawn to scale and was produced using Servier Medical Art.

innate immune cells showing abnormalities such as the constitutive release of cytokines (reviewed in (29)).

In Chapter 6 we show that not only SP-D, but also MBL is a novel ligand for LAIR-1. MBL is the key initiator of the lectin pathway of the complement system, and has up to 6 subunits which each consist of a homo-trimer of the same polypeptide chain. This chain also contains a C-terminal globular domain, coupled to an N-terminal collagen-like region (30).

We show that MBL is able to bind LAIR-1 and mediate ligation of the receptor in a NFAT-GFP reporter assay. Fraser *et al.* showed that MBL and C1q can influence cytokine levels produced by monocytes (31). Sustained LPS activation of monocytes in vitro can lead to the production of anti-inflammatory IL-10 (32). Stimulation with C1q or MBL further enhances IL-10 mRNA while the effect is even more robust on protein level. Furthermore, monocytes produce less of the pro-inflammatory cytokines IL-1α and IL-1β; levels were diminished and more MCP-1 was produced in vitro after exposure to C1q or MBL (31).

It is interesting to speculate that LAIR-1 might play a role in the up regulation of anti-inflammatory and down regulation of pro-inflammatory cytokines in monocytes, as LAIR-1 is known to influence cytokine production on B cells (33).

MBL double knockout (knockout for MBL-A chain and MBL-C chain) mice on mixed genetic background (SV129EvSv x C57BL/6) produce higher antibody titres in a hepatitis B surface antigen immunization study than wild-type littermates, suggesting that MBL plays a role in a negative feedback regulation of adaptive immunity (34). A similar finding was done by Guttormsen *et al.*, who demonstrated that MBL deficient mice (on a C57BL/6 background) have enhanced antigen specific IgG production following immunization with Group B streptococcus (35). It is tempting to speculate that this over-activation of the immune system reflects a lack of negative feedback via LAIR-1 signalling. However, the modulating effect of MBL in the study of Ruseva *et al.* was dependent on the genetic background of the mouse strain, giving opposite effects in C57BL/6 vs SV129EvSv mice (34). Guttormsen *et al.* did not study different genetic backgrounds.

Over all, there seems to be a role for SP-D, C1q and MBL in modulating the immune system, which in part could be mediated by LAIR-1, for instance in the bloodstream (Figure 1B) or the periphery. By the constitutive ligation of the receptor by C1q and MBL in the blood and by SP-D in mucosal tissues, the immune system is kept in a quiescent state. Upon infection or injury, danger signals are released and override the inhibition provided by the constitutive ligation of LAIR-1. This ensures that the activation level of the immune system is raised to clear the infection.

SOLUBLE LAIR PROTEINS

As discussed above, there are several ligands for LAIR-1, which all can contribute to the regulation of the immune system. The amount and nature of the newly discovered ligands ensue a constant immune inhibition via LAIR-1. However, certain situations such

as infections or injuries require a more active state of the immune system. Invading pathogens need to be cleared. The inflammatory mediators produced upon an infection or injury can counter-act the inhibition mediated by LAIR-1, ensuring activation of the proper immune cells and clearance of the pathogen. The balance between activating and inhibitory signals is not only determined by the availability of the ligand and the amount of inflammatory mediators which are produced. To ensure continued activation of the individual immune cell, LAIR-1 can be shed of the surface to produce soluble LAIR-1. In Chapter 3 we show that it is unlikely that soluble LAIR-1 can function as an anti-inhibitory molecule by blocking the LAIR-1 binding sites on ligands because of the low affinity. Shedding LAIR-1 of the cell surface will however, lead to a reduced expression and thus signalling of the receptor, thereby leading to a lower threshold for activation of the cell. In Chapter 3 we also show that activated T cells produce LAIR-2. LAIR-2 has a higher affinity for collagen than LAIR-1, and can function as a competitor for collagen binding (Figure 2), thereby blocking access to the ligand for LAIR-1 and reducing the signalling which will lead to a more activated immune system. Thus, LAIR-2 is a pro-inflammatory protein, which will probably exert its actions locally. High levels of LAIR-2 in for instance synovial fluid, may lead to blockage of the LAIR-1 binding sites on exposed collagen. Similarly, in the lung, locally produced LAIR-2 might function to block binding sites on SP-D. However, the concentration of LAIR-2 in circulation is less than 350 ng/ml. This is so low, that it is unlikely that sufficient binding sites on C1q and MBL in the blood will be blocked to obtain an effect on immune regulation.

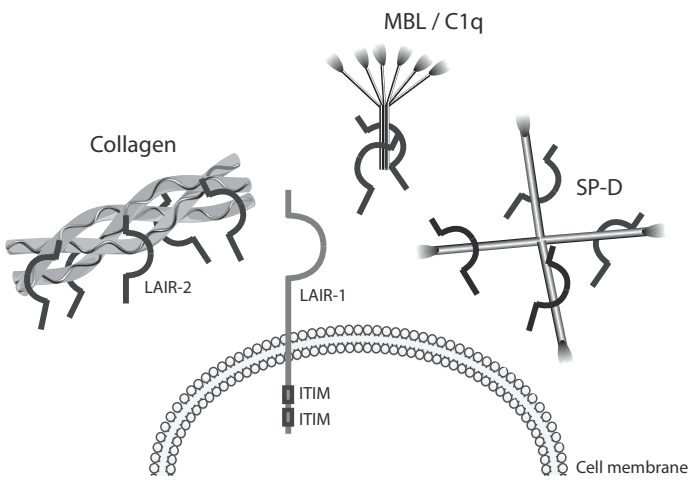


Figure 2: Immune regulation by LAIR-2. Schematic representation of immune regulation by LAIR-2. By blocking the LAIR-1 binding sites at collagen or the collagenous domains of proteins, LAIR-1 is unable to ligate and mediate inhibition, rendering the immune cell to a more activated state. Figure is not drawn to scale and was produced using Servier Medical Art.

Several inhibitory immune receptors have soluble variants, either generated by alternative splicing or shedding of the cell membrane, to compete for ligand binding. CTLA-4, for instance, is expressed by activated T cells. A soluble form of CTLA-4 is generated through alternative splicing. Soluble CTLA-4 has the extracellular structure and the ability to bind the ligand of CTLA-4, B7. Resting T cells produce the soluble variant of CTLA-4, and production is suppressed by T-cell activation (36). Like CTLA-4, the programmed death (PD)-1 receptor is also highly up regulated on T-cells. PD-L1 and PD-L2 function as ligands for PD-1 and their interactions inhibit T cell effector functions such as IFN-γ production in an antigen-specific manner (37). There are four soluble variants of PD-1 that are obtained by alternative splicing. The soluble receptors can bind PD-L1 and PD-L2 and therefore antagonize the actions of membrane bound PD-1 (38). Our studies indicate that the production of a soluble competitor for LAIR-1 binding sites by cells that express LAIR-1, leads to a site- and even cell specific type of immune regulation. This ensures the proper activation status of the immune system.

SOLUBLE INHIBITORY RECEPTORS AS BIOMARKERS FOR DISEASE

Soluble derivatives of inhibitory receptors often function to inhibit the actions of their cellular counterparts, but might also be used as biomarkers for various diseases. High serum levels of the above discussed sCTLA-4 are associated with disease severity in spondylarthropathy, Graves disease, Hashimoto thyroiditis, myasthenia gravis, systemic lupus erythematosus and systemic sclerosis (39-43). sPD-1 levels are elevated in sera of aplastic anemia patients, and elevated sPECAM-1 levels in human sera are found in HIV, multiple sclerosis and acute myocardial infarction patients (38;44-46). Previously, our group showed that levels of LAIR-2 are elevated in the synovial fluid of rheumatoid arthritis (RA) patients as compared to osteoarthritis (OA) patients (47). In Chapter 3 of this thesis we show that levels of sLAIR-1 in synovial fluid of RA patients are significantly increased. Furthermore, we show that sLAIR-1 and LAIR-2 are significantly increased in the urine of RA patients, as compared to healthy controls, while we found no difference between patients and controls regarding plasma levels of sLAIR-1 and LAIR-2. This might be explained by the detection limit of the ELISA that was used in these experiments. Currently, the sLAIR-1 and LAIR-2 ELISA are in the process of conversion to a multiplex assay in order to increase the sensitivity. An additional advantage of the multiplex assay is the amount of inflammatory markers that can be measured in the same sample. In Chapter 3 we show that the amount of urinary LAIR-2 in RA patients correlated significantly with the erythrocyte sedimentation rate (ESR), which is a well known marker of inflammation. The ability to measure sLAIR-1 and LAIR-2 in a multiplex assay can expand the number of inflammatory markers measured in the samples. Correlation of these markers with levels of sLAIR-1 and LAIR-2 will gain insights about the function of the immune system and the feasibility of using LAIR-1 and/or LAIR-2 as a biomarker for disease.

sLAIR-1 IN AMNIOTIC FLUID

In Chapter 4 we show that sLAIR-1 correlates with compliance of the total respiratory system at age one month, when measured in amniotic fluid of term pregnancies. Additionally, children with a high level of amniotic fluid sLAIR-1 and / or high airway compliance have a lower risk of rhinovirus lower respiratory tract infections and recurrent wheeze during the first year of life. sLAIR-1 in amniotic fluids is of fetal origin, as the first urine of newborns contains higher levels of sLAIR-1 than amniotic fluid. There is no correlation between sLAIR-1 levels in cord blood and amniotic fluid.

Soluble inhibitory receptors may have a regulatory function in amniotic fluid. In a recent study, Kacerovsky *et al.* show that CD200, the ligand for the inhibitory immune receptor CD200R, is constituently present in amniotic fluid during uncomplicated pregnancies, while the levels increase during labor. CD200 levels were higher in pregnancies complicated by funisitis, which is an inflammation of the connective tissue of the umbilical cord. They suggest that amniotic fluid CD200 acts as a negative regulator for preventing over activation of the innate immune system (48).

As discussed above, the affinity of monomeric soluble LAIR-1 for collagen is so low that it is not likely to block collagen binding sites in vivo, but decreased expression of LAIR-1 after shedding from the cell will lower the threshold for activation. There is a large body of evidence that supports the contribution of in utero immune activation to lung maturation (49-52). We propose that amniotic fluid LAIR-1 reflects perinatal in utero immune activation. Interestingly, amniotic fluid LAIR-1 levels correlate poorly with, and are distinct from, other cytokines measured which resemble the 'acute inflammation gene expression signature' such as IL-17, IL-18, IL23 and TNF-alpha (53). This might indicate that LAIR-1 is not 'just another' inflammatory biomarker, but reflects the activation state of the immune system in a distinct manner. The exact function of soluble LAIR proteins and their usefulness as biomarkers needs to be further investigated, in large cohorts and in a more sensitive assay such as the multiplex assay. Further correlations with (anti) inflammatory markers need to be made in order to understand the exact role of soluble LAIR proteins in the total regulation of the immune system.

LAIR-2-FC IN COMPLEMENT REGULATION

In Chapter 6 we show that MBL can function as a ligand for LAIR-1. We also show high affinity binding of recombinant dimeric LAIR-2. Binding of LAIR-2 to C1q and MBL inhibits the activation of the classical and lectin pathway of the complement system. Although the complement system is essential to provide protection against invading micro-organisms, it can also cause serious tissue damage after for instance ischemia/ reperfusion injuries or organ transplantation (54;55). LAIR-2-Fc is also an effective inhibitor of platelet aggregation (56). In 1997 Fujiwara *et al.* showed that inhibition of the complement cascade by soluble complement receptor type one and inhibition of coagulation by anti-thrombin-III had a synergistic effect on xenograft survival (57).

In vivo studies are needed to investigate whether LAIR-2 can mach the synergistic effect of the complement inhibitor and the anti-coagulant of the study of Fujiwara. However, simultaneously inhibiting coagulation and the two major complement pathways with one drug (fusion protein) seems a promising way to improve transplanted organ survival. Another promising therapeutic strategy for LAIR-2-Fc might be to apply it as a coating for biomaterials, for instance on stents inserted in coronary arteries after ischemia/reperfusion injury to the heart. Biomaterial surfaces lacking regulators will likely trigger amplification of the immune response (58). Heparin coatings to absorb factor H show varying results and alternative sources of regulators which can be applied to biomaterials are desired (58). It would be of interest to investigate the suitability of LAIR-2 in this field.

CONCLUDING REMARKS

In this thesis we investigated immune regulation by cell expressed LAIR-1 and soluble LAIR proteins. We found that LAIR-1 ligation can be mediated by several ligands, some of which are present in the circulation, and show for the first time that ligation of the receptor is able to inhibit the oxidative burst. Furthermore, we investigated the potential use of sLAIR-1 and LAIR-2 as biomarkers in inflammatory disease or amniotic fluid. In addition, we show that recombinant LAIR-2 is able to inhibit the two major pathways of the complement cascade and might be a potential therapeutic. All these data combined show that although there are many inhibitory immune receptors, they are involved in many different processes and one can make a major difference in immune regulation.

REFERENCE LIST

1. Pritchard NR, Smith KG. B cell inhibitory receptors and autoimmunity. Immunology 2003 Mar;108:263-73.
2. Daeron M, Jaeger S, Du Pasquier L, Vivier E. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol Rev 2008;224:11-43.
3. Verbrugge A, De Ruiter T, Geest C, Coffer PJ, Meyaard L. Differential expression of Leukocyte Associated Ig-like Receptor-1 during neutrophil differentiation and activation. J Leukoc Biol 2006;79:828-36.
4. Jansen CA, Cruijsen C, De Ruiter T, Nanlohy N, Willems N, Janssens-Corpela P, *et al.* Regulated expression of the inhibitory receptor LAIR-1 on human peripheral T cells during T cell activation and differentiation. Eur J Immmunol 2007 May 1;37:914-24.
5. Steevels TAM, Lebbink RJ, Westerlaken GHA, Coffer PJ, Meyaard L. Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) is a novel functional inhibitory immune receptor expressed on human phagocytes. J Immunol 2010;184:4741-8.
6. Plagnol V, Howson JM, Smyth DJ, Walker N, Hafler JP, Wallace C, *et al.* Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases. PLoS Genet 2011 Aug;7(8):e1002216.
7. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, Thomson BP, *et al.* Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet 2010 Jun;42(6):508-14.
8. Gregersen PK, Amos CI, Lee AT, Lu Y, Remmers EF, Kastner DL, *et al.* REL, encoding a member of the NF-kappaB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis. Nat Genet 2009 Jul;41(7):820-3.
9. Franke A, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, *et al.* Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat Genet 2010 Dec;42(12):1118-25.
10. Verbrugge A, Rijkers ESK, De Ruiter T, Meyaard L. Leukocyte-associated Ig-like receptor-1 has SH-2 domain-containing phosphatase-independent function and recruits C-terminal Src kinase. Eur J Immmunol 2006 Jan;36:190-8.
11. Sayos J, Martinez-Barriocanal A, Kitzig F, Bellon T, Lopez-Botet M. Recruitment of C-terminal Src kinase by the leukocyte inhibitory receptor CD85j. Biochem Biophys Res Commun 2004 Nov 12;324:640-7.
12. Veillette A, Thibaudeau E, Latour S. High expression of inhibitory receptor SHPS-1 and its association with protein-tyrosine phosphatase SHP-1 in macrophages. J Biol Chem 1998;273:22719-28.
13. Meyaard L, Adema GJ, Chang C, Woollatt E, Sutherland GR, Lanier LL, *et al.* LAIR-1, a novel inhibitory receptor expressed on human mononuclear leukocytes. Immunity 1997 Aug;7(2):283-90.
14. Meyaard L. The inhibitory collagen receptor LAIR-1 (CD305). J Leukoc Biol 2007 Dec 6.
15. Lebbink RJ, van den Berg MCW, De Ruiter T, Raynal N, van Roon JAG, Lenting PJ, *et al.* The soluble LAIR-2 antagonizes the collagen/LAIR-1 inhibitory immune interaction. J Immunol 2008;180:1662-9.
16. Lebbink RJ, De Ruiter T, Adelmeijer J, Brenkman AB, van Helvoort JM, Koch M, *et al.* Collagens are functional, high-affinity ligands for the inhibitory immune receptor LAIR-1. J Exp Med 2006;203:1419-25.
17. Poggi A, Tomasello E, Ferrero E, Zocchi MR, Moretta L. p40/LAIR-1 regulates the differentiation of peripheral blood precursors to dendritic cells induced by granulocyte-monocyte colony-stimulating factor. Eur J Immunol 1998;28:2086-91.
18. Son M, Santiago-Schwarz F, Al-Abed Y, Diamond B. C1q limits dendritic cell differentiation and activation by engaging LAIR-1. Proc Natl Acad Sci U S A 2012 Oct 23.

19. Verbrugge A, De Ruiter T, Clevers H, Meyaard L. Differential contribution of the Immunoreceptor tyrosine-based inhibitory motifs of human leukocyte-associated Ig-like receptor-1 to inhibitory function and phosphatase recruitment. Int Immunol 2003;15:1349-58.
20. Sheppard FR, Kelher MR, Moore EE, McLaughlin NJ, Banerjee A, Silliman CC. Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. J Leukoc Biol 2005 Nov;78(5):1025-42.
21. Bellavite P, Cross AR, Serra MC, Davoli A, Jones OT, Rossi F. The cytochrome b and flavin content and properties of the O2- -forming NADPH oxidase solubilized from activated neutrophils. Biochim Biophys Acta 1983 Jul 28;746(1-2):40-7.
22. van Beek EM, Zarate JA, van BR, Schornagel K, Tool AT, Matozaki T, *et al.* SIRPalpha controls the activity of the phagocyte NADPH oxidase by restricting the expression of gp91(phox). Cell Rep 2012 Oct 25;2(4):748-55.
23. Steevels TA, Van AK, Westerlaken GH, Stalpers F, Walk J, Bont L, *et al.* Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) negatively regulates the oxidative burst in human phagocytes. Eur J Immunol 2013 Feb 22.
24. Forget G, Gregory DJ, Whitcombe LA, Olivier M. Role of host protein tyrosine phosphatase SHP-1 in Leishmania donovani-induced inhibition of nitric oxide production. Infect Immun 2006 Nov;74(11):6272-9.
25. Haagsman HP, Hogenkamp A, van EM, Veldhuizen EJ. Surfactant collectins and innate immunity. Neonatology 2008;93(4):288-94.
26. Herias MV, Hogenkamp A, van Asten AJ, Tersteeg MH, van EM, Haagsman HP. Expression sites of the collectin SP-D suggest its importance in first line host defence: power of combining in situ hybridisation, RT-PCR and immunohistochemistry. Mol Immunol 2007 Jul;44(13):3324-32.
27. Eddens T, Kolls JK. Host defenses against bacterial lower respiratory tract infection. Curr Opin Immunol 2012 Aug;24(4):424-30.
28. Voorhout WF, Veenendaal T, Kuroki Y, Ogasawara Y, van Golde LM, Geuze HJ. Immunocytochemical localization of surfactant protein D (SP-D) in type II cells, Clara cells, and alveolar macrophages of rat lung. J Histochem Cytochem 1992 Oct;40(10):1589-97.
29. Orgeig S, Hiemstra PS, Veldhuizen EJ, Casals C, Clark HW, Haczku A, *et al.* Recent advances in alveolar biology: evolution and function of alveolar proteins. Respir Physiol Neurobiol 2010 Aug 31;173 Suppl:S43-S54.
30. Sacks SH, Zhou W. The role of complement in the early immune response to transplantation. Nat Rev Immunol 2012 Jun;12(6):431-42.
31. Fraser DA, Bohlson SS, Jasinskiene N, Rawal N, Palmarini G, Ruiz S, *et al.* C1q and MBL, components of the innate immune system, influence monocyte cytokine expression. J Leukoc Biol 2006 Jul;80(1):107-16.
32. de Waal MR, Abrams J, Bennett B, Figdor CG, De Vries JE. Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 1991 Nov 1;174(5):1209-20.
33. Van der Vuurst de Vries A, Clevers H, Logtenberg T, Meyaard L. Leukocyte Associated Ig-like Receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. Eur J Immunol 1999;29:3160-7.
34. Ruseva M, Kolev M, gnaes-Hansen F, Hansen SB, Takahashi K, Ezekowitz A, *et al.* Mannan-binding lectin deficiency modulates the humoral immune response dependent on the genetic environment. Immunology 2009 Jun;127(2):279-88.
35. Guttormsen HK, Stuart LM, Shi L, Carroll MC, Chen J, Kasper DL, *et al.* Deficiency of mannose-binding lectin greatly increases antibody response in a mouse model of vaccination. Clin Immunol 2009 Mar;130(3):264-71.

36. Magistrelli G, Jeannin P, Herbault N, oit De CA, Gauchat JF, Bonnefoy JY, *et al.* A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. Eur J Immunol 1999 Nov;29(11):3596-602.
37. Fife BT, Pauken KE. The role of the PD-1 pathway in autoimmunity and peripheral tolerance. Ann N Y Acad Sci 2011 Jan;1217:45-59.
38. Wu H, Miao M, Zhang G, Hu Y, Ming Z, Zhang X. Soluble PD-1 is associated with aberrant regulation of T cells activation in aplastic anemia. Immunol Invest 2009;38(5):408-21.
39. Liu MF, Wang CR, Chen PC, Fung LL. Increased expression of soluble cytotoxic T-lymphocyte-associated antigen-4 molecule in patients with systemic lupus erythematosus. Scand J Immunol 2003 Jun;57(6):568-72.
40. Wang XB, Kakoulidou M, Giscombe R, Qiu Q, Huang D, Pirskanen R, *et al.* Abnormal expression of CTLA-4 by T cells from patients with myasthenia gravis: effect of an AT-rich gene sequence. J Neuroimmunol 2002 Sep;130(1-2):224-32.
41. Wong CK, Lit LC, Tam LS, Li EK, Lam CW. Aberrant production of soluble costimulatory molecules CTLA-4, CD28, CD80 and CD86 in patients with systemic lupus erythematosus. Rheumatology (Oxford) 2005 Aug;44(8):989-94.
42. Sato S, Fujimoto M, Hasegawa M, Komura K, Yanaba K, Hayakawa I, *et al.* Serum soluble CTLA-4 levels are increased in diffuse cutaneous systemic sclerosis. Rheumatology (Oxford) 2004 Oct;43(10):1261-6.
43. Oaks MK, Hallett KM. Cutting edge: a soluble form of CTLA-4 in patients with autoimmune thyroid disease. J Immunol 2000 May 15;164(10):5015-8.
44. Eugenin EA, Gamss R, Buckner C, Buono D, Klein RS, Schoenbaum EE, *et al.* Shedding of PECAM-1 during HIV infection: a potential role for soluble PECAM-1 in the pathogenesis of NeuroAIDS. J Leukoc Biol 2006 Mar;79(3):444-52.
45. Losy J, Niezgoda A, Wender M. Increased serum levels of soluble PECAM-1 in multiple sclerosis patients with brain gadolinium-enhancing lesions. J Neuroimmunol 1999 Oct 29;99(2):169-72.
46. Serebruany VL, Murugesan SR, Pothula A, Semaan H, Gurbel PA. Soluble PECAM-1, but not P-selectin, nor osteonectin identify acute myocardial infarction in patients presenting with chest pain. Cardiology 1999;91(1):50-5.
47. Lebbink RJ, van den Berg MC, de RT, Raynal N, van Roon JA, Lenting PJ, *et al.* The soluble leukocyte-associated Ig-like receptor (LAIR)-2 antagonizes the collagen/LAIR-1 inhibitory immune interaction. J Immunol 2008 Feb 1;180(3):1662-9.
48. Kacerovsky M, Drahosova M, Krejsek J, Musilova I, Hornychova H, Matula V, *et al.* Amniotic fluid CD200 levels in pregnancies complicated by preterm prelabor rupture of the membranes. J Matern Fetal Neonatal Med 2013 Mar 14.
49. Kramer BW, Moss TJ, Willet KE, Newnham JP, Sly PD, Kallapur SG, *et al.* Dose and time response after intraamniotic endotoxin in preterm lambs. Am J Respir Crit Care Med 2001 Sep 15;164(6):982-8.
50. Newnham JP, Moss TJ, Kramer BW, Nitsos I, Ikegami M, Jobe AH. The fetal maturational and inflammatory responses to different routes of endotoxin infusion in sheep. Am J Obstet Gynecol 2002 May;186(5):1062-8.
51. Smith R. Parturition. N Engl J Med 2007 Jan 18;356(3):271-83.
52. Lakwijk N, Van Strien RT, Doekes G, Brunekreef B, Gerritsen J. Validation of a screening questionnaire for atopy with serum IgE tests in a population of pregnant Dutch women. Clin Exp Allergy 1998 Apr;28(4):454-8.
53. Haddad R, Tromp G, Kuivaniemi H, Chaiworapongsa T, Kim YM, Mazor M, *et al.* Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature. Am J Obstet Gynecol 2006 Aug;195(2):394-24.
54. Diepenhorst GM, van Gulik TM, Hack CE. Complement-mediated ischemia-reperfusion injury: lessons learned from animal and clinical studies. Ann Surg 2009 Jun;249(6):889-99.

55. Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010 Sep;11(9):785-97.
56. Lenting PJ, Westerlaken GHA, Denis CV, Akkerman J.W., Meyaard L. Efficient inhibition of collagen-induced platelet activation and adhesion by LAIR-2, a soluble Ig-like receptor family member. Plos One 2010;5:e12174.
57. Fujiwara I, Nakajima H, Akioka K, Matsuda T, Oka T. Soluble complement receptor type 1 and antithrombin-III combination therapy prolongs xenograft survival: the role of thrombin and prostacyclin in hyperacute rejection. Transplant Proc 1997 Feb;29(1-2):935-7.
58. Ekdahl KN, Lambris JD, Elwing H, Ricklin D, Nilsson PH, Teramura Y, *et al.* Innate immunity activation on biomaterial surfaces: a mechanistic model and coping strategies. Adv Drug Deliv Rev 2011 Sep 16;63(12):1042-50.

&

APPENDICES

NEDERLANDSE SAMENVATTING VOOR NIET INGEWIJDEN

DANKWOORD

CURRICULUM VITAE

LIST OF PUBLICATIONS

NEDERLANDSE SAMENVATTING VOOR NIET INGEWIJDEN

INLEIDING

Ons lichaam wordt dagelijks blootgesteld aan vele ziekteverwekkers zoals bacteriën, virussen, schimmels en parasieten. Om zichzelf te beschermen beschikt het lichaam over een uiterst verfijnd verdedigingsmechanisme: het immuunsysteem. Een goed functionerend immuunsysteem houdt ons lichaam gezond door deze ziekteverwekkers op te ruimen. Het immuunsysteem bestaat uit twee gedeeltes: het aangeboren immuunsysteem en het verworven immuunsysteem. Beide delen van het immuunsysteem bevatten een cellulaire en humorale component. De cellulaire component wordt gevormd door de cellen die voor het immuunsysteem werken om ziekteverwekkers op te ruimen, bijvoorbeeld de witte bloedcellen. De humorale component wordt gevormd door eiwitten en enzymen die zich in lichaamsvloeistoffen zoals bloed bevinden. De eiwitten en enzymen kunnen ziekte verwekkers opruimen én de eerder genoemde cellen van het immuunsysteem aansporen om ziekteverwekkers op te ruimen.

Het aangeboren immuunsysteem

Het aangeboren immuunsysteem werkt hard om ziekteverwekkers buiten het lichaam te houden. Het herkent een groot aantal ziekteverwekkers en is snel geactiveerd. De huid en slijmvliezen van bijvoorbeeld de mond en neus vormen een eerstelijns afweer om ziekteverwekkers buiten het lichaam te houden. Mocht het een ziekteverwekker toch lukken om het lichaam binnen te dringen, dan wordt deze herkend door verschillende receptoren op de witte bloedcellen (de cellulaire component) van het aangeboren immuunsysteem. Voorbeelden van deze witte bloedcellen zijn: macrofagen, monocyten, dendritische cellen en neutrofiele granulocyten. Deze cellen ruimen ziekteverwekkers op door ze op te eten en te vernietigen met bijvoorbeeld zuurstof radicalen. Vervolgens kunnen sommige van deze witte bloedcellen van het aangeboren immuunsysteem, bijvoorbeeld de dendritische cellen en de monocyten, de witte bloedcellen van het verworven immuunsysteem activeren.

De humorale component van het aangeboren immuunsysteem speelt ook een grote rol in het opruimen van ziekteverwekkers. Een van de belangrijkste onderdelen van het humorale deel van het aangeboren immuunsysteem is het complement systeem. Deze groep van eiwitten die voorkomt in het bloed vormt een kettingreactie zodra een ziekteverwekker herkend wordt, zo'n ketting reactie wordt ook wel een cascade genoemd. Deze cascade kan ervoor zorgen dat de ziekteverwekker dood gaat, of dat de cellulaire component van het aangeboren (of zelfs het verworven) immuunsysteem de ziekteverwekker makkelijker kan herkennen en opruimen. Het complement systeem kan ook gestresste cellen (bijvoorbeeld cellen die een tijdje zonder zuurstof hebben gezeten) in het lichaam herkennen en opruimen.

Het verworven immuunsysteem

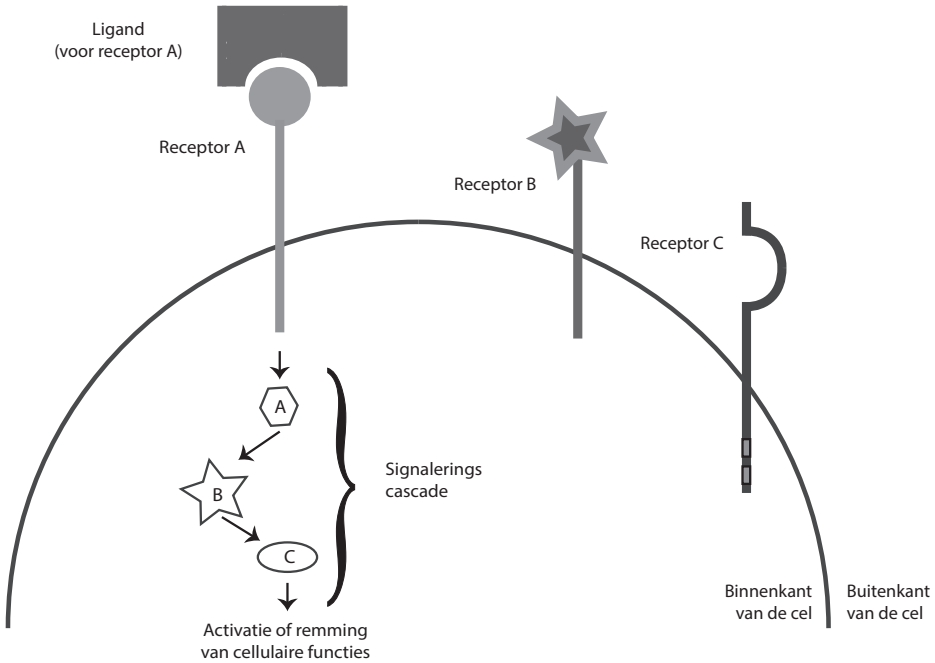
De cellen van het verworven immuunsysteem, de witte bloedcellen die ook vaak de T-cellen en B-cellen worden genoemd, worden geproduceerd door stamcellen in het beenmerg. Deze witte bloedcellen ontwikkelen zich verder in het beenmerg zelf, of in de zogenoemde lymfoïde organen: de thymus en de lymfeklieren.

Het verworven immuunsysteem heeft langer nodig om op gang te komen dan het aangeboren immuunsysteem en is pas effectief na ongeveer 7 tot 10 dagen nadat de ziekteverwekker herkend is door de witte bloedcellen van het aangeboren imuunsysteem. Zodra een ziekteverwekker herkend is, ontwikkeld zich een soort geheugen dat kenmerkend is voor het verworven immuunsysteem. Bij een volgende infectie met dezelfde ziekteverwekker zal de ziekteverwekker razendsnel overwonnen worden door het geheugen van het verworven immuunsysteem; het lichaam is immuun geworden voor deze ziekteverwekker.

De humorale component van het verworven immuunsysteem speelt een belangrijke rol in de immuniteit tegen ziekteverwekkers. B-cellen produceren eiwitten (antistoffen) die onderscheid kunnen maken tussen wat tot ons eigen lichaam behoort, en wat lichaamsvreemd is. Deze antistoffen kunnen aan ziekteverwekkers binden en zo een 'immuuncomplex' vormen. Deze immuuncomplexen kunnen witte bloedcellen of het complement systeem (van het aangeboren immuunsysteem) activeren, wat tot de verwijdering van de ziekteverwekker zal leiden.

BALANS

Het is van groot belang dat ziekteverwekkers zo snel mogelijk na het binnendringen van het lichaam worden opgeruimd. Wat echter net zo belangrijk is, is dat de reactie van het immuunsysteem, de immuunrespons, ook zo snel mogelijk na het opruimen van de ziekteverwekker beëindigd wordt. Sterker nog, al tijdens de immuunrespons moet de sterkte van de respons gereguleerd worden om schade aan het lichaam te voorkomen. Voor het opruimen van ziekteverwekkers zoals bacteriën en virussen worden stoffen aangemaakt (zoals zuurstof radicalen) die ook schade kunnen toebrengen aan het eigen lichaam. Om de juiste balans te vinden tussen de productie van schadelijke stoffen om de ziekteverwekker op te ruimen, en het belang van het lichaam om zichzelf te beschermen, zijn er activerende en remmende signalen die de immuunrespons reguleren. Witte bloedcellen bevatten receptoren die ziekteverwekkers kunnen detecteren en de immuunrespons in gang zetten, dit zijn activerende receptoren. Dezelfde witte bloedcellen bevatten ook remmende receptoren die de immuunrespons kunnen beperken. Als activerende of remmende receptoren in contact komen met bepaalde structuren in het lichaam (die we 'liganden' noemen), wordt er een cascade van signaleringseiwitten binnen in de cel aangezet, die de functie van de cel kunnen remmen (zie Figuur 1). Door de combinatie van activerende en remmende signalen ontstaat er een balans die ervoor zorgt dat de immuunrespons precies de juiste sterkte



Figuur 1: Receptoren op een cel. Witte bloedcellen kunnen verschillende receptoren tot expressie brengen, bijvoorbeeld receptor A, receptor B en receptor C. Als receptor A in aanraking komt met een structuur die precies bij de receptor past, ook wel een ligand genoemd, wordt de signalerings cascade van de receptor in werking gezet. Het resultaat van de signalerings cascade kan activatie of remming van een cel zijn, dat verschilt per receptor.

heeft om de ziekteverwekker op te ruimen terwijl er zo min mogelijk schade aan het eigen lichaam ontstaat. Als de balans tussen de activatie en de remming van de immuunrespons verstoord is, kun je daar last van krijgen. Als er te veel activatie of te weinig remming is, loopt het lichaam meer schade op door de immuunrespons dan door de ziekteverwekker en is er kans op de ontwikkeling van chronische ontstekingen of zelfs auto-immuunziekte ziekten. Bij te weinig activatie of te veel remming krijgen ziekteverwekkers de kans om grote infecties te veroorzaken en zullen kwaadaardige (kanker)cellen niet opgeruimd worden door het immuunsysteem. Het is dus van groot belang dat de regulatie van de immuunrespons op de juiste manier verloopt.

Inhibitoire immuunreceptoren

De remmende receptoren noemen we ook wel inhibitoire immuunreceptoren. Genetisch onderzoek heeft uitgewezen dat er waarschijnlijk ongeveer 300 van deze receptoren zijn. Van deze receptoren zijn er ongeveer 60 onderzocht en beschreven. Elke receptor herkent een bepaalde structuur (ligand), en kan op deze manier het functioneren van een witte bloedcel beïnvloeden. Niet alle receptoren komen op dezelfde cel voor. Er

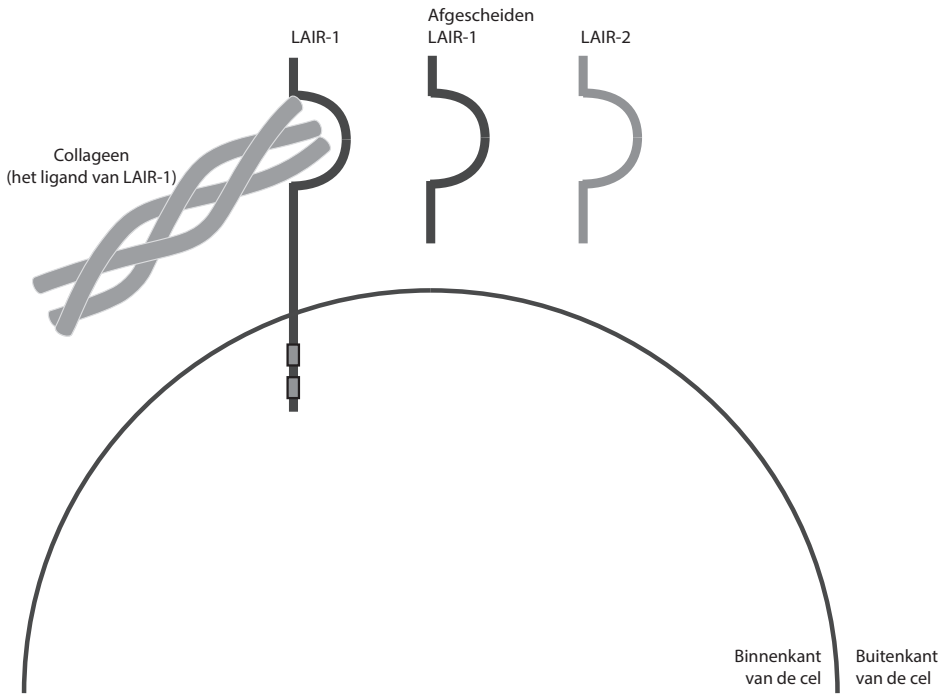
zijn receptoren die bijvoorbeeld alleen op B-cellen voorkomen, terwijl andere juist op een groot aantal verschillende cellen zoals monocyten, dendritische cellen, neutrofielen en T-cellen zitten. Elke specifieke witte bloedcel kan meerdere verschillende inhibitoire immuunreceptoren hebben. Het maken van deze (verschillende) receptoren door een cel noemen we 'tot expressie brengen'. Tijdens de uitrijping en activatie van cellen kan het aantal inhibitoire immuunreceptoren ook nog verschillen. De variërende expressiepatronen en de binding aan verschillende liganden die op diverse plekken in het lichaam voorkomen leiden tot een activatie van het immuunsysteem die precies op maat gemaakt is voor de situatie. Elke inhibitoire immuunreceptor heeft zo zijn eigen specifieke en unieke rol in de regulatie van het immuunsysteem.

DIT PROEFSCHRIFT

Het onderzoek in dit proefschrift richt zich vooral op de inhibitoire immuunreceptor genaamd 'Leukocyte-associated Ig-like receptor'-1 (afgekort: LAIR-1). LAIR-1 is een inhibitoire immuunreceptor die veel voorkomt op T-cellen, B-cellen, monocyten, macrofagen en nog een heel aantal andere witte bloedcellen. Een aantal jaar geleden is ontdekt dat LAIR-1 kan binden aan collageen en verschillende functies van de witte bloedcellen kan remmen. Collageen is een van de meest voorkomende eiwitten in in het lichaam en is een belangrijk onderdeel van het bindweefsel dat er voor zorgt dat alles in het lichaam op zijn plek blijft zitten. Het komt ook veel voor in de huid. Collageen is dus het ligand van LAIR-1. Behalve LAIR-1 dat op cellen tot expressie komt, bestaat er ook nog een variant van deze receptor die niet op cellen tot expressie komt. LAIR-2 lijkt erg op het deel van LAIR-1 dat aan de buitenkant van een witte bloedcel zit. Het grootste verschil is dat LAIR-2 een oplosbaar eiwit is dat door witte bloedcellen wordt uitgescheiden in lichaamsvloeistof. Verder is het ook nog mogelijk dat het deel van LAIR-1 wat buiten de cel zit, van de cel afgeknipt wordt. Dit afgeknipte gedeelte komt ook voor in verschillende lichaamsvloeistoffen zoals bloed en urine en noemen we afgescheiden LAIR-1 (zie Figuur 2). In dit proefschrift richten we ons op de vraag hoe deze verschillende LAIR eiwitten de immuunregulatie beïnvloeden.

In Hoofdstuk 1 geven we een korte introductie over het immuunsysteem en beschrijven we de doelstellingen van dit proefschrift.

In Hoofdstuk 2 kijken we kritisch naar de algemene aanname dat inhibitoire immuunreceptoren belangrijk zijn in het voorkomen (of het ontstaan) van auto-immuunziekten. De aanname is dat als een inhibitoire immuunreceptor niet goed functioneert, er niet genoeg remming is om het immuunsysteem in balans te houden en het immuunsysteem overactief zal zijn. Dit zou kunnen leiden tot een immuunreactie gericht tegen het eigen lichaam, zoals het geval is bij auto-immuniteit. Er zijn vele studies met genetisch gemodificeerde muizen die een of meerdere van deze receptoren missen die deze aanname onderschrijven. Muizen die een inhibitoire immuunreceptor missen zijn vaak vatbaarder voor ziektemodellen die veel gebruikt worden om



Figuur 2: De verschillende LAIR eiwitten. LAIR-1 komt voor op witte bloedcellen. Afgescheiden LAIR-1 en LAIR-2 komen voor in verschillende lichaamsvloeistoffen. Afgescheiden LAIR-1 bestaat uit het afgeknipte gedeelte van LAIR-1 dat aan de buitenkant van de cel zit. LAIR-2 is een oplosbaar eiwit dat door de cel wordt geproduceerd. Het ligand van LAIR-1 is Collageen. Afgescheiden LAIR-1 en LAIR-2 kunnen ook aan collageen binden.

auto-immuunziekten te bestuderen. Er is ook veel onderzoek gedaan naar mensen met auto-imuunziekten. Op witte bloedcellen van veel patiënten met reumatoïde artritis of systemische lupus erythematosus, worden afwijkingen in de expressie van verschillende inhibitoire immuunreceptoren gevonden. Daar komen we in Hoofdstuk 3 van dit proefschrift op terug. Soms kan men door middel van experimenten in het laboratorium aantonen dat de functie van bepaalde receptoren op witte bloedcellen van patiënten met een auto-immuunziekte niet goed is. Een gebruikelijke en betrouwbare manier om echt te kijken of deze receptoren belangrijk zijn voor auto-immuunziekten is door genetisch onderzoek te doen op enorme groepen patiënten en de uitkomsten daarvan te vergelijken met genetisch onderzoek op gezonde mensen. Op deze manier kunnen verschillen tussen het genoom van mensen met een auto-immuunziekte en gezonde mensen worden opgespoord. Zulke grote studies heten genoomwijde associatie studies. Een belangrijke kanttekening bij deze genoomwijde associatie studies is dat ze misschien niet geschikt zijn om een eventuele afwijking in

inhibitoire immuunreceptoren op te sporen. Er zijn zo veel verschillende receptoren die allemaal met remming van de cel en de immuunrespons te maken hebben dat het mogelijk is dat er mensen problemen hebben met verschillende receptoren, en toch hetzelfde eindresultaat (namelijk een overactief immuunsysteem) laten zien. De conclusie van dit hoofdstuk is dat we, op enkele uitzonderingen na, niet kunnen aantonen dat er belangrijke verschillen in inhibitoire immuunreceptoren zijn gevonden tussen patiënten met een auto-immuunziekte en gezonde mensen. Dit wil echter niet zeggen dat op individueel niveau deze receptoren geen belangrijke rol kunnen spelen. Bepaalde eiwitten die belangrijk zijn voor signalering van deze receptoren zijn wel verschillend tussen mensen met een auto-immuunziekte en gezonde mensen.

In Hoofdstuk 3 onderzoeken we de LAIR eiwitten die in lichaamsvloeistoffen voorkomen. We tonen aan dat activatie van het immuunsysteem leidt tot de productie van LAIR-2 door witte bloedcellen. LAIR-2 bindt ook aan collageen, en een lokale grote productie van LAIR-2 (zoals bijvoorbeeld in de gewrichtsvloeistof) zou de bindingsplaatsen van collageen kunnen bezetten, en daarmee voorkomen dat LAIR-1 er aan kan binden. Als LAIR-1 niet meer aan collageen kan binden worden de witte bloedcellen dus niet meer geremd. Op deze manier kan de activatie van het immuunsysteem lokaal geregeld worden. Aangezien activatie van het immuunsysteem tot de productie van LAIR-2 leidt, hebben we onderzocht of we de aanwezigheid van LAIR-2 en afgescheiden LAIR-1 (dat van de cellen is afgeknipt) konden meten in lichaamsvloeistoffen zoals urine en bloed en of we dat konden relateren aan de activatie status van het immuunsysteem. Daarnaast hebben we onderzocht of de hoeveelheden afgescheiden LAIR-1 en LAIR-2 in het bloed en urine van mensen met reumatoïde artritis verschilt van de hoeveelheid die we kunnen meten in het bloed en urine van gezonde mensen. De belangrijkste conclusies in dit onderzoek zijn dat reumatoïde artritis patiënten significant meer afgescheiden LAIR-1 en LAIR-2 in hun urine hebben dan gezonde mensen. Bovendien hield de hoeveelheid LAIR-2 in urine van reumatoïde artritis patiënten verband met de bezinkingssnelheid van rode bloedcellen, wat een belangrijke meetwaarde voor ontsteking is. Het is belangrijk om in de toekomst meer onderzoek naar afgescheiden LAIR-1 en LAIR-2 te doen in grotere groepen patiënten en gezonde mensen. Als afgescheiden LAIR-1 en LAIR-2 iets zeggen over de activatie status van het immuunsysteem kunnen we het misschien als een voorspellende waarde gebruiken. Auto-immuunziekten hebben vaak periodes van opleving van ziekteactiviteit en periodes waarin er een relatief rustiger ziektebeeld is. Als we door het eenvoudig meten van deze eiwitten in urine een periode van opleving van de ziekte zouden kunnen voorspellen, zou er eerder met de juiste medicijnen gestart kunnen worden, wat de patiënten een hoop narigheid zou besparen.

In Hoofdstuk 4 laten we zien dat we afgescheiden LAIR-1 kunnen meten in vruchtwater. Bij spontane bevallingen kunnen vaak grote hoeveelheden eiwitten, die samenhangen met immuunactivatie, in het vruchtwater worden gemeten. In dit hoofdstuk laten we zien dat de bron van het afgescheiden LAIR-1 in vruchtwater de foetale urine is.

Bij de kinderen van wie we de hoeveelheid afgescheiden LAIR-1 in het vruchtwater hebben gemeten is ook longfunctie onderzoek gedaan. De hoeveelheid afgescheiden LAIR-1 in vruchtwater hangt positief samen met de gemeten luchtwegweerstand van deze kinderen. Hoe hoger de hoeveelheid afgescheiden LAIR-1 des te beter de luchtwegweerstand. Wij concluderen dat de hoge hoeveelheid afgescheiden LAIR-1 in het vruchtwater een reflectie is van de activatie van het immuunsysteem (in de baarmoeder, voor de bevalling) en dat de hoeveelheid misschien iets zegt over de rijping van de longen van het kind.

We weten sinds een aantal jaren dat collagenen de eiwitten zijn die als ligand van LAIR-1 functioneren. Nadat LAIR-1 aan collageen heeft gebonden, wordt er een cascade van signaleringseiwitten in de cel in werking gezet die de functie van de cel vervolgens remmen. Er zijn in het lichaam nog tal van andere eiwitten die qua structuur op collageen lijken. Een van deze eiwitten is surfactant eiwit-D (afgekort: SP-D). SP-D komt onder andere voor in de longen en heeft onder andere als functie om het opeten van bacteriën door witte bloedcellen gemakkelijker te maken. SP-D speelt ook een belangrijke rol in de regulatie van het immuunsysteem, al is nog niet helemaal bekend op welke manier precies.

In Hoofdstuk 5 tonen we aan dat SP-D ook als ligand van LAIR-1 kan functioneren. We laten op verschillende manieren zien dat LAIR-1 aan SP-D kan binden en dat die binding afhankelijk is van het gedeelte van SP-D dat op collageen lijkt. Vervolgens laten we zien dat binding van LAIR-1 aan SP-D de cascade van signaleringseiwitten die voor de remming van de witte bloedcel kunnen zorgen in gang kan zetten; we laten dus zien dat SP-D als ligand voor LAIR-1 kan dienen. Tot slot laten we zien dat binding van SP-D aan cellen die LAIR-1 tot expressie brengen, de productie van zuurstofradicalen door die cellen kan verminderen. De zuurstofradicalen worden geproduceerd om ziekteverwekkers (doorgaans bacteriën) die het lichaam zijn binnengedrongen te doden. Te veel van deze zuurstof radicalen kunnen schade aan het eigen weefsel toebrengen. Waarschijnlijk dient de vermindering van de productie door LAIR-1 als bescherming voor de eigen weefsels. We hebben in Hoofdstuk 5 dus een nieuw ligand van LAIR-1 gevonden, wat tevens (voor een gedeelte) zou kunnen verklaren op welke manier SP-D het immuunsysteem reguleert.

Andere eiwitten met stukken die qua structuur op collageen lijken zijn C1q en MBL. Dit zijn twee van de belangrijkste schakels in de complement cascade, die aan het begin van deze samenvatting besproken is. Beide eiwitten staan aan de start van de cascade en zijn essentieel voor het functioneren van het complement systeem.

In Hoofdstuk 6 laten we zien dat LAIR-1 en LAIR-2 allebei aan C1q en MBL kunnen binden. Recent onderzoek heeft al aangetoond dat C1q als ligand voor LAIR-1 kan dienen, en wij breiden deze bevinding in dit hoofdstuk uit naar MBL. In dit hoofdstuk werken we ook met een aangepaste vorm van deze eiwitten. In plaats van met een enkel eiwit te werken, hebben we er 2 aan elkaar gemaakt. Zo krijg je dus een soort dubbele LAIR-1 en dubbele LAIR-2. Een belangrijke vondst in dit hoofdstuk is dat de

dubbele vorm van LAIR-2 zo stevig aan MBL en C1q kan binden dat deze eiwitten hun functie niet meer goed kunnen uitoefenen en de complement cascade wordt geremd. Nu is de complement cascade van essentieel belang om binnengedrongen ziekteverwekkers te bestrijden maar in bepaalde gevallen kan de complement cascade echter voor grote problemen zorgen. Als er een deel van het weefsel een tijdje zonder vers bloed, en dus zonder zuurstof is geweest (bijvoorbeeld na een hartaanval of een orgaantransplantatie) raakt het gestresst. Gestresste cellen brengen andere eiwitten tot expressie dan gezonde cellen, en het complement systeem speelt een belangrijke rol in het opruimen van deze gestresste cellen. Na een hartaanval of een orgaantransplantatie kan het complement systeem echter voor grote schade aan deze organen zorgen omdat het de gestresste cellen wil opruimen. Een getransplanteerd orgaan kan daardoor zelfs afgestoten worden door het lichaam. In deze gevallen zou het een groot voordeel voor patiënten zijn als we het complement systeem tijdelijk en lokaal konden remmen. Toekomstig onderzoek moet uitwijzen of LAIR-2 hiervoor geschikt is, maar het is een bijzonder interessante optie.

De resultaten van dit proefschrift worden bediscussieerd in Hoofdstuk 7. We plaatsten de resultaten in de context van huidig onderzoek en bespreken het belang van LAIR eiwitten in de immuunregulatie.

Onze bevindingen tonen aan dat de regulatie van het immuunsysteem op vele manieren geregeld wordt. Zelfs een enkele receptor zoals LAIR-1 kan daar op verschillende manieren aan bijdragen en LAIR eiwitten kunnen mogelijk gebruikt worden om het immuunsysteem te monitoren en zelfs te beïnvloeden. Ons onderzoek draagt bij aan het inzicht in immuunregulatie door inhibitoire immuunreceptoren.

DANKWOORD

Zo, dit is het dan. Het laatste stuk van het boekje dat geschreven moet worden. Dit is tevens een van de moeilijkste stukken. Er hebben zo veel mensen op zo veel verschillende manieren iets bijgedragen aan dit boekje, dat het moeilijk is om iedereen te noemen. Mocht je een bijdrage hebben geleverd aan dit boekje (direct of indirect), en ik noem je niet persoonlijk: Sorry! Vul hier je naam in, en je staat erbij:

_____, bedankt voor alles!

Laat ik beginnen aan de start van mijn wetenschappelijke carrière:
Timo, bedankt dat ik in jouw groep mijn eerste stage mocht lopen. Ik heb ontzettend veel van je geleerd, en wil je daar graag voor bedanken. Zoals je kunt zien, heeft mijn stage in het veld van inhibitoire immuun receptoren grote indruk gemaakt, en heb ik nog jaren lang met heel veel plezier aan dit onderwerp gewerkt. Ellen en Karin, jullie ook bedankt voor de goede begeleiding, de gezelligheid en alle tips over het werken op een lab.

Jos, bedankt dat ik mijn tweede stage bij jou mocht lopen. Ik heb ontzettend genoten van de tijd op het lab, de gezellige etentjes met de groep en de lunches bij het pannenkoekenhuis. Bedankt dat ik nog een tijdje als analist in je lab mocht werken nadat ik afgestudeerd was, zodat ik rustig kon zoeken naar een geschikte promotieplek. Alle gesprekken die we over de toekomst gevoerd hebben, hebben er zeker aan bijgedragen dat ik met het volste vertrouwen de keuze heb gemaakt om te gaan promoveren. Andreas, bedankt voor de dagelijkse begeleiding in het lab.

Na mijn stages ging ik op zoek naar een leuke promotie plek bij een gezellige groep. Zo kwam ik bij jou terecht, Linde. Ontzettend bedankt voor al je hulp bij de totstandkoming van dit boekje. Ik had me de afgelopen jaren geen betere promotor kunnen wensen. Je enthousiasme werkte aanstekelijk en je eindeloze stroom van ideeën zorgden ervoor dat we de meeste projecten tot een prachtig einde hebben kunnen brengen. Je bijzondere Engelse opmerkingen tijdens de werkbesprekingen (zoals: 'There comes the monkey out of the sleeve' of 'That's too short through the bend') zorgen ervoor dat ik straks in Oxford goed beslagen ten ijs kom. :) Bedankt voor alle gezellige feestjes en etentjes (vaak bij jou thuis) die de banden in de groep versterkten en altijd voor een leuke sfeer zorgden. Eigenlijk gewoon: bedankt voor alles!

Alle mensen van de afdeling Immunologie (tegenwoordig LTI), ontzettend bedankt voor de goede sfeer op de afdeling, de interesse in elkaars werk en de werkbesprekingen met vaak vele hulpvaardige tips en goede adviezen. Ik heb de afgelopen jaren met heel veel

plezier op deze afdeling gewerkt, en vele mensen hebben daar aan bijgedragen. Al kan ik niet iedereen afzonderlijk noemen; allemaal bedankt!

Een aantal mensen wil ik graag in het bijzonder noemen:
Groep Linde: zonder al mijn leuke, gezellige collega's had ik lang niet zo veel plezier in mijn werk gehad. Bij deze wil ik dan ook de oud-leden van groep Linde bedanken: Tomek, Mirjam, Femke, Ellen, Tessa, Talitha, Willemien, Nathalie en Guru, bedankt voor jullie hulp met van alles en nog wat en alle gezelligheid. De huidige leden van groep Linde: Margreet, Michiel, Kristof, Amelia (voor ons hoor je nog steeds bij groep Linde), Maarten, Louis, Marije en Anita; bedankt voor alle lol op het lab, de gezellige werkbesprekingen (vaak met koekjes en taart zonder dat we daar een goede reden voor konden verzinnen) en de leuke uitjes. Zonne en Saskia, mijn studenten, bedankt voor de lol die we in de weken van jullie stage hebben gehad. Ik vond het erg leuk dat ik jullie begeleider mocht zijn. Ik hoop dat jullie in de toekomst net zo veel lol in jullie werk zullen hebben als ik de afgelopen jaren heb gehad. Alle studenten die de afgelopen jaren groep Linde versterkt hebben (met in het bijzonder Carien en Luuk, de huidige studenten): bedankt! Jullie hebben altijd een grote bijdrage geleverd aan de gezelligheid en de sfeer in de groep. Guru and Kristof: It was nice to do our PhD together; I hope we'll stay in touch. Michiel, ik zal bij elk Hammond orgeltje aan je denken. Amelia, bedankt voor al je leuke verhalen en gezelligheid! Mirjam, zonder jou was het niet meer hetzelfde op het lab. Ik vond het super gezellig om bench buurtjes te zijn, en ben blij dat we zulke goede vriendinnen zijn. Femke, bedankt voor alles gezelligheid in ons U-tje! Louis, ik vind het erg leuk dat we als co-auteurs op twee van de hoofdstukken in mijn proefschrift staan, bedankt voor al je hulp en advies (ook bij de andere hoofdstukken). Ik heb veel van je geleerd en ben blij dat ik altijd met al mijn (statistiek) vragen bij je terecht kon. Margreet, bedankt voor alles wat we de afgelopen jaren samen hebben gedaan, ondanks dat we officieel niet samenwerkten. Ik vond het ontzettend leuk dat we altijd zo goed op de hoogte waren van elkaars proeven, en dat we daarom vaak voor elkaar konden bijspringen. Bedankt voor alle lol en de gezellige gesprekken. We gaan onze vrijdagmiddag mails gewoon volhouden hoor! Zonder jou waren de afgelopen jaren niet wat ze zijn geweest. Ik ben ontzettend blij dat je mijn paranimf wilt zijn!

Mijn lieve kamergenootjes (en oud-kamergenootjes) hebben met de vele kopjes thee, koekjes en snoepjes, luisterende oren, advies, gezellige kamer-etentjes, leuke verhalen, gesprekken over series en gedeelde frustraties ook een belangrijke bijdrage geleverd aan mijn beleving van de afgelopen jaren. Nening, Vera, Kirsten, Michiel, Jennifer, Dan, Kim, Tomek, Guru, Ana, Marieke en Ineke (ook al waren we maar heel even kamergenootjes): ontzettend bedankt voor de afgelopen jaren. Ik ga jullie verschrikkelijk missen.

Alle mensen op het leuke, drukke kleine lab: bedankt voor alle lol die we op het lab hebben gehad, de gezellige gesprekken over en weer tussen de verschillende U-tjes

(bij deze: sorry groep Jeanette dat jullie daar vaak klem tussen zaten) en alle advies. Peter en Marco, bedankt voor alle hulp met het plannen (en het uitvoeren van) de muizenproeven. Helaas hebben ze het niet gehaald tot in de paper, maar ik vond het een fijne samenwerking en ben blij dat jullie co-auteurs op het stuk zijn. Maaike, mijn leuke overbuurvrouw, bedankt voor alle gezelligheid.

Ik wil graag Kiki en Erik (Kalkhoven) bedanken dat ze mijn AIO commissie wilden vormen. Joël, zonder jou had ik het nooit aangedurfd om hoofdstuk 3 naar Arthritis en Rheumatism te sturen! Bedankt voor je optimisme, hulp, advies, samples en de fijne samenwerking. Max, ook al hebben we nooit tegelijk in groep Linde gewerkt, ik vind het geweldig dat een deel van jouw stageverslag als idee en basis kon dienen voor hoofdstuk 3. Bedankt!

Erik (Hack), bedankt voor je onmisbare bijdrage aan hoofdstuk 6. Zonder jouw expertise en advies was het nooit zo'n mooi stuk geworden! Ik ben blij met je samengewerkt te hebben. Jef, zonder jou waren een groot deel van de proeven in hoofdstuk 6 er niet geweest. Bedankt voor je inzet, hulp, snelle reacties op e-mailtjes en optimisme. Ik vond het erg leuk om met je samen te werken! Jeanette, bedankt voor de hulp bij hoofdstuk 6!

Michiel (Houben), bedankt voor de mooie samenwerking op hoofdstuk 4. Dank voor al je harde werk om het tot een mooi stuk af te ronden!

Sigrid en Gerrit, bedankt voor alle hulp bij verschillende soorten problemen. Het was super handig dat jullie altijd overal wel een oplossing voor wisten te verzinnen!

Saskia en Yvonne, enorm bedankt voor al jullie advies! Of het nu om formulieren ging of om andere praktische zaken, jullie stonden altijd met raad en daad klaar en dat heb ik enorm gewaardeerd.

Behalve samenwerkingen met mensen van de afdeling LTI, heb ik ook heel veel te danken aan samenwerkingen met mensen van andere afdelingen.

Rolf, bedankt dat je me hebt leren werken met de BIAcore en voor al je advies en tips over het reilen en zeilen op het lab. Ik heb ontzettend veel van je geleerd, en ben blij dat ik met je heb mogen samenwerken. Dat het een bijzonder vruchtbare samenwerking was, blijkt wel uit het feit dat je co-auteur bent op 3 hoofdstukken uit mijn proefschrift!

Martin en Henk, zonder jullie was hoofdstuk 5 er niet geweest! Bedankt voor de bijzondere samenwerking, alle input, adviezen en medeleven. Ik vond het enorm gezellig om met jullie samen te werken! Martin, weinig mensen zullen zo'n snelle, efficiënte en toch gezellig communicatie hebben gehad als wij.

Bobby, dank voor al het harde werk om hoofdstuk 2 tot een goed einde te brengen! Je input was onmisbaar, en ik ben heel blij dat het zo'n mooi stuk is geworden.

Ik wil ook al mijn lieve vrienden bedanken die indirect ook zeker hebben bijgedragen aan dit boekje.

Flavio, bedankt voor al je steun en enthousiasme. Ook al zien we elkaar niet meer zo vaak, ik vind het heerlijk hoe we altijd aan een half woord genoeg hebben. Bedankt dat je er altijd voor me bent, je bent een schat!

Maarten en Lieke, ik kan niet eens meer tellen hoe vaak we de afgelopen jaren wel niet bij elkaar hebben gegeten. Ik vond het altijd super gezellig om het wel en wee op het lab met jullie te mogen delen, en naar jullie verhalen te luisteren. Jammer dat jullie niet mee verhuizen naar Oxford... ik ga jullie verschrikkelijk missen! Ontzettend bedankt voor alle gezelligheid en steun de afgelopen jaren!

Sietske, bedankt voor wie je bent en hoe goed je me kent, voor alle goede gesprekken en je vriendschap!

Lieneke en Gwen, dank voor alle gezellige kopjes thee en etentjes waarin ik mijn hart kon luchten. Het was fijn en herkenbaar om te horen dat er op elk lab dezelfde frustraties spelen.

Henk, Rina en Esther, door de gezelligheid in de bus leek het stuk van Amersfoort naar Utrecht altijd veel korter dan het was. Bedankt voor alle leuke gesprekken!

Marleen, zullen we proberen eerder bij te kletsen dan over 3 jaar in de muntenkassa?

Alle vrienden uit Haaksbergen (en omgeving): Tim, Kjeld, Joost, Luke & Inge (en Jesse & Aiden), Ronnie & Angelica, Sebass & Asankha en Mark & Marion, bedankt voor alle gezellige etentjes, barbecues en verjaardagen! Zonder jullie waren de weekenden en feestdagen de afgelopen jaren een stuk saaier geweest!

Zonder mijn familie was ik niet geweest waar (en wie) ik nu ben. Ik wil iedereen bedanken voor het luisteren naar al mijn verhalen (ook al hadden jullie vast vaak geen idee waar ik het over had) en de eindeloze interesse.

Familie Tebroke, bedankt voor de gezellige familiedagen, het eieren zoeken en alle andere leuke dingen die we samen doen. Ik heb nu al zin in het volgende kerstdiner en ben benieuwd wie er wat zal maken.

Familie Olde Nordkamp, bedankt voor de gezellige maandag middagen op de kermis, de leuke familiedagen, verjaardagen en alle andere ongein! Rene, bedankt dat je zo'n geweldige peetoom bent! Alwie en Agnes, bedankt voor alle peultjes! Erik, bedankt voor alle gezellige, ontspannen zaterdagen buiten bij de dieren en de uitjes naar van der Poel voor de nodige verkoeling.

Familie Pietersen, ik had me geen leukere, gezelligere schoonfamilie kunnen wensen. Theo en Mariëtte, bedankt voor al jullie belangstelling de afgelopen jaren. Heerlijk dat ik bij jullie zo 'thuis' ben. Jullie zijn schatten! Saskia en Erwin (en Wout, Koen en Lieke), bedankt voor alle gezelligheid! Het is altijd leuk om bij jullie te zijn!

Dana, je bent mijn grote zus en mijn beste vriendin. Ik heb zo veel respect voor jouw kracht en de manier waarop je in het leven staat. Je bent een bron van inspiratie. Bedankt voor je luisterende oor, al je advies, en het corrigeren van de Nederlandse delen in dit boekje. Ik ga je niet missen de komende jaren omdat we zo veel contact gaan houden! Edwin, je bent een super leuke zwager (eigenlijk meer een grote broer). Bedankt voor al jullie interesse in mijn werk en de eindeloze kopjes thee die we samen gedronken hebben.

Line, mijn lieve petekindje. Je bent echt een schatje. Wie had gedacht dat zo'n klein meisje zo vertederend kon zijn?

Michel, bedankt dat je zo'n geweldige broer bent! Super fijn dat er altijd iemand was die wel begreep waar ik het over had, als ik weer eens iets over het lab kwijt moest. Ik ben ontzettend trots op het feit dat je zo goed bent in wat je doet en ben heel blij dat je mijn paranimf wilt zijn! Sanne, mijn leuke schone zus, bedankt voor het luisteren naar al mijn verhalen, en alle tips over taarten bakken en versieren!

Pap en Mam, jullie zijn fantastische ouders en ik hou ontzettend veel van jullie! Bedankt dat jullie altijd achter me staan en me stimuleren om het beste uit mezelf te halen! Ik hoop dat ik ooit zo slim als jullie zal worden!

En dan het lekkerste voor het laatste: Marco, zonder jou had ik dit niet gekund. Ontzettend bedankt voor al je liefde en je steun. Ik weet dat jij de afgelopen jaren degene was die er het meeste last van had wanneer ik moe of druk was. Bedankt dat je daar zo goed mee om bent gegaan. Ik geniet altijd enorm van alle leuke dingen die we samen doen en zie uit naar ons avontuur in Oxford. Je bent een schat (mijn schat). Ik hou van je...

CURRICULUM VITAE

Marloes Olde Nordkamp werd op 12 november 1983 geboren in Hengelo. Na het behalen van haar HAVO diploma aan het Assink Lyceum in Haaksbergen begon zij in 2001 aan de leraren opleiding Biologie aan het Windesheim te Zwolle. In 2005 behaalde zij haar Bachelor of Education en begon ze aan een pre-master jaar aan de Vrije Universiteit te Amsterdam. In 2006 startte zij daar haar Master studie in Biomedische Wetenschappen, met als specialisaties Immunologie en Infectieziekten. Haar eerste stage volgde zij bij dr. Timo van den Berg bij de afdeling Blood Cell research van Sanquin te Amsterdam. Het onderzoek aan de inhibitoire immuun receptor SIRP-α was haar kennismaking met het veld van inhibitiore immuun receptoren. Haar tweede stage over het effect van small molecule inhibitors op *Campylobacter* voerde ze uit bij Prof. dr. Jos van Putten van de divisie infectiebiologie aan de faculteit diergeneeskunde van de Universiteit van Utrecht. Na het behalen van haar Master of Science in 2008 keerde zij in 2009 terug naar het veld van de inhibitoire immuun receptoren en starte zij haar promotie onderzoek getiteld 'Immune regulation by LAIR proteins' aan de afdeling Immunologie van het Universitair Medisch Centrum Utrecht, onder begeleiding van Prof. dr. Linde Meyaard. De bevindingen van haar onderzoek zijn beschreven in dit proefschrift. Tegenwoordig werkt ze als postdoctoraal onderzoeker in de groep van Prof. dr. Alison Banham aan de Nuffield Division of Clinical and Laboratory Sciences aan de Universiteit van Oxford, waar zij zich in samenwerking met dr. Demin Li richt op de ontwikkeling van een vaccin tegen borstkanker.

LIST OF PUBLICATIONS

Enhanced secretion of Leukocyte-Associated Immunoglobulin-like Receptor (LAIR)-2 and soluble LAIR-1 in Rheumatoid Arthritis: LAIR-2 is a more efficient antagonist of the LAIR-1-collagen inhibitory interaction than is soluble LAIR -1
Marloes J.M. Olde Nordkamp, Joël A.G. van Roon, Max Douwes, Talitha de Ruiter, Rolf T. Urbanus and Linde Meyaard
Arthritis and Rheumatism 2011 Dec;63(12):3749-57

Do Inhibitory Immune Receptors play a role in the Etiology of Autoimmune Disease?
Marloes J.M. Olde Nordkamp, Bobby P. Koeleman and Linde Meyaard
Submitted

sLAIR-1 in Amniotic Fluid is of Fetal origin and positively associates with Lung Compliance
Michiel L. Houben, Marloes J.M. Olde Nordkamp, Peter Nikkels, Cornelis K. van der End, Linde Meyaard and Louis J. Bont
Submitted

Leukocyte Associated Ig-like Receptor-1 is a novel Inhibitory Receptor for Surfactant Protein D
Marloes J.M. Olde Nordkamp, Martin van Eijk, Rolf T. Urbanus, Louis Bont, Henk P. Haagsman and Linde Meyaard
Submitted

Inhibition of the Classical and Lectin pathway of the Complement System by recombinant LAIR-2
Marloes J.M. Olde Nordkamp, Peter Boross, Cafer Yildiz, J.H. Marco Jansen, Jeanette H.W. Leusen, Rolf T. Urbanus, C. Erik Hack and Linde Meyaard
Submitted