Immune inhibitory receptors: regulated expression and suppression

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Immune inhibitory receptors: regulated expression and suppression

Immuun inhibitoire receptoren: gereguleerde expressie en suppressie

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

General Introduction

CHAPTER 1

The immune system protects against disease by identifying and eliminating pathogens and tumor cells, while leaving healthy host cells unaffected. Immune responses should only be initiated when necessary and require timely termination, since inappropriate immune activation can result in persistent inflammatory diseases or autoimmunity.[1;2] To prevent improper immune activation it is crucial that immune cells can distinguish between healthy host tissue and transformed cells or pathogens. Signals generated through multiple activating receptors may be integrated to mount a potent immune response.[3] Such activating receptors include pattern recognition receptors (PRR), such as Toll-like receptors (TLR) and c-type lectins, which recognize pathogen-associated molecular patterns (PAMPs) and induce among others inflammatory cytokine production. Furthermore, pathogens may be opsonized by complement and antibodies, and resulting immunoglobulin (Ig) complexes can activate Fc Receptors (FcR).[4] FcR are expressed on multiple leukocyte subsets and are involved in many cellular processes, such as phagocytosis, degranulation, oxidative burst, cytokine production and antibody-dependent cell-mediated cytotoxiticy.[5] Activating natural killer (NK) cell receptors, including natural cytotoxicity receptors (NRC), NKG2D and 2B4, lead to cytotoxic killing of virally infected cells.[3;6] The T cell receptor recognizes presented antigens and mediates an immune response, which can consist of inflammatory cytokine production and cytotoxicity. Finally, the B cell receptor recognizes foreign antigens and mediates an immune response consisting of cytokine and antibody production. Many of these immune receptors signal through immunoreceptor tyrosine-based activation motifs (ITAMs), including the T and B cell receptor,[7] NK cell receptors,[8] c-type lectins[9] and FcR.[5] Importantly, the immune response resulting from collective activation signals is kept in check by specific inhibitory signals, together leading to effective defense against pathogens without damaging healthy tissues.[2]

Just as multiple activating signals are integrated to mount an immune response, multiple regulatory mechanisms are required to prevent excess or inappropriate immune cell activation and to ultimately terminate the immune response, thereby restoring homeostasis. Regulatory mechanisms include production of anti-inflammatory cytokines such as transforming growth factor-β (TGF-β) and interleukin-10 (IL-10), immune cell apoptosis, and expression of immune inhibitory receptors. The latter group of regulators suppresses activation signals initiated by immune receptors.

Thus far, approximately 60 immune inhibitory receptors have been described,[10] each of which has a specific expression pattern. Some are broadly expressed, whereas others are expressed by only a specific leukocyte subtype. The expression of such inhibitory receptors

is also regulated during the immune response. Inhibitory receptors that are up-regulated during the immune response may regulate its termination, while receptors that are highly expressed on immune cells in steady-state conditions may create an immune activation threshold.

In general, inhibitory receptors require interaction with cognate ligand before mediating their inhibitory effects. Upon ligand binding, immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the cytoplasmic tail are phosphorylated by Src family tyrosine kinases, which also phosphorylate and activate SH2 domain-containing phosphatases SHP-1, SHP-2 or SHIP. These phosphatases are subsequently recruited and can mediate suppression of cellular activation by dephosphorylation of effector molecules,[11] although recruitment of phosphatases is not always a prerequisite for inhibitory receptors to mediate cellular inhibition.[12] While signaling through several types of activating receptors, including TLRs and cytokine receptors, may be inhibited by ITIM-bearing receptors, the inhibitory capacity of ITIM-bearing receptors in inhibiting ITAM signaling is particularly well established.[11]

The first inhibitory receptor to be described was FcγRIIb, which is the only FcR family member containing an ITIM.[10] Co-ligation of FcγRIIb with activating FcR or with the B cell receptor inhibits activating signals relayed through these receptors.[13] Thereafter, research focused on inhibitory receptors on NK cells. An important study described that NK cells can kill tumor cells that lack major histocompatibility complex (MHC) I, while sparing the same cells when expressing MHC I.[14] This observation led to the formulation of the 'missing self' hypothesis, which states that NK cells attack cells that do not express autologous MHC I, while ignoring cells that express normal levels of MHC I.[14] The identification of MHC I-binding inhibitory receptors provided the molecular mechanism behind the 'missing self' hypothesis, upon ligand binding these receptors could inhibit NK cell cytotoxicity.[15] The first MHC-specific inhibitory receptors to be discovered were the c-type lectin Ly49 receptors in rodents, while the killer cell Ig-like receptors (KIRs) family were the first identified MHC-specific receptors in humans.[15] Subsequently, the Leukocyte Ig-like receptors (LILRs, also known as Ig-like transcripts (ILTs)) were identified as MHC I-binding receptors.[16-20] Other immune inhibitory receptors have different types of ligands. For example, the Sialic-acid-binding Ig-like lectins (Siglecs) bind Sialic acids,[21] platelet endothelial cell adhesion molecule (PECAM)-1 and carcinoembryonic antigen-related cell adhesion molecule1 (CEACAM1) are involved in homophilic interactions, and KLRG1 binds Cadherins.[22] For many other immune inhibitory receptors the ligand remains to be identified.

Although inhibitory receptors all signal through ITIMs and recruit similar phosphatases, their function is non-redundant. This is best demonstrated by studies using immune inhibitory receptor knock-out mice, which are often prone to autoimmune disease, or develop an uncontrolled and frequently lethal inflammation in response to infection. For example, FcγRIIb deficient mice are susceptible to autoimmune disease that resembles systemic lupus erythematosus (SLE) and show increased disease severity in mouse models of arthritis.[13] PD-1, an inhibitory receptor specifically expressed on activated T cells, is a negative regulator of autoimmunity. C57BL/6 mice deficient for PD-1 develop progressive arthritis and SLE-like glomerulonephritis disease, while NOD mice deficient in PD-1 develop accelerated autoimmune diabetes.[23] Mice deficient for another T cell-specific inhibitory receptor, CTLA-4, develop lymphoproliferative disease and die within 3–4 weeks of birth.[23] Finally, mice deficient in CD200, the ligand for CD200R, succumb to enhanced, fatal inflammation after influenza infection, which may be due to excessive leukocyte activation.[24;25] Specificity is achieved through regulated expression of the immune inhibitory receptors, expression of the ligand, recruitment of specific phosphatases and the affinity with which these are recruited. In addition, some inhibitory receptors recruit alternative molecules to mediate the inhibitory effect. For example, in addition to SHP-1 and SHP-2, LAIR-1, SIRP-α and LILRB1 can recruit C-terminal Src kinase (Csk)[12;26;27] and CD33 and Siglec-7 can recruit suppressor of cytokine signaling 3 (SOCS3).[21] CD200R does not contain ITIMs, but instead is capable of recruiting the adapter proteins Dok-1 and Dok-2 to its phosphorylated tyrosines.[28] Recruitment of these additional molecules may play a role in determining specific receptor output.

Research has traditionally focused on studying the function of inhibitory receptors on NK, B and T cells, whereas less attention is has been paid to the expression of inhibitory receptors on phagocytes such as neutrophils and macrophages. Importantly, a number of inhibitory receptors have recently been identified on phagocytes, and there may have an equally important regulatory role in the activation of these cells. This thesis centers on the characterization and function of the novel immune inhibitory receptor termed signal inhibitory receptor on leukocytes-1 (SIRL-1), expressed exclusively on phagocytes, while the second section focuses on the expression and function of the inhibitory receptor leukocyte-associated Ig-like receptor-1 (LAIR-1).

The Leukocyte Receptor Complex

A number of immune inhibitory receptor families have now been identified and characterized. Most inhibitory receptors, including LAIR-1, are type I transmembrane proteins and Ig superfamily (IgSF) members. Other groups consist of Siglecs[21] and type II transmembrane proteins, which include c-type lectins.[8] LAIR-1 consists of 287 amino acids, contains a single extracellular Ig-like domain[29] and is structurally related to several other inhibitory IgSF members, including KIRs and LILRs.[29] *LAIRs, KIRs and LILRs* are all localized to the leukocyte receptor complex (LRC) on human chromosome 19q13.4, suggesting that these molecules have evolved from a common ancestral gene.[30;31] In mice, chromosome 7 is the syntenic chromosome of human chromosome 19q13.4, containing *LAIR* and *paired Ig-like receptors (PIRs)*. LRC regions in both mice and men show extensive gene duplications and a considerable degree of genetic polymorphism.[31;32] Duplication and evolutionary divergence of an ancestral gene have also been shown for other multigene families, including MHC I genes.[32] The fact that many of the divergent, duplicated genes have remained functional indicates this has been influenced by positive selection processes,[32] a functionally diverse balanced repertoire of inhibitory receptors apparently contributes to a regulated immune response.

Leukocyte-Associated Ig-like Receptor-1

LAIR-1 is expressed on a majority of peripheral blood leukocytes, including NK cells, T cells, B cells, monocytes, eosinophils and dendritic cells.[29;33] Activation of T cells results in a down-regulation of plasma membrane LAIR-1 expression,[34] whereas cell surface LAIR-1 expression on neutrophils is induced upon G-CSF stimulation,[33] and is absent from neutrophils in steady-state conditions. LAIR-1 is also expressed on CD34+ hematopoietic progenitor cells.[33] The function of LAIR-1 on these cells is unclear.

LAIR-1 contains two ITIMs, and receptor cross-linking results in ITIM phosphorylation by Src family kinases and recruitment of the SH2 domain-containing phosphatases SHP-1, SHP-2 and the Csk.[12] *In vitro*, a strong LAIR-1-mediated inhibition of cytotoxicity and calcium mobilization has been demonstrated in NK cells, T cells and B cells respectively.[29;35;36] In addition, LAIR-1 may inhibit dendritic cell differentiation *in vitro*.[37]

We have recently demonstrated that collagens are high-affinity ligands for LAIR-1, and that binding of collagen to LAIR-1 results in inhibition of FcγRI-induced degranulation in a basophilic cell line.[38] Collagens are among the most abundant proteins in the human

body.[39] While not normally exposed in the vasculature, collagens may become so after tissue damage or mechanical injury and they have usually been studied in the context of thrombosis and hemostasis.[40] Since the bone marrow environment is also rich in collagens, the expression of LAIR-1 on CD34+ hematopoietic stem and progenitor cells is of particular interest. LAIR-1 expressed on these cells could be constitutively active, as hematopoiesis takes place in the bone marrow. So far, the role of LAIR-1 on hematopoietic progenitor cells has not been elucidated, and its expression has only been studied in detail during granulocyte differentiation, where LAIR-1 expression decreases during differentiation.[33]

While several activating collagen receptors have been recognized on mammalian cells,[41] LAIR-1 is the only inhibitory collagen receptor identified thus far. Activating collagen receptors include $\alpha_2\beta_1$ and glycoprotein VI (GPVI),[41] both of which play a crucial role in platelet aggregation in response to collagen.[42] Although the intracellular tail of GPVI associates with the ITAM-containing FcRγ,[43-45] GPVI is structurally related to LAIR-1[38] and the genes encoding LAIR-1 and GPVI are both localized to the LRC region. The genomic proximity and structural homology between the two receptors suggest that LAIR-1 and GPVI have a common origin and the collagen-binding site in LAIR-1 and GPVI overlaps between the two receptors.[46-48] Furthermore, LAIR-1 cross-linking abrogates collagen-induced GPVI signaling when both receptors are ectopically expressed on the same cell.[49] Thus, co-expression of both collagen receptors on primary cells could determine their responsiveness to collagen. However, since GPVI is regarded as a platelet-specific receptor and LAIR-1 is broadly expressed on leukocytes, GPVI and LAIR-1 expression appear mutually exclusive. A detailed analysis of LAIR-1 and GPVI expression during megakaryocytopoiesis could perhaps result in the identification of a cell type that expresses both collagen receptors.

Several splice variants of the LAIR-family have been identified (Figure 1).[50] LAIR-1b lacks 17 amino acids in the stalk region between the transmembrane and Ig-like domain compared to full-length LAIR-1a, LAIR-1c is identical to LAIR-1b except for a single amino acid change in the extracellular domain, and LAIR-1d lacks part of the intracellular tail. LAIR-2a and LAIR-2b are both soluble proteins, lacking the transmembrane and intracellular regions of LAIR-1.[29] Surprisingly few studies have investigated the function of splice variants of immune inhibitory receptors, while regulated splicing can have important functional consequences.[51] It would therefore be of particular interest to investigate the specific expression pattern and function of the LAIR-1 splice variants. So far, differential expression of LAIR-1a and LAIR-1b has only been demonstrated in NK and T cells,[35] and the biological relevance

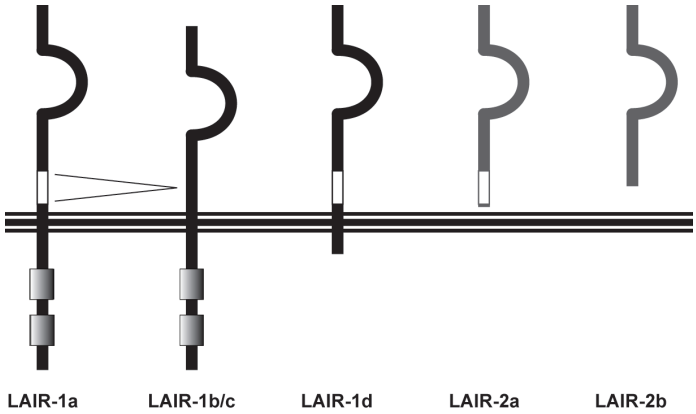

Figure 1. Several splice variants of LAIR-1 (black) and LAIR-2 (grey) have been reported. LAIR-1a differs from LAIR-1b by the presence of 17 amino acids located in the extracellular region (indicated in white). LAIR-1c is identical to LAIR-1b except for a single amino acid change in the extracellular domain (not depicted). LAIR-1d lacks part of the intracellular tail and contains no ITIMs (indicated as grey boxes). LAIR-2a and LAIR-2b are both soluble proteins, lacking the transmembrane and intracellular regions.

of these findings remains unclear as it is not known whether LAIR-1a and LAIR-1b are distinct with regard to collagen binding and collagen-induced signaling capacity. Further studies are required to investigate whether LAIR-1 splice variants are distinct with regard to expression pattern and function.

Signal Inhibitory Receptor on Leukocytes-1

Utilizing an *in silico* search for novel inhibitory receptors we identified SIRL-1, the presence of conserved ITIM sequences suggesting it was a functional inhibitory receptor. SIRL-1 is a type I transmembrane protein and IgSF member, with LAIR-1b being its closest homolog, although the homology is not extensive (30% overall identity). *VSTM1*, which encodes SIRL-1 is located close to the LRC region of human chromosome 19q13.4, which as already discussed encodes many other ITIM-bearing receptors. The relationship between SIRL-1, LAIRs, GPVI and several LILR and KIR family members can be visualized in a phylogenetic tree (Figure 2).

The human LRC region is also rich in uncharacterized ITIM-containing molecules. Thus far, approximately 60 of the 300 predicted ITIM-containing genes in the human genome

have been characterized.[10] The identification of novel ITIM-bearing receptors will advance the current view of the role of inhibitory receptors in immune regulation.

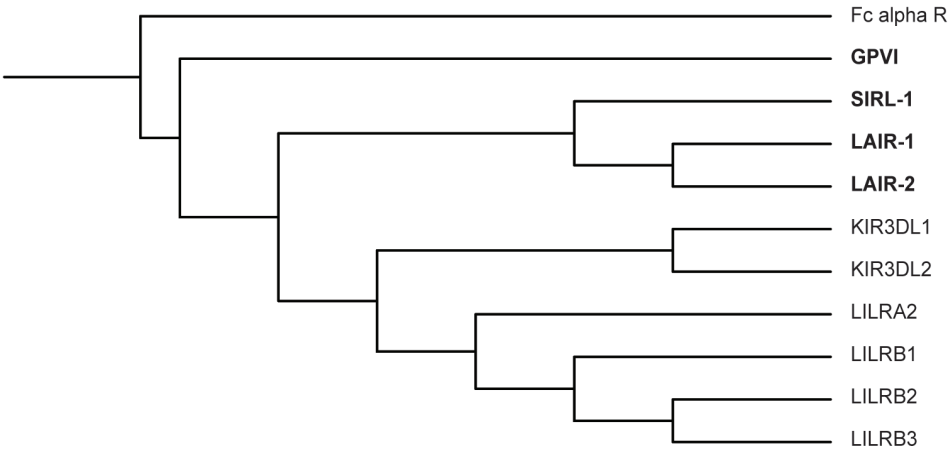

Figure 2. Phylogenetic tree of SIRL-1, LAIR, GPVI and several LILR and KIR family members. The tree was computed using Neighbour Joining (Kimura distance), and depicted as a cladogram. The Fc alpha receptor was used as an out-group. SIRL-1, LAIRs and GPVI are indicated in bold.

Scope of this thesis

Further research on inhibitory receptors is essential to fully understand their distinct role in infection or inflammation and during the various stages of the immune response. In the first part of this thesis we focus on the identification and characterization of the novel immune inhibitory receptor SIRL-1. The initial characterization of SIRL-1 is described in Chapter 2 and comprises expression pattern, phosphatase recruitment and *in vitro* inhibitory capacity. In Chapter 3, the distinct role of SIRL-1 in regulating ROS production by primary phagocytes upon challenge is elucidated. Chapter 4 reviews the role of immune inhibitory receptors, including SIRL-1, in the regulation of phagocyte function. In the next chapters we focus on the inhibitory receptor LAIR-1. The structural similarity of LAIR-1 and GPVI, and the observation that they share a common ligand is intriguing and suggests LAIR-1 may have a role in regulating GPVI signaling. In addition, LAIR-1 splice variants may be differentially expressed by distinct leukocyte subtypes and may thereby mediate distinct cellular functions. Chapter 5 describes the co-expression of the activating collagen receptor GPVI and the inhibitory collagen receptor LAIR-1 on a subset of megakaryoblasts. Chapter 6 describes expression pattern and adhesion differences between the isoforms LAIR-1a and LAIR-1b. Finally, the implications of the findings presented in this thesis are discussed in Chapter 7. Taken together, this work contributes to the understanding of the role of inhibitory receptors in immune regulation.

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Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) is a novel functional inhibitory immune receptor expressed on human phagocytes

CHAPTER 2

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Abstract

Myeloid cells play a crucial role in controlling infection. Activation of these cells needs to be tightly regulated, since their potent effector functions can damage host tissue. Inhibitory receptors expressed by immune cells play an important role in restricting immune cell activation. Here, we have characterized a hitherto unidentified ITIM-bearing receptor that is highly expressed on human neutrophils and monocytes: Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1). The chromosomal location of SIRL-1 is adjacent to the human Leukocyte Receptor Complex on chromosome 19q13.4 and contains two Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) in its cytoplasmic tail. As a classical ITIM-bearing receptor, SIRL-1 is capable of inhibiting FcεRI-mediated signaling and can recruit the SH2 domain-containing phosphatases SHP-1 and SHP-2. To investigate the specific involvement of the individual ITIMs herein, mutational analysis was performed, which revealed that both ITIMs are crucial for SIRL-1 inhibitory function and phosphatase recruitment. When primary cells were stimulated *in vitro*, SIRL-1^{high} monocytes produce less TNF-α than SIRL-1^{low} monocytes. Thus, SIRL-1 is a novel inhibitory immune receptor belonging to the growing family of ITIM-bearing receptors that is implied in the regulation of phagocytes.

Introduction

Neutrophils and cells of the monocytic lineage are the most important effector cells of the innate immune response. After infection, they are immediately activated and recruited to the site of infection, where they rapidly control the replication of pathogens by phagocytosis and secretion of antimicrobial peptides (1). In addition, they secrete proinflammatory mediators to recruit additional immune cells to the site of infection and to activate the adaptive immune system (2, 3). The secretion of proinflammatory mediators is not without danger for the host, since overproduction can lead to uncontrolled influx of inflammatory cells (2) resulting in severe tissue damage, or even induce lethal septic shock (4). Hence, to protect the host, the immune system has developed multiple mechanisms to regulate these potentially harmful effects of an overactive immune response.

One important mechanism to ensure a balanced immune response is the expression of inhibitory receptors by immune cells. Upon ligand binding these receptors relay inhibitory signals that increase the threshold for cellular activation. Their suppression of cell function is usually mediated via Immuno receptor Tyrosine-based Inhibitory Motifs (ITIMs) in the intracellular tail with the consensus sequence V/L/I/SxYxxV/L/I, where x denotes any amino acid (5). ITIMs are phosphorylated upon receptor ligation, usually by Src-family kinases, and can consequently recruit the SH2-domain containing tyrosine phosphatases SHP-1, SHP-2, the inositol phosphatase SHIP or C-terminal Src kinase (Csk) to mediate their inhibitory function (6, 7).

Multiple inhibitory receptors are expressed simultaneously on all immune cell types. While some inhibitory receptors are expressed on multiple cell types, the expression pattern of others is more restricted. For example LAIR-1 is expressed on most immune cells (8), whereas ILT3 is expressed exclusively by cells of the monocytic lineage and dendritic cells (9). Although inhibitory receptors show a large overlap in recruited phosphatases, their function is non-redundant, as illustrated by studies with mice deficient in a single inhibitory receptor (10). Regulated and/or localized expression of both ligand and receptor will dictate which aspect of the immune response is modulated. Furthermore, the capacity to inhibit cellular functions will be determined by the particular set of recruited down-stream molecules and the affinity with which these are recruited. The family of immune inhibitory receptors is still expanding: only ~one fifth of the 300 potential type I and type II ITIM-containing molecules in the human genome is recognized as such (11). The Leukocyte Receptor Complex (LRC) region on chromosome 19q13.4, which contains numerous immunoglobulin superfamily (IgSF) members, is particularly rich in putative ITIM-containing molecules (11).

Here, we characterize Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1), a novel inhibitory receptor of the IgSF expressed by human neutrophils and monocytes, as a new potential inhibitor of the innate immune response.

Materials and Methods

Computer based analysis

Identification of SIRL-1 (NP_940883 or VSTM1) was achieved by homology search in protein databases of the National Center of Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi). NCBI entrez gene was used to study SIRL-1 gene orientation and localization (http://www.ncbi.nlm.nih.gov/gene). Protein sequences were aligned using Clustal multiple alignment from BioEdit sequence alignment editor and the similarity matrix PAM250. The Center for Biological Sequence analysis site from the Technical University of Denmark was used to study protein characteristics (http://www.cbs.dtu.dk/services). The SignalP (12), NetNGlyc (13), and TMHMM servers were used to predict signal sequences, glycosylation sites, and transmembrane regions respectively.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Peripheral blood was obtained from healthy volunteers and mononuclear cells were isolated by Ficoll-Histopaque density gradient centrifugation. From these cells, total RNA was isolated using Trizol and converted to cDNA with oligo$(dT)_{18}$ primers and murine leukemia virus (MuLV) reverse transcriptase using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA). The cDNA-mixtures were amplified by PCR using SIRL-1-specific forward (5'- TTGGTTCTGGCAGAAGGGACG) and reverse (5'- AGTATCTGCATCTCCAGATTTCC) primers and the Amplitaq® Gold DNA Polymerase system (Applied Biosystems, Foster City, CA). Each amplification reaction underwent 40 cycles of denaturation at 96ºC for 30 s, annealing for 30 s at 58ºC, and elongation at 72ºC for 1 min. SIRL-1 cDNA obtained from these samples was cloned into pGEM®-T Easy vectors using the pGEM®-T Easy vector system (Promega, Madison, WI) and subsequently sequenced to examine cDNA sequence. Compared to the reference sequence GI:145580633 a silent mutation (GCC>GCT) was found in the extracellular domain at amino acid position 41. The obtained sequence was added to the EMBL-EBI database (http://www.ebi.ac.uk/embl/) using the accession number FN398145.

cDNA constructs

SIRL-1 cDNA was cloned into a retroviral pMX vector using SIRL-specific forward (5'-CGGGATCCCACCATGACCGCAGATTCCTCTC) and reverse (5'-TTTTCAATTGCTACACTTTCAGTGCCGC) primers. To create N-terminal flag-tagged SIRL-1, SIRL-1 cDNA lacking a leader sequence was ligated in a pMX vector containing a CD8-leader-flag construct using the SIRL-1-specific forward primer (5'- CCATCGATGAAGATGAGAAAAAGAATGAG). Tyrosines at positions Y206 and Y231, both part of a (semi) ITIM, were mutated to phenylalanines using quick change PCR (VTYAEL to VTFAEL and HEYAAL to HEFAAL). Sequencing of the constructs confirmed the substitution. To obtain stable SIRL-1 expression, retroviral pMX vectors containing SIRL-1 cDNAs were packaged by the pCL-ampho system (14) and virus was used to infect target cells. A chimeric SIRL-1-hIg protein was created by fusing the extracellular domain of SIRL-1, consisting of amino acids (aa) 1-132, to the Fc region of human IgG1. SIRL-1-hIg was cloned and purified as described in Lebbink et al. (15).

Cell lines

RBL-2H3 is a rat basophilic leukemia cell line that has been described before (16). Other cell lines used are: SP2/0, which is a mouse tumor B cell line and human embryonic kidney (HEK) 293T, which were used for production of infectious particles in retroviral transduction experiments and for production of SIRL-1-hIg. SP2/0 cells were cultured in DMEM, other cells were cultured in RPMI 1640 media (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Integro, Dieren, the Netherlands), and antibiotics.

Generation of monoclonal antibodies

Monoclonal antibodies (MAb) specific for SIRL-1 were produced as described (17). Briefly, 50 µg of purified SIRL-1-hIg fusion protein in PBS was mixed with Complete Freunds adjuvant (Difco, Lawrence, KS) and injected subcutaneously into BALB/c mice. This injection was repeated after two weeks using SIRL-1-hIg fusion protein mixed with Incomplete Freunds adjuvant. One week after the second injection mice were boosted with an injection of 50 µg fusion protein in PBS. Mice were sacrificed three days after the final injection and splenic PBMCs were fused with SP2/0 cells using standard hybridoma technology. The resulting hybridoma clones were screened for the ability to bind SIRL-1 transfected RBL-2H3 cells, but not the non-transfected cell line. Monoclonal hybridoma cells were obtained by limiting dilution. The IgG1 SIRL-specific MAb 1A5 was purified from monoclonal hybridoma cell

supernatant using a HiTrapTM protein G column (GE Healthcare). Part of the antibody was FITC-conjugated (Molecular Probes) to facilitate flow cytometry analysis.

Analysis of SIRL-1 expression on primary peripheral leukocytes and cell lines

Peripheral blood was obtained from healthy volunteers. Mononuclear cells and granulocytes were isolated by Ficoll-Histopaque density gradient centrifugation, and analyzed for SIRL-1 and lineage markers co-expression by flow cytometry. Lymphocytes and granulocytes were gated on base of forward and side scatter and analyzed for expression of CD3, CD11b, CD11c, CD14, CD16, CD19, CD56, CD123 and HLA-DR. Anti-CD3 and anti-HLA-DR were from eBiosciences. Anti-CD123 was from Miltenyi Biotec, and anti-CD16 was from Sanquin (Amsterdam, the Netherlands). FITC-conjugated mouse IgG1 isotype control MAb (BD Biosciences) was used to analyze a-specific staining. Quadrants were set in such a way that isotype control stainings were in the bottom part of the quadrant. Other antibodies were from BD Biosciences.

Immunoprecipitation and Western blotting

Isolated granulocytes were used to study SIRL-1 expression. RBL-2H3 cells transduced with SIRL-1 and SIRL-1 tyrosine-to-phenylalanine (tyr-phe) mutants and monocytes isolated from mononuclear cells using a CD14-positive selection MACS kit (Miltenyi Biotec) were used to study SIRL-1 phosphorylation pattern and intracellular binding partners. Cells were washed twice in PBS and incubated with 50 μM pervanadate in PBS for 15 minutes at 37ºC. After the incubation period samples were kept on ice. Cells were lysed in Triton lysis buffer (1% Triton X-100, 10 mM Tris, and 150 mM NaCl) supplemented with protease inhibitors (complete Mini EDTA-free protease inhibitor cocktail tablets, Roche, Manheim, Germany), 1 mM phenylmethylsulfonyl fluoride and 50 μM pervanadate. For immunoprecipitation anti-flag M2 Affinity Gel (Sigma-Aldrich, Germany) was used, or protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, CA) coated with 2 μg 1A5 anti-SIRL-1 or an isotype control MAb for 2h. Gel or beads were washed with PBS and blocked with 30 μL 10% bovine serum albumin (BSA). Samples were immunoprecipitated for 90 minutes. Immune complexes were washed five times with 0.1% Triton X-100, supplemented with 1 mM phenylmethylsulfonyl fluoride and 250 μM sodium orthovanadate and boiled in non-reducing sample buffer. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blots were incubated

with antibodies specific for phosphotyrosine, SIRL-1, flag M2, SHP-1 and SHP-2, followed by HRP-linked secondary antibodies. Anti-phosphotyrosine 4G10 was from Upstate (Lake Placid, NY), anti-flag M2 HRP was from Sigma, polyclonal rabbit anti-SHP-1 and anti-SHP-2 antibodies were purchased from Santa Cruz Biotechnology. HRP-linked secondary antibodies were from Dako. Enhanced chemiluminescence (Supersignal from Pierce, Rockford, IL) was used for detection.

Degranulation assays

The degranulation assay of RBL-2H3 has been described previously (18). RBL-2H3 cells were stably transduced with SIRL-1 or SIRL-1 tyr-phe mutants. Plates were coated with 3 µg/mL Trinitrophenyl (TNP) coupled to BSA and various concentrations of 1A5 anti-SIRL-1 MAb or an isotype control. Triplicate reactions were used for each condition. The spontaneous release was determined by coating BSA instead of BSA-TNP. The percentage of inhibition of degranulation by SIRL-1 was calculated by [OD_{405} TNP with isotype cross-linking – OD_{405} TNP with SIRL-1 cross-linking]/ [OD_{405} TNP with isotype cross-linking – OD_{405} spontaneous release] *100.

Intracellular cytokine staining

Whole blood was diluted 1:5 in RPMI supplemented with FCS and transferred to plates coated with 1A5 anti-SIRL-1 MAb or an isotype control. TNF-α production was induced by addition of 10 µg/mL Curdlan (Wako Chemicals), or 5 - 10 ng/mL of LPS (Sigma). TNF-α secretion was prevented by addition of GolgiplugTM (BD) to the culture. After 4h stimulation at 37°C, erythrocytes were lysed and cells were stained for surface expression of SIRL-1 and CD14, after which cells were permeabilized with cytofix/perm (BD), stained with anti-TNF-α (BD) and analyzed by flow cytometry.

Statistical analysis

Data were analyzed using SPSS 15.0 software (SPSS, Chigaco, Illinois, USA). Differences between SIRL-1^{high} and SIRL-1^{low} monocytes after stimulation were analyzed using Wilcoxon signed ranks test. A p-value of ≤ 0.05 was considered statistically significant.

Results

SIRL-1 is homologous to LAIR-1b and the SIRL-1 gene is located in close proximity to the human Leukocyte Receptor Complex region

A cDNA encoding a putative novel ITIM-bearing receptor (NP_940883 or VSTM1) was retrieved from the human protein databases (NCBI). We named this putative receptor Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1). SIRL-1 core nucleotide and protein sequences were analyzed and correspond to a putative polypeptide composed of 236 aa. The SIRL-1 polypeptide contains a 16 aa signal peptide, three putative glycosylation sites and a single hydrophobic transmembrane region spanning from aa 135 to aa 157 (Figure 1A). SIRL-1 is a type Ia membrane protein that contains one extracellular variable-like immunoglobulin (IgV) domain, classifying SIRL-1 as a member of the IgSF. SIRL-1 also contains two intracellular tyrosines (Y206 and Y231) that are part of an ITIM (VtYaeL) and a putative ITIM (HeYaaL) respectively. To investigate whether SIRL-1 had close family members or homologues, we searched the NCBI human blast program with a blastp algorithm for sequences homologous with SIRL-1 protein sequence. The protein with the highest aa homology to SIRL-1 was Leukocyte Associated Immunoglobulin-like Receptor (LAIR)-1b, which is approximately 25% identical and 55% similar to full length SIRL-1 (Figure 1A). The *SIRL-1* gene spans ~ 23.1 kb of genomic sequence and consists of nine exons (Figure 1B). The gene is located centromeric to the human LRC region on chromosome 19q13.4 (Figure 1C). The LRC region contains several gene families that are part of the IgSF including *LAIRs, Leukocyte Immunoglobulin-Like Receptors (LILRs)* and *Killer Cell Immunoglobulin-like Receptors (KIRs)* (19).

SIRL-1 is expressed by myeloid, but not lymphoid cells

To investigate the SIRL-1 expression profile, we generated SIRL-1-specific mouse MAb. The monoclonal hybridoma clone 1A5 producing antibody of the IgG1 type was purified and used for our studies (see Materials and Methods). SIRL-1-transfected but not non-transfected RBL-2H3 cells stained with 1A5 anti-SIRL-1-FITC MAb as analyzed by flow cytometry, demonstrating the specificity of the antibody (Figure 2A). In addition, the SIRL-1-specific antibody detected a ~45 kDa and a fainter ~37 kDa protein by Western blot analysis in SIRL-1 transfected, but not in the non-transfected RBL-2H3 cells (Figure 2B).

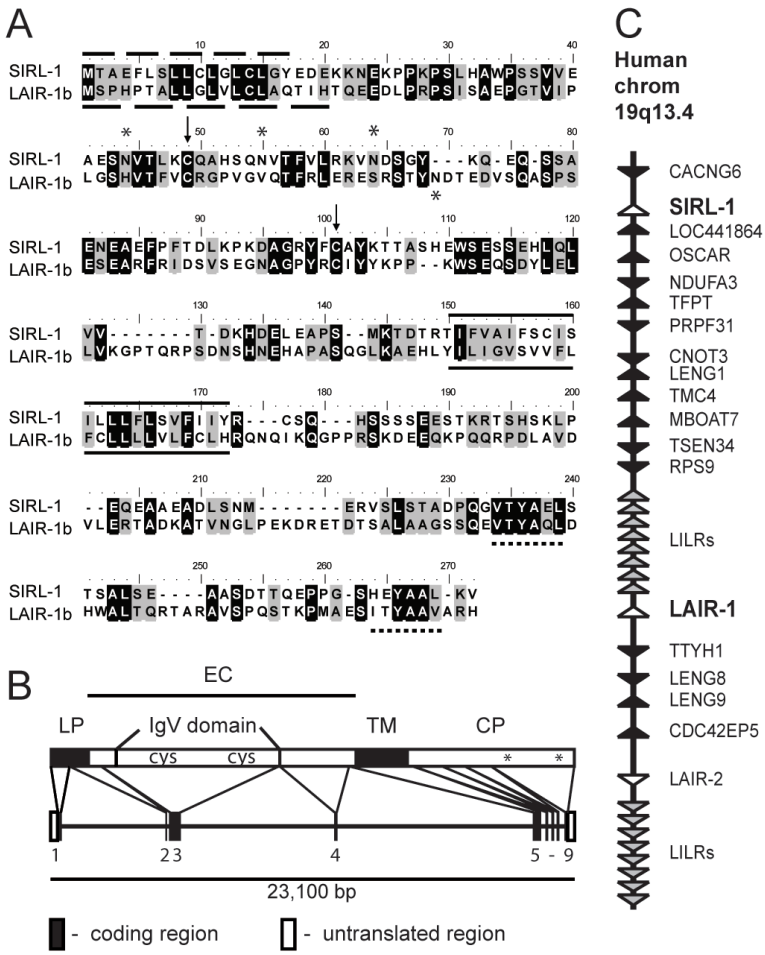

Figure 1. SIRL-1 is homologous to LAIR-1b and SIRL-1 gene is located in close proximity to the human Leukocyte Receptor Complex region. A, Protein sequence alignment of SIRL-1 and LAIR-1b. Black boxes indicate identical amino acids and grey boxes indicate similar amino acids. Gaps introduced to optimize the sequence alignment are indicated by dots. SIRL-1 contains three putative glycosylation sites at N44, N55 and N64, indicated by asterisks. Arrows indicate cysteines that form a disulfide bond and mediate formation of an IgV domain. The single hydrophobic transmembrane of both proteins is underlined. Dashed lines illustrate intracellular tyrosines that are part of putative ITIMs. SIRL-1 protein sequence is available at NBCI under accession number NP_940883.2. B, Schematic overview of SIRL-1 gene and protein. The protein-coding sequences are denoted as closed boxes. The protein structure is subdivided into the leader peptide (LP), the extracellular domain (EC), transmembrane domain (TM), and cytoplasmic domain (CP). The two cysteines (cys) that form disulfide bonds and shape the IgV region are also indicated. Putative ITIM sequences are indicated by asterisks. SIRL-1 contains nine exons. The first two exons encode for SIRL-1 leader peptide. The IgV region, TM region and the two ITIMs are all located on separate exons. C, Schematic organization of the human LRC region on chromosome 19q13.4. SIRL-1 and its closest homolog, LAIR-1b are depicted in bold and indicated by white arrows, which indicate their direction of transcription. Other genes depicted include the large LILR family of genes, which is depicted by grey arrows.

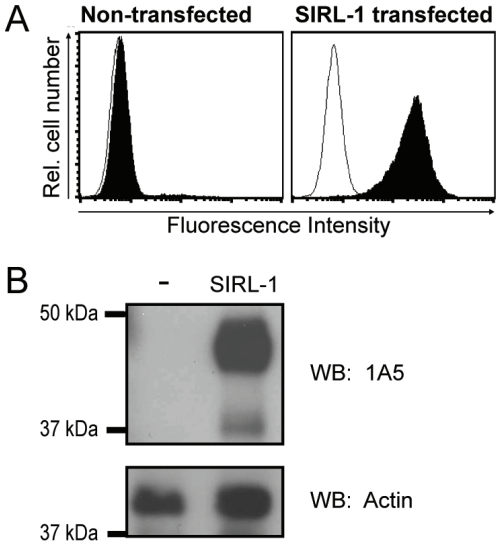

Figure 2. Anti-SIRL-1 monoclonal antibodies specifically bind SIRL-1. A, Non-transfected (-) and SIRL-1 transfected RBL-2H3 cells were stained with FITC-conjugated 1A5 anti-SIRL-1 MAb or an isotype control and analyzed by flow cytometry. The open histogram represents isotype control staining; the filled histogram represents SIRL-1 staining. B, Non-transfected and SIRL-1 transfected RBL-2H3 cells were lysed and analyzed by Western blot. The SIRL-1-specific antibody detected a ~45 kDa band and a fainter ~37 kDa band in the SIRL-1 transfected, but not in the non-transfected RBL-2H3 cells.

We subsequently analyzed SIRL-1 expression on human peripheral blood leukocytes by flow cytometry. As described in *Materials and Methods*, quadrants were set on base of isotype stainings (Figure 3A). We found that SIRL-1 was highly expressed by both $CD11b^+$ $CD16^{high}$ neutrophils and $CD11b^+$ $CD16^{low}$ eosinophils (Figure 3A-B). Likewise, most $CD14^+$ monocytes highly expressed SIRL-1, although a subset of monocytes was low/intermediate for SIRL-1-expression (Figure 3A-B). Expression of SIRL-1 on $CD3^-$ $CD14^-$ $CD19^-$ $CD56^-$ $CD11c^+$ $HLA\text{-}DR^+$ myeloid dendritic cells differed per donor: some donors showed intermediate expression of SIRL-1, while other donors did not express SIRL-1 on the myeloid dendritic cells (Figure 3A-B). In contrast, $CD3^-$ $CD14^-$ $CD19^-$ $CD56^-$ $CD11c^-$ $HLA\text{-}DR^+$ $CD123^+$ plasmacytoid dendritic cells did not express SIRL-1 (data not shown). Lymphocytes, including $CD3^-$ $CD56^+$ natural killer cells, $CD3^-$ $CD19^+$ B cells and $CD3^+$ T also did not express SIRL-1 (Figure 3A-B). We next performed immunoprecipitations with 1A5 anti-SIRL-1 or an isotype control MAb on isolated granulocytes and analyzed Western blots with 1A5 anti-SIRL-1 MAb to detect SIRL-1 expression. SIRL-1 transfected RBL-2H3 cells were loaded as a control. After immunoprecipitation with 1A5 anti-SIRL-1 MAb, a specific band at 37 kDa was detected that was absent in isotype control immunoprecipitations (Figure 3c).

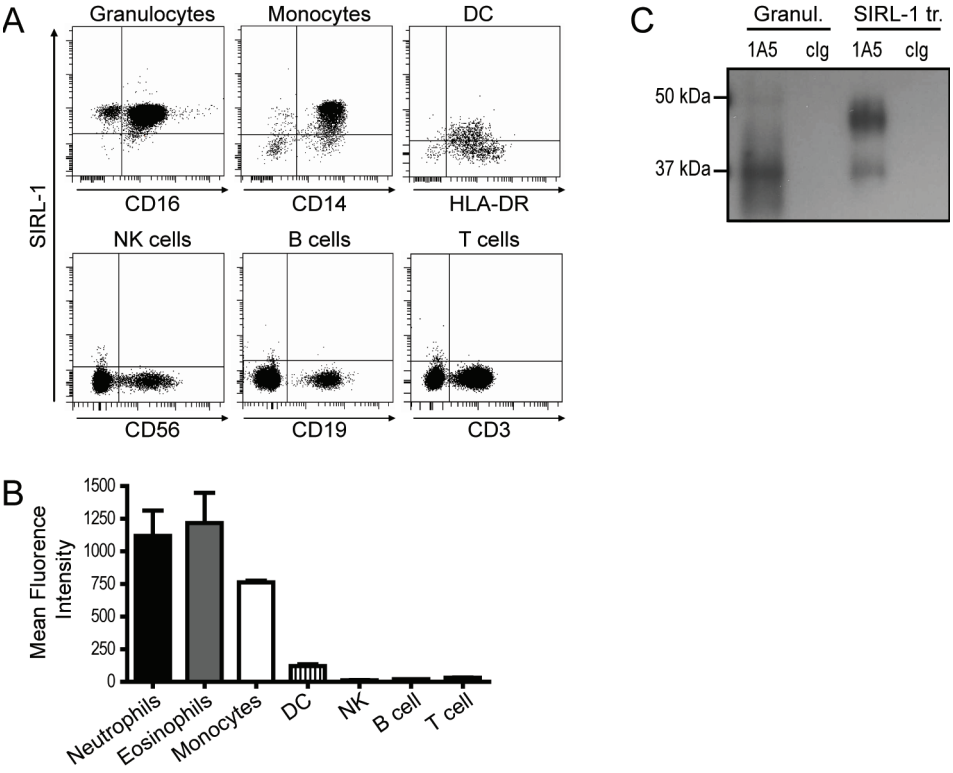

Figure 3. SIRL-1 is expressed on myeloid, but not on lymphoid cells. A, Leukocytes were isolated from human peripheral blood by Ficoll-histopaque density gradient centrifugation and co-expression of SIRL-1 and lineage markers was determined by flow cytometry. Granulocytes were gated on base of forward and side scatter and CD11b expression. SIRL-1 is highly expressed by $CD16^{high}$ neutrophils and $CD16^{low}$ eosinophils (top panel, left). $CD14^{+}$ monocytes (top panel, middle) were gated on base of forward and side scatter. $HLA\text{-}DR^{+}$ myeloid dendritic cells (top panel, right) were gated on base of forward and side scatter and further selected by gating $CD3^{-}$ $CD14^{-}$ CD19- $CD56^{-}$ and $CD11c^{+}$ positive cells. Lymphocytes were gated on base of forward and side scatter. $CD56^{+}$ Natural killer (NK) cells and $CD19^{+}$ B cells were selected by gating $CD3^{-}$ cells. Neither NK cells, B cells nor $CD3^{+}$ T cells expressed SIRL-1 (bottom panels). Quadrants were set on base of isotype stainings. Data shown are representative of at least three different donors analyzed in independent experiments. B, Mean fluorescence intensity of SIRL-1 expression on different peripheral blood cell types averaged for three donors. Error bars represent SEM. C, Immunoprecipitations with 1A5 anti-SIRL-1 (1A5) or an isotype control (clg) MAb were performed on isolated granulocytes and SIRL-1 transfected RBL-2H3 cells. Samples were analyzed by Western blots using 1A5 anti-SIRL-1 MAb followed by HRP-linked secondary antibodies to detect SIRL-1 expression. A specific ~37 kDa band was detected in granulocytes after immunoprecipitation with 1A5 anti-SIRL-1 MAb.

To determine whether additional cell types expressed SIRL-1, we performed flow cytometry analysis on cells obtained from human tonsils, adenoid, spleen, cord blood, bone marrow, blastocysts and hematopoietic and mesenchymal stem cells. In the bone marrow compartment a small subset of $CD11b^+$ $CD16^+$ SIRL-1 positive neutrophils was identified (data not shown), whereas all other samples were negative for SIRL-1 expression.

We next performed flow cytometry analysis of several cell lines including: THP-1, U-937, HL-60 (monocytic cell lines); Daudi, Raji (B lymphoblastic cell lines); CEM, Jurkat (T lymphoblastic lines); YT2c2 (NK cell line); TF-1 (erythroblastic cell line); CHRF-288, Meg-01, Dami (megakaryocytic cell lines); HEK293T (human embryonic kidney cells) and human foreskin fibroblasts (HFF). None of these cell lines expressed SIRL-1 on their membrane (data not shown), suggesting that SIRL-1 expression is restricted to primary myeloid cells.

SIRL-1 requires both ITIMs to recruit SHP-1 and SHP-2

The capacity of inhibitory immune receptors to inhibit cellular activation depends on effector molecules, generally recruited to the ITIMs. Since most phosphorylated ITIMs can recruit the phosphatases SHP-1, SHP-2 or SHIP, we tested whether these molecules were indeed recruited to SIRL-1. Non-transfected and RBL-2H3 cells stably transfected with SIRL-1 were treated with pervanadate to increase tyrosine phosphorylation or left unstimulated. SIRL-1 was immunoprecipitated with 1A5 anti-SIRL-1 MAb. SIRL-1 recruited SHP-1 after pervanadate treatment, but no interaction between SIRL-1 and SHP-2 was found in these cells (Figure 4A). We next assessed the contribution of the individual ITIMs in the recruitment of SHP-1. Tyr-phe mutants of SIRL-1 were generated, in which the central tyrosine of either the N-terminal ITIM (SIRL-1 FY), the C-terminal putative ITIM (SIRL-1 YF) or both ITIMs (SIRL-1 FF) was mutated to a phenylalanine. RBL-2H3 cells were stably transfected with wild type SIRL-1or SIRL-1 tyr-phe mutants, which were expressed at comparable levels on the cell surface (Figure 4B). SIRL-1 and tyr-phe mutants were subsequently immunoprecipitated with 1A5 anti-SIRL-1 MAb and both tyrosine phosphorylation pattern and association of SHP-1 and SHP-2 were determined. Pervanadate treatment resulted in strong phosphorylation of wild type SIRL-1, whereas none of the mutants attained the same degree of phosphorylation (Figure 4C). This indicates that both tyrosines of SIRL-1 can be phosphorylated. Interestingly, in the SIRL-1 mutant with an intact N-terminal ITIM a moderate amount of phosphorylation could still be detected, but not in the mutant with an intact C-terminal ITIM (Figure 4C).

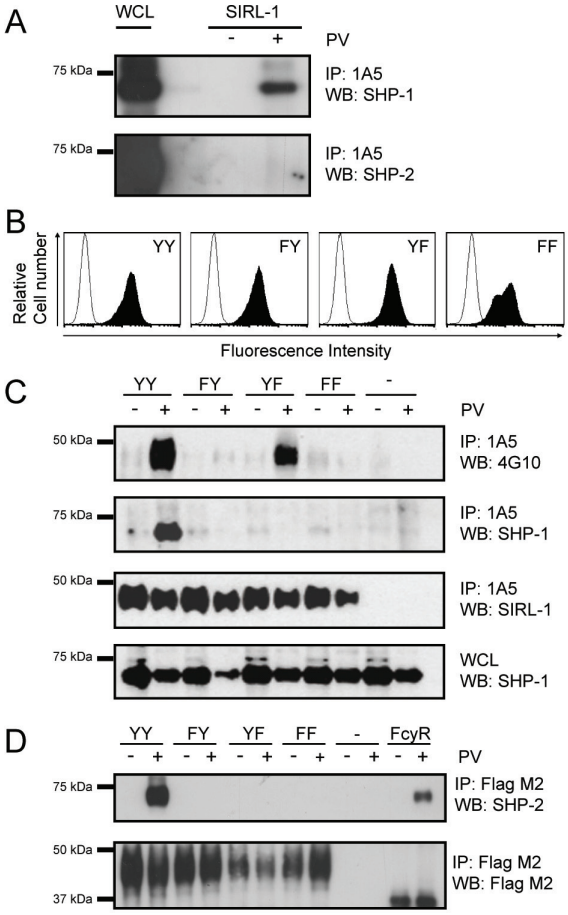

Figure 4. SIRL-1 needs both ITIMs to recruit SHP-1 and SHP-2. A, SIRL-1 recruits SHP-1 but not SHP-2 in RBL-2H3 cells. RBL-2H3 cells transfected with SIRL-1 were treated with 50 µM pervanadate (PV) in PBS, or PBS alone for 15 minutes at 37°C after which cells were lysed in 1% Triton buffer. The immunoprecipitation (IP) was performed with protein A/G PLUS-Agarose beads coupled to 1A5 anti-SIRL-1 MAb. Western blotting (WB) was performed using anti-SHP-1 and anti-SHP-2 antibodies. The results are representative of three independent experiments. B, Expression of SIRL-1 on RBL-2H3 cells stably transfected with wild type SIRL-1- (YY) and SIRL-1 tyr-phe mutants which include mutation of the N-terminal ITIM (FY); the C-terminal ITIM (YF) and both ITIMs (FF). Non-transfected RBL-2H3 cells were taken as a control. Cells were stained with FITC-conjugated SIRL-1 specific mouse MAb 1A5 or an isotype control and analyzed by flow cytometry (solid and open histograms respectively). C, Phosphorylation pattern and recruitment of SHP-1 by SIRL-1 YY and tyr-phe mutants. RBL-2H3 cells transfected with SIRL-1 YY and tyr-phe mutants were treated with pervanadate, lysed and immunoprecipitated as described in *(A)*. Western blotting was performed using anti-SHP-1 (top panel and bottom panel loading control), 4G10 anti-phosphotyrosine (second panel), and 1A5 anti-SIRL-1 antibodies (third panel). The results are representative of five independent experiments. D, Recruitment of SHP-2 by SIRL-1 YY and tyr-phe mutants. HEK293T cells were transiently transfected with flag-tagged SIRL-1 YY and tyr-phe mutants and flag-tagged FcγRIIB. After pervanadate treatment cells were lysed an immunoprecipitated with anti-flag M2 Affinity Gel. Western blotting was performed using anti-SHP-2 (top panel) and anti-flag M2-HRP (bottom panel). The results are representative of two independent experiments. WCL: whole cell lysate.

This may indicate that phosphorylation of the C-terminal ITIM is facilitated by prior phosphorylation of the N-terminal ITIM. As expected, no phosphorylation could be detected in the SIRL-1 FF mutant. Notably, the interaction between SHP-1 and SIRL-1 after pervanadate treatment was abrogated in all SIRL-1 tyr-phe mutants, indicating that both ITIMs of SIRL-1 are required for SHP-1 recruitment.

Since the lack of SHP-2 recruitment to SIRL-1 in RBL cells may be cell line-dependent, we performed additional immunoprecipitations in HEK293T transiently transfected with flag-tagged SIRL-1 and tyr-phe mutants and flag-tagged FcγRIIb as a control. Comparable cell surface expression of all receptors was confirmed by flow cytometry (data not shown). Flag-tagged SIRL-1, tyr-phe mutants and FcγRIIb were immunoprecipitated with anti-flag beads and association with SHP-2 was analyzed. Notably, an interaction between wild type SIRL-1 and SHP-2 was clearly detected in these cells (Figure 4D). Immunoprecipitations using tyr-phe mutants of SIRL-1 once more suggest that two intact ITIMs are required for the interaction, as mutation of either tyrosine disrupted the recruitment. Conversely, no recruitment of SHIP was observed (data not shown). To summarize, both tyrosines of SIRL-1 can be potentially phosphorylated and are required for the interaction of SIRL-1 with SHP-1 and SHP-2.

SIRL-1 requires both ITIMs for full inhibition of FcεRI mediated degranulation

Classically, ITIM-bearing receptors are capable of inhibiting signals mediated by receptors containing Immuno receptor Tyrosine-based Activation Motifs (ITAMs). RBL-2H3 cells express the IgE receptor FcεRI, a well-characterized ITAM-bearing receptor, and FcεRI-mediated degranulation of RBL-2H3 cells is a reputable model to test the inhibitory capacity of ITIM-bearing receptors. We used this model to investigate whether SIRL-1 could inhibit cell activation signals. To stimulate the FcεRI, RBL-2H3 cells were primed with anti-TNP-IgE and transferred to plates coated with TNP. The amount of β-glucuronidase released is used as a measure of the extent of degranulation. Cross-linking of SIRL-1 by coated 1A5 anti-SIRL-1 MAb resulted in the complete inhibition of degranulation, whereas an isotype control had no effect (Figure 5A). We next used SIRL-1 tyr-phe mutants to assess the capacity of individual SIRL-1 ITIMs to suppress FcεRI-mediated degranulation. Interestingly, SIRL-1 receptors with mutated N- or C-terminal ITIM were both able to inhibit degranulation by ~50% compared to the isotype control, demonstrating that both ITIMs have inhibitory potential (Figure 5B). Mutating both tyrosines however resulted in a complete loss of inhibitory potential, suggesting that inhibition in this *in vitro* system is mediated exclusively via SIRL-1 ITIMs. In

conclusion, our data indicate that SIRL-1 can inhibit FcεRI-mediated degranulation and that SIRL-1 requires both ITIMs for its full inhibitory capacity.

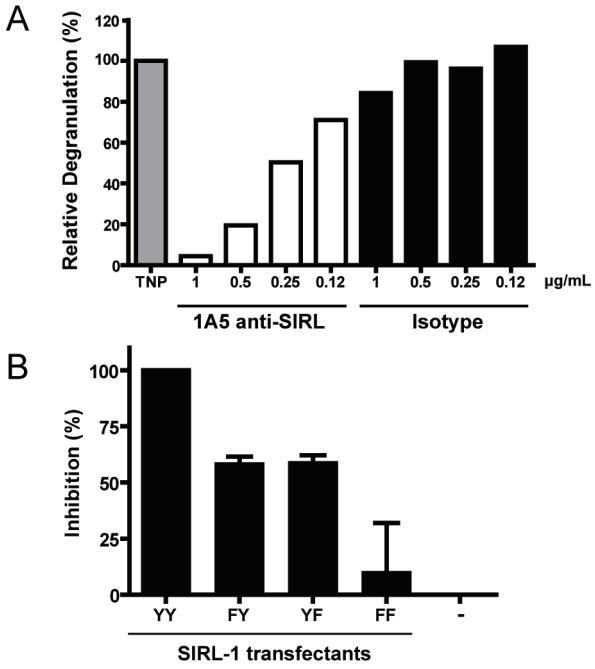

Figure 5. Both ITIMs of SIRL-1 contribute to inhibition of FcεRI-mediated degranulation. A, Inhibition of FcεRI-mediated degranulation by RBL-2H3 cells transfected with SIRL-1. RBL-2H3 cells were primed with IgE anti-TNP, and transferred to plates coated with TNP (3 µg/mL), or TNP combined with 1A5 anti-SIRL-1 MAb or an isotype control (1 to 0.12 µg/mL). The percentage of inhibition of degranulation by SIRL-1 was calculated by [OD405 TNP stimulation – OD405 TNP with SIRL-1 cross-linking]/ [OD405 TNP stimulation – OD405 spontaneous release] *100. B, Inhibition of FcεRI-mediated degranulation by RBL-2H3 cells transfected with wild type SIRL-1- (YY) and SIRL-1 tyr-phe mutants which include mutation of the N-terminal ITIM (FY); the C-terminal ITIM (YF) and both ITIMs (FF). Non-transfected RBL-2H3 cells were taken as a control. RBL-2H3 cells were primed with IgE anti-TNP, and transferred to plates coated with TNP (3 µg/mL) and 1A5 anti-SIRL-1 MAb or an isotype control (1 µg/mL). The percentage of inhibition of degranulation by SIRL-1 was calculated by [OD405 TNP stimulation with isotype cross-linking – OD405 TNP with SIRL-1 cross-linking]/ [OD405 TNP stimulation with isotype cross-linking – OD405 spontaneous release] *100. Error bars represent SEM.

SIRL-1^{high}-monocytes produce less TNF-α than SIRL-1^{low} monocytes

To determine whether SIRL-1 could modulate TNF-α production by primary cells, whole blood was stimulated by adding either LPS or Curdlan, a strong agonist for the pattern recognition receptor Dectin (20), to the culture. Monocyte TNF-α production was measured by

intracellular staining using flow cytometry analysis. Monocytes were gated on base of forward and side scatter and on CD14 expression. TNF-α expression was quantified in human monocytes expressing either low or high SIRL-1 (Figure 6a). Notably, SIRL-1^{high}-monocytes consistently express less TNF-α than SIRL-1^{low} monocytes ($p= 0.018$ for Curdlan-stimulated samples and $p= 0.028$ for LPS-stimulated samples) (Figure 6b). Cross-linking of SIRL-1 by plate-bound 1A5 anti-SIRL MAb did not decrease TNF-α expression in total CD14+ monocytes (Figure 6c), or in SIRL-1^{high} or SIRL-1^{low} monocytes (data not shown). The lack of effect of plate-bound 1A5 anti-SIRL MAb was not due to steric hindrance by other blood cells, since similar results were obtained when we stimulated isolated PBMC and analyzed intracellular TNF-α production in monocytes, nor did plate-bound 1A5 anti-SIRL MAb decrease TNF-α expression in either SIRL-1^{high} or SIRL-1^{low} monocytes (data not shown). Similarly, levels of secreted TNF-α after stimulation of MACS-isolated monocytes were not affected by plate-bound 1A5 anti-SIRL MAb as measured by ELISA (data not shown). We next investigated whether recruitment of phosphatases to SIRL-1 could be detected in monocytes. Immunoprecipitations were performed using 1A5 anti-SIRL-1 MAb in isolated monocytes. Indeed, an interaction of SIRL-1 with SHP-1 was demonstrated (Figure 6d, top panel). Notably, in three out of four donors, this interaction was demonstrated without SIRL-1 stimulation. Conversely, no interaction of SIRL-1 with SHP-2 could be demonstrated, either with or without pervanadate treatment (Figure 6d, bottom panel). In conclusion, SIRL-1 recruits SHP-1 but not SHP-2 in monocytes, and SIRL-1^{high}-monocytes produce less TNF-α than SIRL-1^{low} monocytes.

Discussion

Here, we describe the identification and characterization of the novel ITIM-bearing receptor SIRL-1. This receptor has an extracellular IgV domain and is therefore a member of the IgSF. *SIRL-1* is located close to the human LRC region on chromosome 19q13.4, which contains many genes of the IgSF.

Based on primary amino acid sequence, SIRL-1 is most homologous to the inhibitory receptor LAIR-1b, of which the gene is located close to *SIRL-1* in the human LRC region. LAIR-1 is expressed on almost all immune cells (8), excluding resting neutrophils (21), and is the sole inhibitory receptor described so far that ligates collagen (22). LAIR-1 has a broad modulatory role in many immune cell types, including inhibition of cytotoxic activity of NK cells and effector T cells (reviewed in Meyaard (8)).

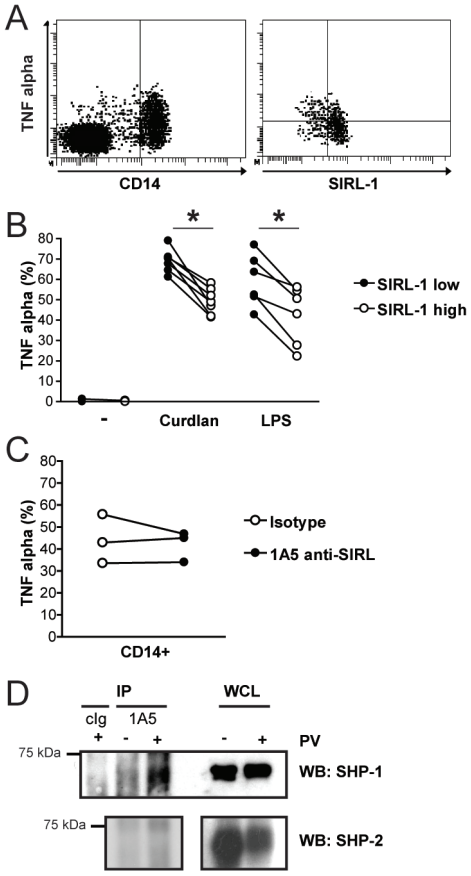

Figure 6. SIRL-1^{high}-monocytes produce less TNFα than SIRL-1^{low} monocytes. Whole blood was diluted 1:5 in RPMI and stimulated for 4h with Curdlan or LPS, after which cells were stained for surface expression of SIRL-1 and CD14, permeabilized and stained with anti-TNFα. Monocytes were gated on base of forward and side scatter. A, TNFα production by SIRL-1^{high} versus SIRL-1^{low} monocytes was analyzed by plotting TNFα production against SIRL-1 expression for CD14+ cells. Quadrants were set on base of unstimulated samples (Y-axis), and to the left of the main SIRL-1^{high} population (X-axis). Representative dot plots are shown. B, Percentage of TNFα production for SIRL-1^{low} and SIRL-1^{high} cells stimulated with 10 µg/mL of Curdlan (n=7) or 10 ng/mL of LPS (n=6). Differences between for SIRL-1^{low} and SIRL-1^{high} cells were analyzed Wilcoxon Signed Ranks Test. p= 0.018 for Curdlan-stimulated samples and p= 0.028 for LPS-stimulated samples. C, Cross-linking with 1A5 anti-SIRL MAb has no effect on TNFα production by CD14+ monocytes. Whole blood was incubated with 10 µg/ml of immobilized 1A5 anti-SIRL or an isotype control for 10 min, after which 10 µg/ml of Curdlan was added to the culture. Percentages TNFα production are shown (n=3). D, SIRL-1 interacts with SHP-1, but not SHP-2. Monocytes were isolated from mononuclear cells using a CD14-positive selection MACS kit. Cells were treated with 50 µM pervanadate (PV) in PBS, or PBS alone for 15 minutes at 37°C after which cells were lysed in 1% Triton buffer. The immunoprecipitations (IP) were performed with protein A/G PLUS-Agarose beads coupled to 1A5 anti-SIRL-1 (1A5) or an isotype control (clg) MAb. Western blotting (WB) was performed using anti-SHP-1 (top panel) and anti-SHP-2 (bottom panel) antibodies. The results are representative of three independent experiments (in total 4 donors). WCL is whole cell lysate.

The broad expression pattern of LAIR-1 is quite dissimilar to the expression profile of SIRL-1, which is restricted to myeloid cells (Figure 3). Furthermore, with no more than 25 percent of the amino acids being identical, the homology between SIRL-1 and LAIR-1 is limited. In addition, we have not detected binding of SIRL-1 to collagen (data not shown), so a physiological ligand for SIRL-1 remains as yet unidentified.

SIRL-1 N-terminal tyrosine is centered in a canonical ITIM sequence, but the C-terminal tyrosine is part of a structurally atypical ITIM sequence (HxYxxL). As previously discussed, ITIMs are structurally defined as V/L/I/SxYxxV/L/I, but the hydrophobic residue at Y-2 is less conserved than at Y+3 (11). Setting up a prediction model for preferential binding of specific ITIMs to SH-2 domain-containing phosphatases, Sweeney and workers (23) reported differential requirements of the SH2 domains of SHP-1, SHP-2 and SHIP. They demonstrate a high preference for hydrophobic residues at Y-2 position for SHP-2 recruitment, but surprisingly, they found that this preference is much weaker for SHP-1 recruitment. Indeed, the C-terminal SH2 domain of SHP-1 has a similar affinity for histidine (present in SIRL-1 C-terminal ITIM) as for isoleucine at the Y-2 in their experiments. Besides the structural definition of ITIMs, ITIMs are functionally defined as being phosphorylated on the central tyrosine, recruiting SH-2 domain-containing phosphatases, and inhibiting ITAM-dependent activation signals (5). By studying tyr-phe mutants of SIRL-1, we were able to determine whether the SIRL-1 C-terminal putative ITIM functions as a true ITIM. We show that two intact tyrosines are required for maximal receptor phosphorylation and recruitment of SHP-1 and SHP-2, suggesting that the second tyrosine participates in SHP-1 and SHP-2 binding (Figure 4). An alternative explanation for the abrogated phosphatase recruitment in the SIRL-1 YF mutant could be that the C-terminal tyrosine is required for phosphorylation of the N-terminal tyrosine without directly binding to SHP-1 or SHP-2 itself. However, our phosphorylation studies using the SIRL-1 YF mutant demonstrate that phosphorylation can take place in the absence of a C-terminal tyrosine and thus strongly disfavor this hypothesis. Furthermore, it has been postulated that two intact ITIMs are necessary for recruitment of SHP-1, since the abrogation of SHP-1 recruitment by disrupting one out of two ITIMs has been demonstrated before (18, 24) supporting the hypothesis of an active participation of the second tyrosine in SHP-1 binding. Finally, in the FcεRI-mediated degranulation model, an independent inhibitory effect of the SIRL-1 FY mutant was observed, and both intact ITIMs were required for SIRL-1 full inhibitory potential (Figure 5). The SIRL-1 FY mutant was found to partly inhibit degranulation despite a lack of detectable tyrosine phosphorylation in this mutant. Most likely, the SIRL-1 FY mutant can be phosphorylated to some extent,

but this phosphorylation was below the detection limit of the phosphotyrosine antibody. Taken together, these results ascertain that the C-terminal tyrosine of SIRL-1 is part of a *bona fide* ITIM, and we propose the inclusion of a histidine residue at the Y-2 position in the definition of the ITIM.

In RBL-2H3 cells an interaction between SIRL-1 and SHP-1 was demonstrated, but no interaction between SIRL-1 and SHP-2. However, in HEK293T cells, which lack SHP-1, SHP-2 recruitment by SIRL-1 was observed (Figure 4). Since the physiological significance of the phosphatase recruitment was unclear, we examined the recruitment potential of SIRL-1 in peripheral blood monocytes. In these cells, an interaction between SIRL-1 and SHP-1, but not SHP-2, was demonstrated (Figure 6d). Additionally, our data suggest that besides SHP-1 and SHP-2, SIRL-1 may recruit other molecules to establish its inhibitory effect, since both SIRL-1 single tyr-phe mutants can partly inhibit degranulation, whereas no SHP-1 or SHP-2 is recruited to these mutants. Similar results were obtained previously by our group, when demonstrating a SHP-independent function of the ITIM-bearing receptor LAIR-1 (7).

Remarkably, SIRL-1 is exclusively expressed by cells of myeloid origin; phagocytes especially have high SIRL-1 expression, whereas SIRL-1 is absent from lymphoid cells. The expression pattern of SIRL-1 resembles that of the inhibitory receptors Signal Regulatory Protein alpha (SIRP-α) (25, 26), Siglec-5 (27) and to a lesser extent CD200R (28, 29). Expression of these receptors is also mainly restricted to myeloid cells of the immune system, although recent papers demonstrate that CD200R is also expressed by human T and B cells (29, 30). Both SIRP-α and CD200R have crucial functions in immune regulation, phagocytosis and in control of bacterial infections. For example, mice deficient in CD200, the ligand for CD200R, are prone to autoimmune disease and have an increased myeloid response to inflammation (31). Indeed, infecting these mice with influenza virus leads to an enhanced, fatal inflammation (32). SIRP-α on the other hand has a well established role in the inhibition of host cell phagocytosis by macrophages (33). Furthermore, SIRP-α cross-linking has shown to inhibit LPS-induced TNF-α production in macrophages (34), whereas knockdown of SIRP-α in mouse macrophages results in increased production of TNF-α, IL-6, nitric oxide and IFN-β in response to LPS. Moreover, transfer of SIRP-α knockdown macrophages into wild type mice results in an increased susceptibility to lethal LPS shock (35).

We here demonstrate that SIRL-1^{high}-monocytes express less TNF-α than SIRL-1^{low} monocytes after LPS or Curdlan stimulation (Figure 6). This could be due to differential SIRL-1 expression on distinct subsets of monocytes. Alternatively, the lower TNF-α production by SIRL-1^{high} monocytes may indicate that SIRL-1 is permanently signaling on these cells, lead-

ing to suppression of cell activation. This notion is supported by the finding that additional antibody-mediated cross-linking of SIRL-1 did not further affect TNF-α expression, and by the finding that SHP-1 is permanently recruited by SIRL-1 in monocytes. An explanation for the constitutive activation of SIRL-1 may be the activation of SIRL-1 by binding to its ligand, the identity of which is presently unknown. The constitutive activation of SIRL-1 by its ligand would suggest an important role for SIRL-1 in the suppression of leukocyte activation or in increasing the activation threshold of myeloid cells. Furthermore, the fact that not only ITAM-containing Dectin signaling is affected by SIRL-1 expression but also TLR4-mediated LPS signaling implies a broad immune modulatory function for SIRL-1.

In conclusion, we have characterized a hitherto unidentified ITIM-bearing receptor, showing expression pattern, recruitment of intracellular phosphatases, inhibitory function, and the particular involvement of individual ITIMs. To further elucidate the biological role of SIRL-1 in the regulation of the innate immune response, more research is necessary. An important step towards unraveling this function would be the identification of SIRL-1 biological ligand.

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Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) selectively regulates the oxidative burst in human phagocytes

CHAPTER 3

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Abstract

The production of reactive oxygen species (ROS) is an important effector mechanism mediating intracellular killing of microbes by phagocytes. Inappropriate or untimely production of ROS can lead to tissue damage, underlining the necessity for tight regulation. Inhibitory receptors are well established negative regulators of the immune response, but few studies have reported a role for these receptors in regulating ROS production. Recently, we characterized signal inhibitory receptor on leukocytes-1 (SIRL-1) as an inhibitory receptor expressed by human phagocytes. Here, we demonstrate that cross-linking of SIRL-1 decreases Fc receptor-induced ROS production both in differentiated monocytic and granulocytic PLB-985 cells and in primary human phagocytes. In accordance, SIRL-1 cross-linking on primary neutrophils results in reduced microbial killing of internalized bacteria. Notably, phagocytosis and cytokine production by phagocytes upon stimulation of Fc receptors are not affected by SIRL-1 cross-linking, pointing to a selective regulation of ROS production. The expression of SIRL-1 is regulated: we found that cytokine- or pattern recognition receptor-induced phagocyte activation leads to a down-regulation of SIRL-1 expression. We propose that SIRL-1 on phagocytes sets an activation threshold to prevent inappropriate production of oxygen radicals. Upon infection, SIRL-1 is down-regulated, allowing microbial killing and clearance of the pathogen.

Introduction

Phagocytes, including neutrophils, monocytes and macrophages, can recognize, phagocytose and eliminate invading pathogens and are consequently crucial in host defense.[1] Pathogens are recognized through pattern recognition receptors (PRR) present on these cells, including Toll-like receptors (TLR) and c-type lectins, which bind specific pathogen-associated molecular patterns (PAMPs), such as LPS for TLR4, and bacterial or fungal β-glucans for the c-type lectin Dectin-1. Stimulation of PRR on immune cells is critical in detecting invading microorganisms, resulting in activation of these cells and leading to production of inflammatory cytokines and chemokines to recruit and activate additional effector cells. Activation of Fc receptors (FcRs) by Ig-opsonized bacteria leads to a particularly well characterized phagocytic response.[2] Microbicidal activity is the key function of phagocytes and is achieved through phagocytosis of the infectious agent followed by fusion of the intracellular formed phagosome with lysosomal granules and production of reactive oxygen species (ROS).[3] The nicotinamide adenine dinucleotide phosphate (NADPH) complex is responsible for ROS production in phagocytes, resulting in generation of hydrogen peroxide and hypochlorous acid.[4]

The key role of ROS in microbial killing is most apparent from the recurrent bacterial infections typical of chronic granulomatous disease (CGD). These patients have mutations in the NADPH oxidase complex resulting in defective ROS production.[5] Although crucial for bacterial killing, ROS production can also be harmful for the host: part of the lysosomal granules and ROS may spill in the extracellular milieu during the immune response, causing severe tissue damage.[6] Besides the potentially adverse effects of excessive ROS production during infection, inappropriate ROS production by phagocytes, for instance induced by non-specific antibody clustering in absence of infection, may also be detrimental and aggravate autoimmune inflammatory diseases.[7] Hence, the production of ROS requires tight regulation by the immune system: excessive ROS production will ensure microbial killing but can result in tissue damage. On the other hand, restrained ROS production may lead to insufficient microbial killing, which could result in life-threatening infections.

We recently described signal inhibitory receptor on leukocytes-1 (SIRL-1 encoded by *VSTM1*), an inhibitory receptor specifically expressed by human monocytes, neutrophils and eosinophils.[8] SIRL-1 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the intracellular tail that adhere to the consensus sequence V/L/I/SxYxxV/L/I.[9] The role of ITIM-bearing receptors in inhibiting signals relayed by immunoreceptor tyrosine-based activation motifs (ITAMs) is particularly well recognized. ITAMs are the principal me-

diators of signal transduction for FcR, c-type lectins and various other receptors. In accordance with the established role for ITIM-bearing receptors in inhibiting ITAM signaling, we have previously demonstrated that SIRL-1 can fully inhibit FcεRI-mediated degranulation.[8] As with most ITIM-bearing receptors,[10;11] SIRL-1 can recruit the SH2-domain containing tyrosine phosphatases SHP-1 and SHP-2 to mediate its inhibitory function.[8]

Here, we demonstrate that cytokine-induced phagocyte activation and microbial recognition through PRR on phagocytes leads to a down-regulation of SIRL-1 expression. Moreover, we show that SIRL-1 can reduce microbial killing by human phagocytes through selective inhibition of FcR -mediated ROS production, demonstrating an important biological role for this receptor in the regulation of phagocyte activity.

Materials and Methods

Antibodies and cDNA constructs

The SIRL-1-specific monoclonal antibody (mAb) 1A5 of the mouse IgG1 type has been described before.[8] Aliquots of the antibody were FITC-conjugated[8] and biotin-conjugated to facilitate flow cytometry analysis. Biotin was purchased from Pierce (Rockford, IL, USA). Streptavidin Pe-Cy7 was purchased from Biolegend (San Diego, CA, USA). FITC- and biotin-conjugated mouse IgG1 isotype control MAb, anti-CD11b APC, anti-FcγRII FITC and anti-FcαR PE were from BD Biosciences (San Jose, CA, USA). Anti-FcγRI FITC was from Immunotech (Praha, Czech Republic). Non-conjugated IgG1 isotype control MAb was from eBiosciences (San Diego, CA, USA). Anti-CD14 was purchased from Biolegend, anti-CD16 and anti-HLA-DR were from eBiosciences. Human IgA was from MP Biomedicals. IV.3 Anti-FcγRII was a kind gift from Dr. Leusen (UMC Utrecht). Anti-Triggering Receptor Expressed on Myeloid cells (TREM) was purchased from R&D systems. Sheep anti-FITC-biotin was from Southern Biotech (Birmingham, AL, USA), streptavidin APC was from BD. SIRL-1 cDNA constructs in retroviral pMX vectors were described before[8] and comprise wild type SIRL-1-(YY) and SIRL-1 tyrosine-phenylalanine (tyr-phe) mutants of the N-terminal ITIM (FY); the C-terminal ITIM (YF) and both ITIMs (FF).

Cell lines

PLB-985 (further referred to as PLB) is a human myeloid leukemia cell line originating from HL-60 cells,[12] and was kindly provided by Dr. Van den Berg (Sanquin, Amsterdam, The

Netherlands). HEK293T cells were used for production of infectious particles in retroviral transduction experiments. All cells were cultured in RPMI 1640 media (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Bodinco, Alkmaar, The Netherlands), and antibiotics. To obtain stable expression of SIRL-1 and SIRL-1 tyr-phe mutants, retroviral pMX vectors containing SIRL-1 cDNAs were packaged by the pCL-ampho system[13] and virus was used to infect target cells. Transfected cell lines were sorted on a FACSaria to obtain comparable SIRL-1 expression. To induce maturation, PLB cells were seeded at a density of $2*10^5$ cells/ml in normal culture media supplemented with 0.65% (v/v) dimethylformamide (DMF) (Sigma-Aldrich, Munich, Germany) for granulocytic differentiation[14] or with 50 nmol/L 1,25-dihydroxyvitamin D3 (Vit D3) (Sigma-Aldrich) for monocytic differentiation[15] for 4-5 days. The differentiation state was verified by flow cytometric analysis of mature neutrophil and monocyte markers such as CD11b, CD14 and FcαR expression (Figure S1). Differentiated PLB were also stained with anti-SIRL or an isotype control mAb. In addition, we examined expression of FcγRI, FcγRII, FcγRIII and FcαR (Figure S2a).

Analysis of SIRL-1 expression on monocyte subsets, and activated monocytes and granulocytes

Peripheral blood was obtained from healthy volunteers. All donors gave written informed consent and protocols were approved by the institutional review board. Mononuclear cells and granulocytes were isolated by Ficoll-Histopaque density gradient centrifugation. Monocytes were isolated from mononuclear cells using a CD14-positive selection MACS kit (Miltenyi Biotec, Auburn, CA, USA). Monocytes and granulocytes were gated on base of forward and side scatter using flow cytometry. To analyze monocyte subsets, cells positive for HLA-DR were further examined for CD16 and CD14 expression. Monocyte subsets comprising CD14- CD16+, CD14+ CD16+ and CD14+ CD16- cells were analyzed for SIRL-1 expression. To analyze SIRL-1 expression on stimulated phagocytes, samples were incubated with LPS ($1*10^{-8}$ ng -1 ng/ml) (Sigma-Aldrich), Curdlan (1 µg -100 µg/ml) (Wako biochemicals), Pam3Cys (100 ng -10 µg/ml) (Bachem biosciences, Weil am Rhein, Germany) and tumor necrosis factor-α (TNF-α) (250 U/ml - $4*10^3$ U/ml) (Peprotech, London, UK) for monocytes, or with Curdlan (1 µg -100 µg/ml) and TNF-α (250 U/ml - $4*10^3$ U/ml) for granulocytes for 0.5 hour to overnight at 37°C. Samples were stained with anti-SIRL-1 or an isotype control mAb, with anti-CD14 for monocytes and analyzed using flow cytometry.

Cytokine analysis

Peripheral blood was obtained from healthy volunteers. Mononuclear cells and granulocytes were isolated by Ficoll-Histopaque density gradient centrifugation. Neutrophils and monocytes were stimulated with LPS (0.1 ng -1 µg/ml), Curdlan (1-30 µg/ml), human IgA (3 µg/ml), anti-FcγRIIa (3 µg/ ml) or anti-TREM (1-10 µg/ml), combined with anti-SIRL-1 (1-10 µg/ml) or isotype control mAb stimulation in the same concentration. After overnight stimulation, culture supernatants were analyzed for TNF-α, interleukin-6 (IL-6) or IL-8 by ELISA (eBioscience, San Diego, USA and Sanquin, Amsterdam, The Netherlands, respectively).
VitD3-differentiated PLB cells were stimulated with 10 µg/ml immobilized human IgA, combined with 10 µg/ml anti-SIRL-1 or isotype control mAb. After overnight stimulation, cell culture supernatants were collected and concentrations of TNF-α and macrophage inflammatory protein-1α (MIP-1α) were measured with the Bio-Plex system in combination with the Bio-Plex Manager software, version 4.0 (Bio-Rad, Hercules, CA), which uses the Luminex xMAP technology as described previously.[16]

Measurement of NADPH-mediated ROS production

ROS production was measured in freshly isolated monocytes or granulocytic or monocytic differentiated PLB cells stably transduced with SIRL-1 or SIRL-1 tyr-phe mutants. NADPH-oxidase activity was assessed as H_2O_2 formation and determined by Amplex Red (Molecular Probes, Breda, The Netherlands), which reacts with H_2O_2 to produce fluorescent resorufin in the presence of horseradish peroxidase (HRP) (Sigma-Aldrich). The assay was performed in microfluor white plates from Thermo. Differentiated cells were resuspended in HEPES buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1,2 mM KH_2PO_4, pH 7.4 supplemented with 0.5% human serum albumin (HSA) (Sigma), 1 mM Ca^{2+} and 5 mM D-glucose) containing 50 µM Amplex Red and 1 U/ml HRP and stimulated ($1*10^6$ cells/ml) with 20 µg/ml human IgG, 10 µg/ml plate-bound human IgA or 10 µg/ml anti-FcγRII and 5 to 10 µg/ml anti-SIRL-1 or an isotype control mAb. Formation of H_2O_2 was measured directly from the start of stimulation and continued for one hour. Fluorescence was measured at one-minute intervals (Ext / Em = 545 / 590 nm). Measurements were corrected for spontaneous H_2O_2 production, as determined by incubation of cells in non-coated wells. The percentage of SIRL-1-mediated inhibition of ROS production was calculated by subtraction of background H_2O_2 production and subsequent calculation of the area under the

curve (AUC) of all samples. The AUC of IgA co-coated with an isotype control mAb was set at a hundred percent and compared to the AUC of IgA co-coated with anti-SIRL-1.

Bactericidal assay and phagocytosis

A stock of *Staphylococcus epidermis (S. epidermis)* was kindly provided by Dr. Ing. Van Kessel (UMC Utrecht, The Netherlands). Bacteria were grown in Luria–Bertani (LB) broth, and frozen in LB supplemented with 15% glycerol. Part of the culture was resuspended in 0.1 M sodium carbonate buffer, pH 9.6 and incubated with 1 mg/ml FITC (Molecular probes) at 37°C for one hour. FITC-labeled bacteria were washed twice with phosphate buffered saline (PBS) and stored at -20°C for future use. The buffer used for both bactericidal and phagocytosis assays consisted of RPMI supplemented with 0.05% HSA. To cross-link SIRL-1 on these samples, neutrophils were incubated with 20 µg/ml anti-SIRL-1 mAb, or as negative controls with 20 µg/ml anti-Major Histocompatibility Complex (MHC) I or an isotype control mAb for 20 minutes on ice. Samples were washed twice with PBS and bound mAbs were cross-linked using a final concentration of 20 µg/ml goat anti-mouse F(ab)2 fragments. Phagocytosis was assessed by flow cytometry. FITC-labeled *S. epidermis* were opsonized with 1% heat-inactivated human pooled serum and incubated with neutrophils for 15 minutes (Multiplicity of Infection (MOI) = 20), after which samples were placed on ice. To discriminate between intracellular and extracellular bacteria, neutrophils were stained with sheep anti-FITC-biotin followed by streptavidin APC. Percentages internalized bacteria were calculated by [percentage FITC+APC- cells] / [percentage FITC+APC+ cells] *100. The mean fluorescent intensity (MFI) of FITC+APC- cells was used as an indicator for the number of internalized bacteria.

For bactericidal assays, neutrophils were incubated with opsonized live *S. epidermis* (MOI = 20), for 15 minutes at 37°C, after which 100 µg/ml gentamicin was added and incubated for 20 minutes at 37°C. Neutrophils were washed in PBS and lysed in 0.1% Triton X-100. Lysate dilutions were plated on LB agar plates and incubated overnight at 37°C. Numbers of colonies were used as an indicator for the number of viable bacteria.

Statistical analysis

The indicated data were analyzed using SPSS 15.0 software (SPSS, Chigaco, Illinois, USA). A p-value of ≤ 0.05 was considered statistically significant.

Results

SIRL-1 expression is down-regulated upon phagocyte activation

Most CD14+ monocytes express high levels of SIRL-1, although a subset of monocytes is typically low/intermediate for SIRL-1 expression.[8] We previously reported that, without antibody-mediated SIRL-1 cross-linking, monocytes that exhibit low/intermediate SIRL-1 expression produce increased amounts of TNF-α upon stimulation with LPS or the Dectin-1 agonist Curdlan compared to monocytes with high SIRL-1 expression.[8] We tested whether this could be explained by differential SIRL-1 expression by monocyte subsets. By far the largest monocyte subset includes CD14+CD16- cells, which consist of 80-90% of the total monocyte population in peripheral blood. Smaller subsets consist of CD14+CD16+ and CD14-CD16+ monocytes.[17] The CD16+ monocytes are considered pro-inflammatory and increased numbers are found during acute inflammation, with these cells producing high levels of TNF-α upon LPS stimulation.[17] Besides monocytes, neutrophils and NK cells also express CD16. To exclude these cells from the analysis, HLA-DR+ cells were gated and further analyzed for CD16, CD14 and SIRL-1 expression (Figure 1A, left panel). We found that CD16+ inflammatory monocyte subsets had a lower expression of SIRL-1 than the CD16-CD14+ population (Figure 1A, right panel).

We next determined whether SIRL-1 expression was affected by cell activation. CD14+ mononuclear cells and isolated granulocytes from peripheral blood were monitored for SIRL-1 expression after stimulation with Curdlan. We observed a rapid down-regulation of SIRL-1 expression for both monocytes (Figure 1B left panel) and granulocytes (Figure 1B right panel). We then determined whether specific activation signals had a differential effect on SIRL-1 expression. Mononuclear cells were stimulated with Curdlan, the TLR2 agonist Pam3Cys, LPS or TNF-α, and isolated granulocytes were stimulated with Curdlan or TNF-α for the indicated time points and CD14+ mononuclear cells and granulocytes were monitored for SIRL-1 expression. For both monocytes and granulocytes, all stimulations resulted in a rapid down-regulation of SIRL-1 expression (Figure 1C and Figure 1D respectively). SIRL-1 expression progressively decreased up to four hours after addition of the stimulus in both monocytes and granulocytes (Figure 1B-D). To determine whether the down-regulation was concentration dependent, we next incubated monocytes with various concentrations of LPS and Curdlan. Indeed, increased concentrations of stimulus resulted in progressively decreased SIRL-1 expression (Figure 1E). Also in granulocytes, down-regulation of SIRL-1 expression was dose-dependent for both Curdlan and TNF-α (Figure 1F).

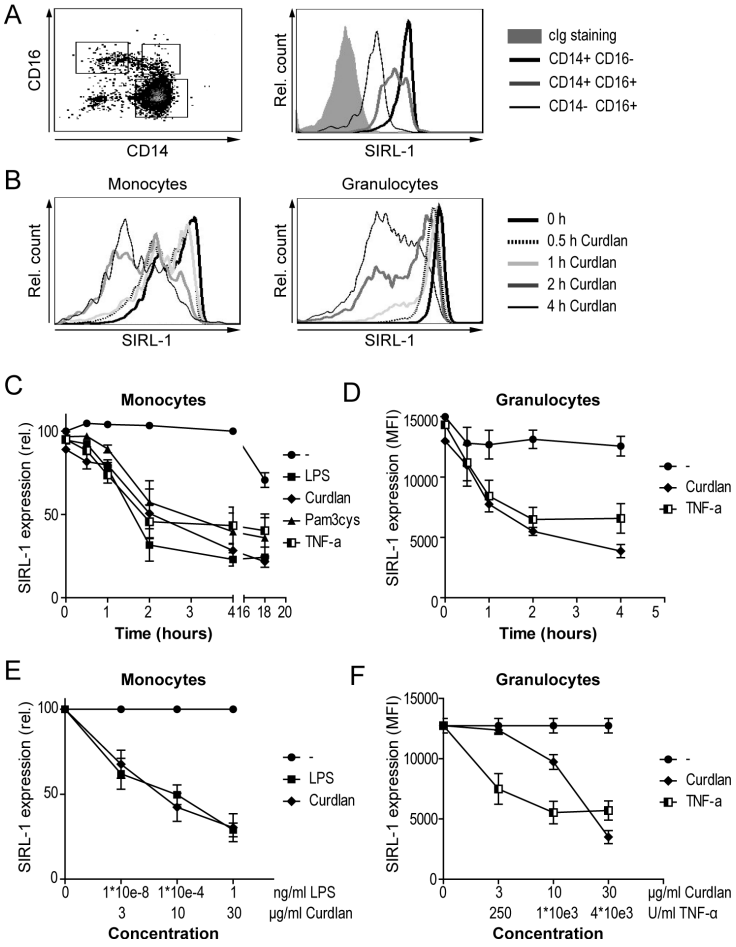

Figure 1. SIRL-1 is differentially expressed by monocyte subsets and down-regulated after phagocyte stimulation. (A) PBMC were isolated from human peripheral blood by Ficoll density gradient centrifugation and analyzed by flow cytometry. Monocytes were gated on base of forward and side scatter, cells positive for HLA-DR were further examined for CD16 and CD14 expression (left panel). Subpopulations of CD14- CD16+, CD14+ CD16+ and CD14+ CD16- cells were analyzed for SIRL-1 and HLA-DR expression (right panel). The grey histogram represents the isotype control and showed comparable fluorescence intensity for all monocyte subsets. A representative example of 3 donors is shown. (B) Using flow cytometric analysis, isolated mononuclear cells and granulocytes were gated on base of forward and side scatter, and monocytes were further gated on CD14 expression. Cells were stimulated with 1 µg/ml Curdlan and intensity of SIRL-1 expression was determined at indicated time-points. A representative donor is shown for monocytes (left panel) and granulocytes (right panel). (C) Isolated mononuclear cells were stimulated with 1 µg/ml Curdlan, 1 ng/ml LPS, 1 µg/ml Pam3Cys or $1*10^3$ U/ml TNF-α for the indicated time points. Intensity of SIRL-1 expression was determined on CD14+ monocytes. SIRL-1 expression at t=0 was set on a hundred percent. (D) Isolated granulocytes were stimulated with 1 µg/ml Curdlan or $1*10^3$ U/ml TNF-α for the indicated time points and intensity of SIRL-1 expression was determined. (E) CD14+ monocytes were stimulated for four hours with the indicated concentrations of Curdlan and LPS. (F) Isolated granulocytes were stimulated for four hours with the indicated concentrations of Curdlan and TNF-α. N=3 for all experiments. Error bars represent SEM.

Concluding, SIRL-1 expression is lower on inflammatory monocyte subsets and is rapidly down-regulated upon activation of phagocytes by PRR or cytokines.

SIRL-1 cross-linking does not affect cytokine or chemokine production

We previously showed that cross-linking SIRL-1 on monocytes upon stimulation of Dectin-1 did not influence the production of TNF-α.[8] To further evaluate whether cytokine production is indeed unaffected by SIRL-1 activation, we transfected SIRL-1 in the human myeloid cell line PLB, lacking endogenous SIRL-1 expression (Figure 2A), and differentiated cells towards a monocytic phenotype as described in *Material and Methods*. Differentiated PLB express high levels of FcαR, lower levels of FcγRII, and hardly any FcγRI and FcγRIII (Figure S2A). Thus, to investigate whether SIRL-1 could affect FcR signaling we stimulated cells with human IgA and anti-SIRL or isotype control mAb, and monitored production of TNF-α and MIP-1α. No difference in FcαR-induced cytokine production was found between wild type PLB and SIRL-1 transfected PLB, nor was the production affected by SIRL-1 cross-linking (Figure 2B). Similarly in primary monocytes and granulocytes, SIRL-1 cross-linking did not affect TNF-α, IL-8 or IL-6 production induced by ITAM-bearing receptors such as FcαR, FcγRIIa or TREM (data not shown). Since it has been recently reported that immune inhibitory receptors can regulate TLR-induced cytokine production,[18-20] we next tested whether SIRL-1 could play a similar role. LPS-induced TNF-α and IL-8 production in monocytes and granulocytes was also unaffected by antibody-mediated SIRL-1 cross-linking (Figure 2C). Together, these results demonstrate that SIRL-1 cross-linking has no effect on inflammatory cytokine or chemokine production by phagocytes upon ITAM- or TLR-mediated activation.

SIRL-1 cross-linking inhibits ROS production in PLB cells and primary monocytes

An important aspect of the phagocyte effector function is the rapid elimination of pathogens, mediated by NADPH oxidase-dependent ROS production.[3] Monocyte differentiated PLB cells generate ROS in response to FcαR stimulation (Figure 3A). Concomitant SIRL-1 cross-linking inhibited ROS production in SIRL-1 transfected PLB cells (Figure 3A-B). We also observed ROS production after stimulation with IgG (Figure S2B), although to a lower extent than found in IgA-stimulated PLB cells. Again, SIRL-1 cross-linking inhibited ROS production (Figure S2B), indicating that SIRL-1 can inhibit both FcαR- and FcγR-mediated ROS production. To determine the role of individual SIRL-1 ITIMs in the inhibitory effect, we transfected PLB cells with SIRL-1 tyr-phe mutants including mutation of the

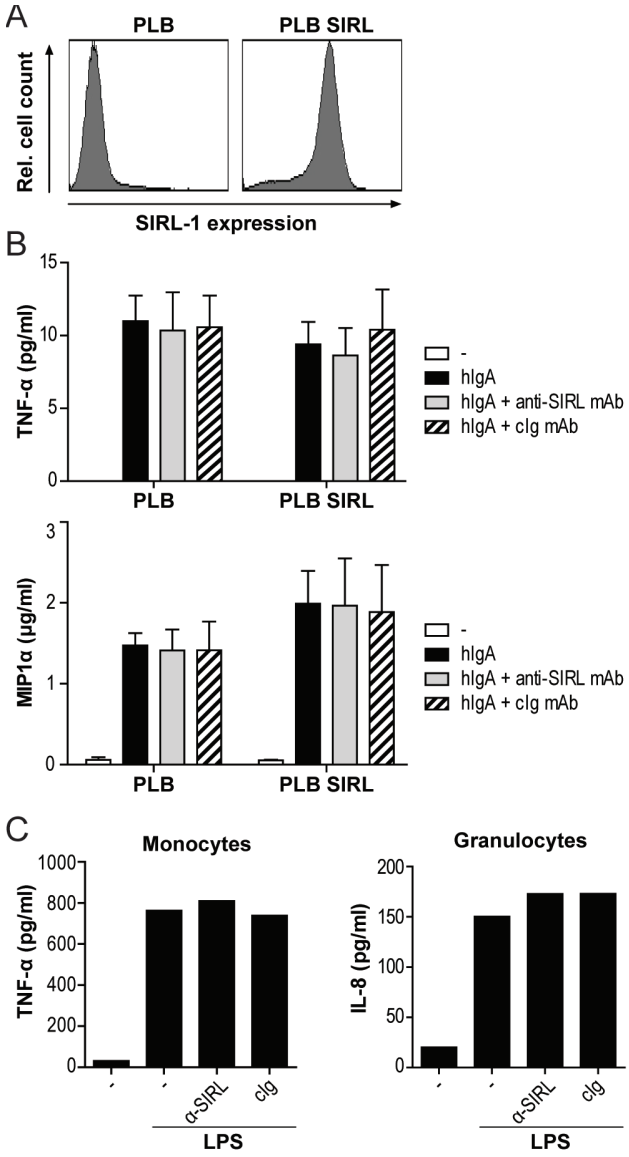

Figure 2. SIRL-1 signaling does not affect cytokine production by phagocytes. (A) wild type and SIRL-1 transfected PLB cells were matured towards a monocytic phenotype and stained for SIRL-1 expression. (B) Differentiated cells were stimulated with 10 µg/ml of immobilized IgA and anti-SIRL mAb. After 24h, supernatants were collected and concentrations of TNF-α and macrophage inflammatory protein 1α (MIP-1α) were measured with the Bio-Plex system. Error bars represent SEM, n=3. (C) Mononuclear cells and granulocytes were isolated from human peripheral blood by Ficoll-Histopaque density gradient centrifugation. Monocytes were enriched by anti-CD14 MACS separation. Various concentrations of LPS were used in 5 different experiments. In the representative sample depicted, monocytes and neutrophils were stimulated with 0.3 µg/ml LPS and SIRL-1 was cross-linked by 10 µg/ml immobilized anti-SIRL mAb. After overnight incubation, supernatants were collected and analyzed for TNF-α and IL-8 production.

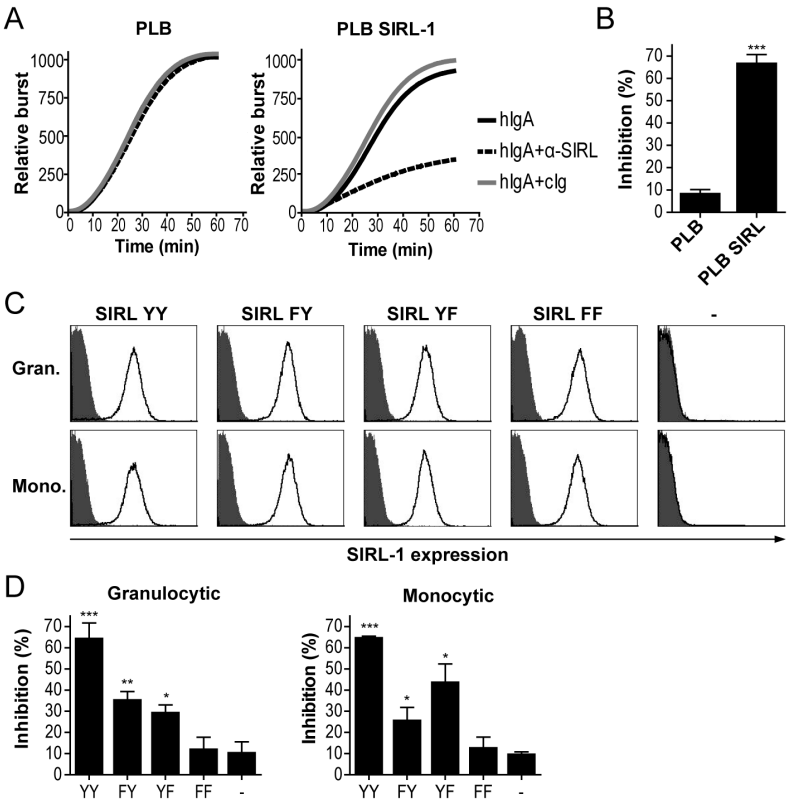

Figure 3. SIRL-1 ligation abrogates ROS production in PLB cells. Non-transfected and SIRL-1 transfected PLB cells were matured towards a monocytic phenotype. Cells were subsequently stimulated with 10 µg/ml plate-bound human IgA (hIgA) and 10 µg/ml anti-SIRL-1 or an isotype control mAb. Measurements were corrected for spontaneous H_2O_2 production. (A) Plots represent the background-corrected cumulative ROS production for WT and SIRL-1 transfected PLB with and without SIRL-1 cross-linking. A representative example is shown (n=5). (B) Inhibition of IgA-induced ROS production by monocyte-differentiated PLB cells and PLB cells transfected with SIRL-1. The percentage of SIRL-1-mediated inhibition of ROS production was calculated by subtraction of background H_2O_2 production and subsequent calculation of the area under the curve (AUC) of all samples. The AUC of hIgA co-coated with an isotype control mAb was set at a hundred percent and compared to the AUC of hIgA co-coated with anti-SIRL. The IgA and anti-SIRL mAb stimulated conditions were analyzed using an independent samples T test. Relative ROS production of SIRL-1 was compared to non-transfected PLB cells (*** = $P \leq 0.001$). Error bars represent SEM (n=5). (C) Expression of SIRL-1 on PLB cells stably transfected with wild type SIRL-1- (YY) and SIRL-1 tyr-phe mutants which include mutation of the N-terminal ITIM (FY); the C-terminal ITIM (YF) and both ITIMs (FF). Non-transfected PLB cells were taken as a control. Cells were matured towards a neutrophilic phenotype or towards a monocytic phenotype. Cells were analyzed by flow cytometry after staining with anti-SIRL or an isotype control mAb (open histograms and grey histograms respectively). (D) Inhibition of IgA-induced ROS production by PLB cells transfected with WT SIRL-1 and mutants and differentiated towards a granulocytic or monocytic phenotype. The percentage of SIRL-1-mediated inhibition of ROS production was calculated as described in *(B)*. The IgA and anti-SIRL mAb stimulated conditions were analyzed using an independent samples T test. Relative ROS production of SIRL-1 and SIRL-1 tyr-phe mutants was compared to non-transfected PLB cells (* = p-value (P) ≤ 0.05; ** = $P \leq 0.01$; *** = $P \leq 0.001$). Error bars represent SEM (n=3).

N-terminal ITIM (FY); the C-terminal ITIM (YF) and both ITIMs (FF). Comparable expression of all mutants was confirmed by flow cytometry (Figure 3C). PLB cells can also be differentiated towards a granulocytic phenotype, enabling an analysis of the effect of SIRL-1 signaling in both cell lineages. SIRL-1 transfected PLB cells were differentiated and subsequently stimulated with IgA combined with anti-SIRL or an isotype control mAb. Cross-linking of either of the SIRL-1 single tyr-phe mutants (FY and YF) resulted in a partial inhibition of ROS production, whereas the FF mutant completely lost its inhibitory capacity (Figure 3D). Thus, SIRL-1 ITIM-mediated signaling can inhibit FcR-mediated ROS production in both granulocytic and monocytic cell types, and both of the SIRL-1 ITIMs contribute to this effect.

We next tested whether SIRL-1 stimulation could likewise affect ROS production in primary human immune cells. Freshly isolated monocytes from healthy donors were stimulated with IgA (Figure 4, top panels) together with anti-SIRL or an isotype control mAb. Although variable, an inhibitory effect of SIRL-1 cross-linking was demonstrated in all donors tested. We next investigated whether SIRL-1 cross-linking could also inhibit FcγR-induced

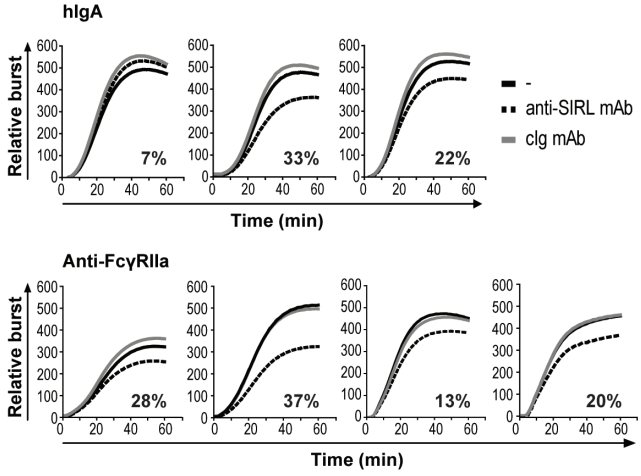

Figure 4. SIRL-1 cross-linking reduces ROS production in primary monocytes. PBMC were isolated from human peripheral blood by Ficoll density gradient centrifugation and enriched for monocytes by anti-CD14 MACS separation. Monocytes were stimulated by 10 µg/ml plate-bound human IgA (hIgA) (n=3) or IV.3 anti-human FcγRIIa (n=4) and 5 µg/ml anti-SIRL-1 or an isotype control mAb. Formation of H_2O_2 was measured directly from the start of stimulation and continued for one hour. Measurements were corrected for spontaneous H_2O_2 production, as determined by incubation of cells in non-coated plates. Plots represent the background-corrected cumulative ROS production per donor with and without SIRL-1 cross-linking. The percentage of SIRL-1-mediated inhibition of ROS production was calculated by subtraction of background H_2O_2 production and subsequent calculation of the area under the curve (AUC) of all samples. The AUC of hIgA or anti-human FcγRII co-coated with an isotype control mAb was set at a hundred percent and compared to the AUC of hIgA co-coated with anti-SIRL. Percentage inhibition is indicated per plot (n=6 donors).

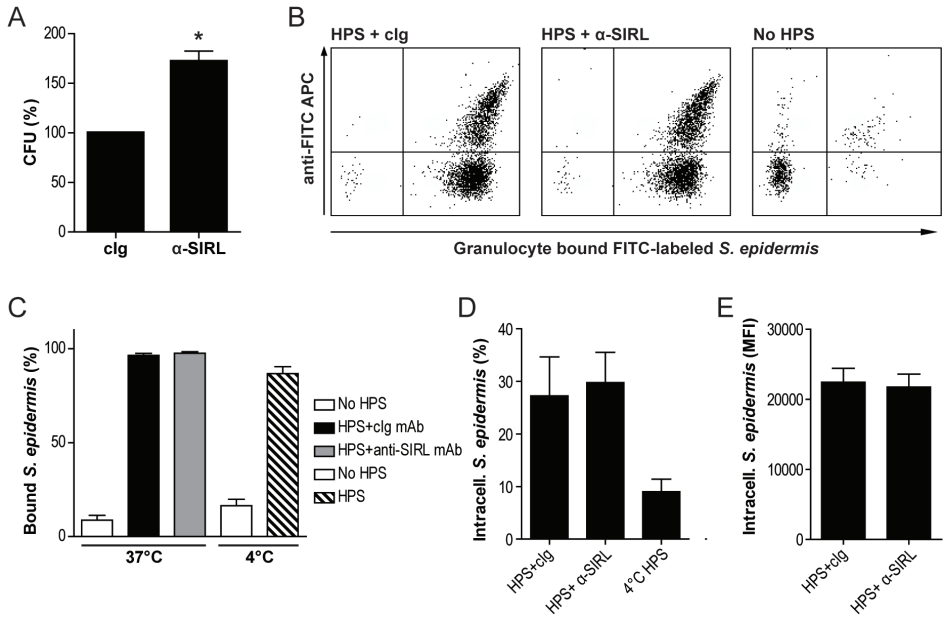

Figure 5. SIRL-1 cross-linking hampers microbial killing. Granulocytes were incubated with anti-SIRL or an isotype control mAb. After washing, samples were cross-linked using anti mouse F(ab)2 fragments and incubated with heat-inactivated human pooled serum (HPS)-opsonized *S. epidermis* (MOI= 20) for 15 min. (A) Extracellular bacteria were killed by incubation with gentamicin for 20 min, granulocytes were lysed, and lysate dilutions were plated and left overnight. The number of colony forming units (CFU) for each sample was determined. The number of CFU after anti-SIRL mAb treatment is shown relative to the isotype control, which is set on a 100%. Isotype and anti-SIRL mAb treated conditions were analyzed using a Wilcoxon signed ranks test, * = p-value (P) ≤ 0.05. Mean results of 6 donors analyzed in two independent experiments are shown. Error bars represent SD. (B) Phagocytosis experiments were performed as described above, using FITC-labeled *S. epidermis* to distinguish granulocyte-interacting bacteria by flow cytometry. To discriminate between intracellular and extracellular bacteria, granulocytes were stained with anti-FITC-biotin followed by streptavidin APC. Representative dotplots are shown. (C) Percentages of neutrophil-bound FITC-labeled *S. epidermis*, with and without opsonization with HPS and after anti-SIRL or isotype control mAb treatment. Incubations at 4°C were taken as a control. (D) Percentage of intracellular FITC-labeled *S. epidermis* after anti-SIRL or isotype control mAb treatment. Incubations at 4°C were taken as a control. (E) FITC mean fluorescence intensity (MFI) of intracellular FITC-labeled *S. epidermis* after anti-SIRL or isotype control mAb treatment.

ROS production in primary monocytes. Monocytes can express FcγRI, FcγRIIa, the inhibitory FcγRIIb, and FcγRIII.[21] We stimulated monocytes using IV.3 mAb, which specifically recognizes FcγRIIa (Figure 4, bottom panels), together with anti-SIRL or an isotype control mAb. Again, an inhibitory effect of SIRL-1 cross-linking was demonstrated in all donors tested. These data demonstrate that SIRL-1 cross-linking inhibits FcR-mediated ROS production in freshly isolated monocytes.

SIRL-1 inhibits killing of microbes without affecting phagocytosis

Since the oxidative burst is bactericidal, one would expect that reduced ROS production due to SIRL-1 activation results in reduced killing of phagocytosed bacteria. To test this, we determined the colony-forming capacity of serum-opsonized *S. epidermis* that were first phagocytosed and used this as an index for bacterial viability. Indeed, a significantly increased number of viable bacteria were retrieved from granulocytes after SIRL-1 cross-linking compared to controls (Figure 5A). To rule out the possibility that this effect was due to enhanced binding of the bacteria to granulocytes via anti-SIRL mAb rather than through SIRL-1 signaling, we also incubated granulocytes with a control anti-MHC I mAb. Similar bacterial counts were obtained with anti-MHC I incubation as found using the isotype control mAb (data not shown), indicating that the reduced killing is SIRL-1 dependent. To exclude the possibility that the increased number of viable bacteria resulted from increased phagocytosis by granulocytes after SIRL-1 cross-linking, we used FITC-labeled *S. epidermis* to determine percentages of phagocytosed particles by flow cytometry (Figure 5B). For a large part, FcRs mediated the bacterial binding, as this depended on serum opsonization (Figure 5C). No differences in percentages of phagocytosed *S. epidermis* were observed after anti-SIRL stimulation of granulocytes compared with an isotype control mAb (Figure 5D), nor was the mean bacterial load per cell different between anti-SIRL or control mAb cross-linking (Figure 5E) as determined by FITC MFI. This suggests that the increased bacterial counts resulted from reduced microbicidal activity by granulocytes. In conclusion, SIRL-1 does not affect phagocytosis, but reduces bacterial killing through inhibition of ROS production in human phagocytes.

Discussion

Here we demonstrate that SIRL-1 activation reduces microbial killing through inhibition of the oxidative burst. Stimulation of PRR on immune cells is critical in detecting invading microorganisms, resulting in activation of these cells and leading to production of inflammatory cytokines such as TNF-α to activate additional effector cells. Detection of bacterial products by PRR expressed on phagocytes, such as TLRs or c-type lectins, or activation of phagocytes by TNF-α results in down-regulation of SIRL-1 expression. Our data imply a role for SIRL-1 in setting the threshold for the oxidative burst in phagocytes, most likely to prevent damaging effects to tissues by inappropriate ROS production. Upon bacterial

infection, when ROS production is called for, PAMPs will induce down-regulation of SIRL-1 expression allowing the cell to mediate intracellular killing for instance in response to FcR-mediated signaling. However, stimulation of cells via PRR does not lead to a complete loss of SIRL-1 expression. A low level of SIRL-1 expression remains detectable on the cell surface, indicating that SIRL-1 may still modulate ROS production. Controlled ROS production during infection would contribute to a balanced immune response.

Up- or down-regulation of inhibitory receptor expression upon cell activation is an important level of regulation for inhibitory receptor function. For example, SIRP-α expression on macrophages is also down-regulated after LPS stimulation,[19] while expression of CD200R is up-regulated after cell activation.[20] The inhibitory receptor LAIR-1 is absent from resting neutrophils, but expression is rapidly induced upon cell activation.[22] Regulated expression thus is an important tool to tune the inflammatory response. Inhibitory receptors that are initially highly expressed may create an immune activation threshold, while receptors that are up-regulated after cell stimulation may function in the termination of the immune response.

We show that SIRL-1 can inhibit ROS production induced by FcRs, which signal via ITAMs. It is well conceivable that SIRL-1 will also inhibit ROS production mediated by other ITAM-bearing receptors, such as c-type lectins or TREMs, both of which may play a role in bacterial recognition.[23;24] A limited number of studies have reported a role for inhibitory receptors in regulating ROS production. CD300a has been reported to inhibit FcγRIIa-mediated ROS production,[25] and a similar capacity has been described for Siglec-5 and Siglec-9.[26;27] However, other studies have found an opposite role for Siglecs in enhancing ROS production.[28] The selective inhibition of ROS production by SIRL points to a specific role for this receptor in modulating phagocyte function. It remains to be determined whether other inhibitory receptors have similar capacities

Through what mechanism does SIRL-1 activation lead to reduced FcR-induced ROS production, without it affecting phagocytosis or cytokine production? Ligand-mediated activation of FcRs results in ITAM phosphorylation by Src family kinases, which also phosphorylate and activate Syk.[29] Syk is a central player, and activation eventually results in calcium influx, ROS production and phagocytosis, as well as cytokine production.[30] Through Syk, ITAM-mediated signals activate MAPK and the adaptor protein CARD9, leading to cytokine production in myeloid cells.[31-33] Although the data are not unambiguous,[32;34] these pathways may be quite distinct from pathways leading to the oxidative burst.[33;35] In contrast, the intracellular pathways leading to ROS production and phagocytosis are closely related.

Further studies are required to pin-point at what level these pathways diverge and through what signaling molecule SIRL-1 mediates its inhibitory action.

The specific inhibition of ROS production demonstrated in this study sheds new light on the classical view of inhibitory receptors that dephosphorylate upstream signaling molecules via recruitment of SHP-1 and/or SHP-2 and consequently inhibit all subsequent events.[36] Alternative effector molecules recruited by SIRL-1, SHP-1- and SHP-2-mediated dephosphorylation of molecules further downstream of Syk or kinetics of SHP-1/ SHP-2 dephosphorylation may provide an explanation for these results. In support of the first option, SIRL-1 single tyr-phe mutants can still partially inhibit oxidative burst, while earlier findings demonstrated that SHP-1 recruitment by these SIRL-1 mutants was abrogated.[8] This indicates that SIRL-1 recruits alternative molecules that contribute to the inhibitory effect.

Also for other inhibitory receptors, inhibition of specific signal transduction pathways has been reported. For example, the immune inhibitory receptor CD300a can inhibit Eotaxin-induced migration, but not Eotaxin-induced calcium influx in eosinophils.[37] It was demonstrated that CD300a signaling inhibits Eotaxin-induced JAK2, ERK and p38 phosphorylation, which could be responsible for the effects on migration. Studies with mice deficient for a single inhibitory receptor demonstrated that their function is non-redundant,[38] despite multiple inhibitory receptors being expressed simultaneously on the same immune cell. Regulation of specific effector functions may greatly contribute to the specificity of inhibitory receptors.

In conclusion, the production of tissue destructive components such as ROS by phagocytes is under tight regulation. SIRL-1 may function to determine whether the risk of tissue damage is out-ruled by the risk of infection. Excess ROS production may play an important role in the pathogenesis of diseases characterized by persistent inflammation, such as atherosclerosis, where endothelial cell-derived ROS promotes inflammation, and COPD.[39] It would be interesting to study SIRL-1 expression in these conditions. It has been proposed that macrophages and neutrophils contribute to COPD pathology, and ROS production by these cells may aggravate inflammation.[40] Intriguingly, tobacco smoke was shown to directly activate TLR4/IL-1R signaling pathways,[41] which could lead to down-regulation of SIRL-1 expression. In Epidermolysis bullosa acquisita, a chronic autoimmune subepidermal blistering disease of the skin characterized by the presence of IgG autoantibodies, the neutrophil-mediated oxidative burst also contributes to autoantibody-induced tissue damage.[7] It is conceivable that locally produced inflammatory cytokines lead to down-regulation of

SIRL-1 expression, thus facilitating ROS production by phagocytes. The identification of the physiological ligand for SIRL-1 would further improve our understanding of the biological role of SIRL-1 during infection and inflammatory disease.

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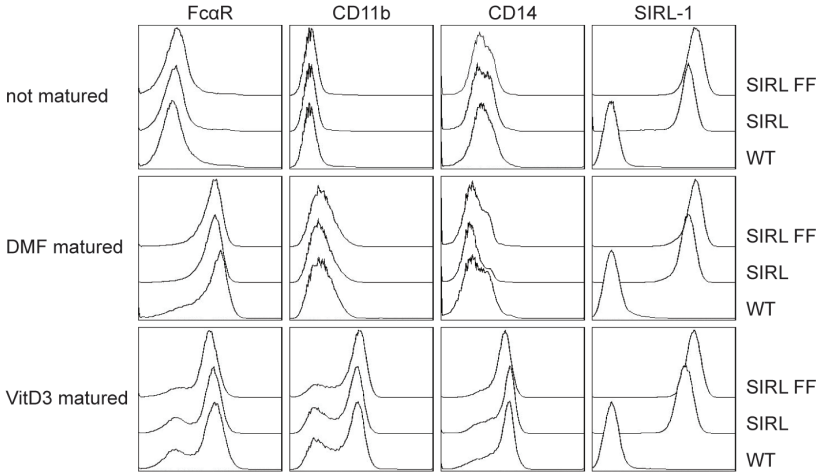

Figure S1. PLB maturation was confirmed by flow cytometric analysis of maturation markers. PLB cells were matured with Dimethylformamide (DMF) to induce a neutrophilic phenotype or with vitamin D3 (Vit D3) to induce a monocytic phenotype and analyzed after 4-5 days for FcαR, CD11b, CD14 and SIRL expression.

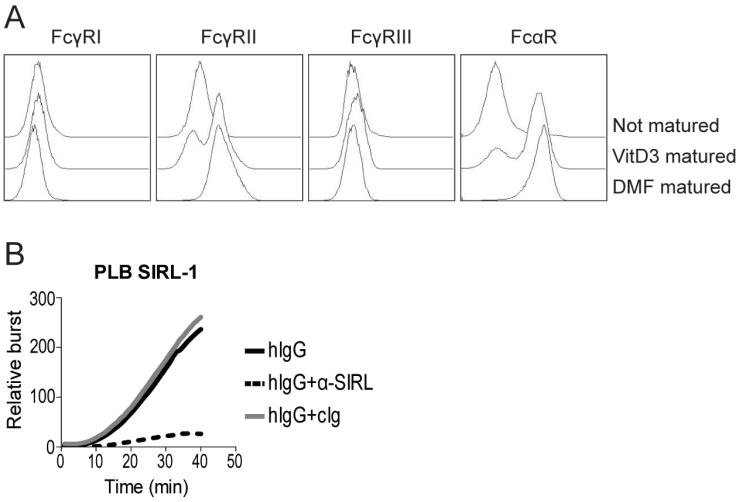

Figure S2. Expression of FcγRs and FcγR-mediated ROS production in PLB cells. (A) PLB cells were matured with Dimethylformamide (DMF) to induce a neutrophilic phenotype or with vitamin D3 (Vit D3) to induce a monocytic phenotype and analyzed after 4-5 days for FcγRI, FcγRII, FcγRIII and FcαR expression. (B) SIRL-1 transfected PLB cells were stimulated with 20 µg/ml plate-bound human IgG (hIgG) and 10 µg/ml anti-SIRL-1 or an isotype control mAb. Measurements were corrected for spontaneous H_2O_2 production. Plots represent the background-corrected cumulative ROS production with and without SIRL-1 cross-linking. Either neutrophilic differentiated or monocytic differentiated PLB cells were used (n=3 in total). The representative example shows ROS production in monocyte-differentiated PLB.

Immune inhibitory receptors: essential regulators of phagocyte function

CHAPTER 4

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Abstract

Phagocytes, including neutrophils, monocytes and macrophages, play a crucial role in host defense by recognition and elimination of invading pathogens. Phagocytic cells produce reactive oxygen species (ROS), inflammatory cytokines and chemokines, leading to bacterial killing and to recruitment and activation of additional immune cells. However, inflammatory mediators are potentially harmful for the host and their production is therefore tightly controlled by multiple regulatory mechanisms. One such mechanism is immune suppression by immune inhibitory receptors, which are increasingly acknowledged as potent regulators of the immune response. So far, research has focused on the role of these receptors in the regulation of natural killer cells, B cells and T cells. Importantly, an accumulating number of inhibitory receptors have been identified on phagocytes. Here, we review the role of inhibitory receptors in the regulation of phagocyte cytokine production, migration, apoptosis, ROS production and phagocytosis. Furthermore, we will discuss intracellular mechanisms utilized by distinct inhibitory receptors to regulate specific phagocyte functions. We demonstrate that inhibitory receptors are important regulators of the immune response, which bacteria can use to their advantage.

Introduction

Phagocytes, including neutrophils, monocytes and macrophages, can recognize, phagocytose and eliminate invading pathogens and thus have a crucial role in host defense [1]. Inherent to their killing capacity, these cells contain numerous molecules that are capable of damaging host tissue. In the process of microbial killing, lysosomal granules and reactive oxygen species (ROS) can spill in the extracellular milieu, causing severe tissue damage [2]. Excess ROS production for example plays an important role in the pathogenesis of many diseases characterized by persistent inflammation, such as atherosclerosis and chronic obstructive pulmonary disease [3]. Furthermore, bacterial infections and trauma can lead to hyper production of inflammatory cytokines, the so-called 'cytokine storm', which can rapidly result in life-threatening conditions such as septic shock. Indeed, severe sepsis is frequently fatal and annually causes as many deaths as acute myocardial infarction [4]. It is therefore not surprising that many regulatory mechanisms are required to control the inflammatory response by preventing inappropriate activation, or by timely terminating the immune response.

Inhibitory receptors on phagocytes

Immune inhibitory receptors are well established negative regulators of the immune response, with the inhibitory signal usually transduced through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) located in the intracellular tail of the receptor with the consensus sequence V/L/I/SxYxxV/L/I [5]. In recent years an expanding number of immune inhibitory receptors has been documented, and their role in B cell, natural killer (NK) cell and T cell regulation has likewise become increasingly clear. Importantly, an accumulating number of inhibitory receptors have been identified on phagocytes (Table I), and emerging evidence suggests they have an equally important regulatory role in the activation of these leukocyte populations. Here, we will discuss the state of the art on the role of inhibitory receptors in the regulation of phagocyte cytokine production, migration, apoptosis, ROS production and phagocytosis (Figure 1). Next, we will discuss intracellular mechanisms in this interplay (Figure 2) and pathogenic strategies to manipulate inhibitory receptor activation.

Regulation of cytokine production by inhibitory receptors

Micro-organisms are recognized by pathogen-associated molecular patterns (PAMPs), which can bind and activate pattern recognition receptors (PRRs) on phagocytes [6]. Patho-

Table I

Expression pattern of inhibitory receptors on phagocytes

Inhibitory receptor	N	Mo	Mφ	Ligand	ref
Ig Superfamily					
CD200R	+	+	+	CD200	[86;87]
CD300a (IRp60)	+	+	+	ND	[88]
CD300f (IREM-1)	+	+	+	ND	[89]
CEACAM1 (CD66a)	+	+	ND	CEACAM1	[33;90]
FcγRIIb	+	+	+	IgG	[91]
ILT-2 (LIR-1; LILRB1; CD85j)	-	+	+	MHC I	[92;93]
ILT-3 (LIR-5; CD85k; LILRB4)	-	+	+	MHC I	[94]
ILT-4 (LIR-2; LILRB2)	+	+	+	MHC I	[87]
ILT-5 (LIR-3; LILRB3)	+	+	+	MHC I	[95]
LAIR-1	-	+	ND	Collagen	[96]
PECAM-1 (CD31)	+	+	ND	PECAM-1 / CD177/ CD38	[8;9;97]
PILR-α (FDF03)	+	+	+	CD99-like protein HSV-1:Glycoprot B	[98-100]
PIR-B (mouse)	+	+	+	MHC I	[101-104]
SIRL-1	+	+	ND	ND	[36]
SIRP-α	+	+	+	CD47, SP-A, SP-D	[37;39]
Siglecs					
CD33	-	+	-	Sialic acid	
Siglec-5	+	+	ND	Sialic acid	[105]
Siglec-7	-	+	ND	Sialic acid	[106]
Siglec-9	+	+	ND	Sialic acid	[107]
Siglec-10	-	+	ND	Sialic acid	[19]
Siglec-11	-	+	+	Sialic acid	[108]
C-type lectins					
DCIR	+	+	+	ND	[109]
Ly49Q	+	+	+	MHC I	[110]
MICL	+	+	+	MHC I	[111]

N= neutrophil, Mo= monocyte, Mφ= macrophage, ND= not determined.

gen recognition by phagocytes induces nuclear factor kappa B (NF-κB) activation and consequently the release of chemokines and inflammatory cytokines. The chemokine interleukin (IL)-8 is highly produced by activated neutrophils, as well as other innate immune cells, and contributes to leukocyte infiltration at the site of infection. Additionally, the inflammatory cytokines tumor necrosis factor (TNF)-α, IL-1β and IL-6 will stimulate the acute phase response, induce the sensation of illness, and activate other immune cells. The role of Toll-like receptors (TLR) in inducing cytokine production has been particularly well studied.

Studies using mice deficient in a single inhibitory receptor have been helpful to characterize the role of these receptors in controlling cytokine production induced by TLR signaling.

For example, LPS administration to mice lacking the signal-regulatory protein (SIRP)-α [7] or platelet endothelial cell adhesion molecule (PECAM)-1 [8-10] results in an increased production of TNF-α, IL-6 and interferon (IFN)-β, most likely by macrophages, and these mice easily succumb to septic shock [11;12]. Both SIRP-α and PECAM-1 directly inhibit TLR4 signaling [11;13]. Studies using mice deficient in a single inhibitory receptor have been helpful to characterize the role of these receptors in controlling cytokine production induced by TLR signaling.

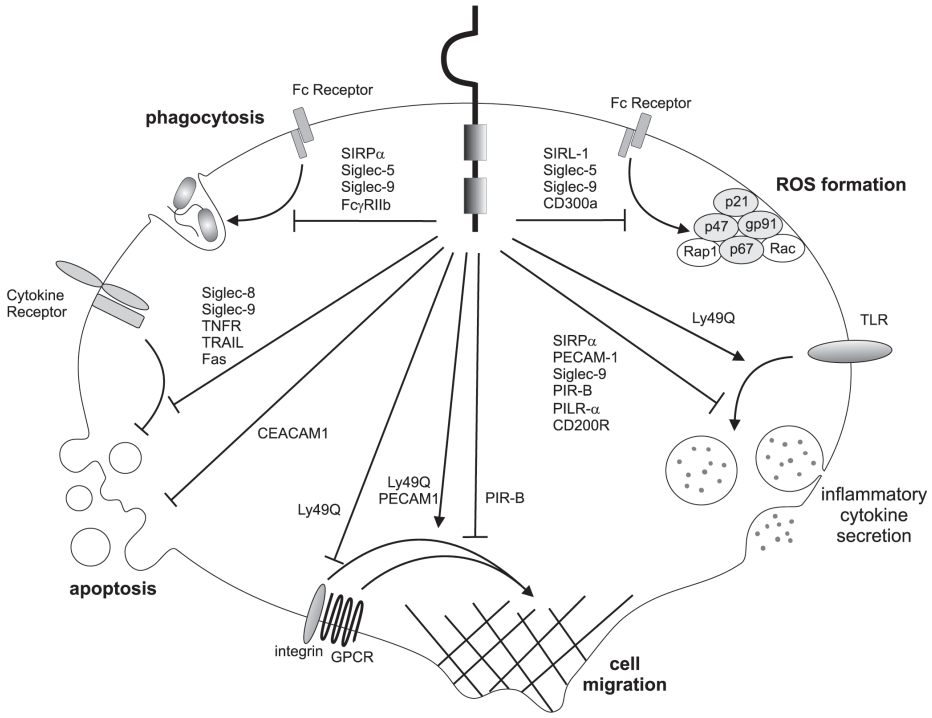

Figure 1. Inhibitory receptors can affect various phagocyte effector pathways, including ROS production, cytokine secretion, migration, apoptosis and phagocytosis, and are specific in the types of activating signals they modulate. SIRL-1 and CD300a inhibit Fc receptor-mediated ROS production in phagocytes, and a similar role has been proposed for Siglec-5 and Siglec-9. SIRP-α, PECAM-1, Siglec-5, PIR-B, PILRα, or CD200R activation leads to impaired Toll-like receptor (TLR)-induced cytokine secretion. In contrast, Ly49Q augments cytokine production after CpG DNA administration. PIR-B inhibits chemotactic responses, whereas Ly49Q and PECAM-1 are both positively involved in chemokine GPCR-induced migration in neutrophils. Neutrophil adhesion on the other hand is prevented by Ly49Q. Cytokine or growth factor-induced survival signals can be suppressed by Siglec-8, Siglec-9, TNFR, Fas and TRAIL. Conversely, CEACAM1 signaling enhances granulocyte and monocyte survival. Finally, inhibition of Fc receptor-mediated phagocytosis is demonstrated for SIRP-α, Siglec-5 and FcγRIIB.

For example, LPS administration to mice lacking the signal-regulatory protein (SIRP)-α [7] or platelet endothelial cell adhesion molecule (PECAM)-1 [8-10] results in an increased production of TNF-α, IL-6 and interferon (IFN)-β, most likely by macrophages, and these mice easily succumb to septic shock [11;12]. Both SIRP-α and PECAM-1 directly inhibit TLR4 signaling [11;13]. In contrast to the apparently similar function of these two receptors, their expression on immune cells after LPS challenge is differentially regulated. Macrophage stimulation with LPS leads to down-regulation of SIRP-α [14], whereas it results in an up-regulation of PECAM-1. This may indicate that SIRP-α and PECAM-1 regulate distinct stages of the immune response upon challenge. SIRP-α may provide an initial activation threshold to prevent activation under steady-state conditions or to prevent an excessive anti-bacterial response, whereas PECAM-1 may be more important in the termination of the immune response after the pathogen has been eliminated. Mice deficient in CD200, the ligand for CD200R, also have an increased myeloid response to inflammation and stimulation of alveolar macrophages with LPS results in an increased production of TNF-α and IL-6 by CD200 deficient mice [15]. More importantly, Influenza infection leads to an enhanced, fatal inflammation in these mice, possibly due to increased production of inflammatory mediators, such as MIP-1α, IL-6, TNF-α and IFN--γ by lung macrophages [15], although T cells also play an important role in the development of disease symptoms [16]. Another recent study showed that ligation of CD200R by CD200 can protect the host from a lethal response to Meningococcal Septicemia by inhibiting PRR-induced inflammatory cytokine production in macrophages [17]. In addition, it was shown that PRR such as TLR or nucleotide oligomerization domain 2 (NOD2) differentially up-regulate CD200 and down-regulate CD200R expression on macrophages through the NF-κB family transcription factor c-Rel [17], demonstrating that CD200R and ligand expression are tightly regulated during the immune response to ensure a proper immune response.

In contrast to these immune suppressive effects, some inhibitory receptors enhance inflammatory cytokine production. For example, the mouse inhibitory receptor Ly49Q enhances TLR9-mediated IFN-β and IL-6 production in mouse macrophages [18]. Other inhibitory receptors may specifically regulate production of inflammatory cytokines depending on the nature of the activation signal received by the cell. For example, Sialic-acid-binding immunoglobulin-like lectin (Siglec)-10 is expressed by monocytes [19]. This inhibitory receptors can inhibit inflammatory cytokine production induced by danger associated molecular pattern (DAMP) signaling through binding of CD24, whereas signaling via PAMPs is unaffected [20]. This specific regulation protects the host against a lethal response to

cell death, while allowing a potent immune response upon infection. Besides regulating pro-inflammatory cytokine production, inhibitory receptors may also regulate production of anti-inflammatory cytokines. For example, upon TLR activation, Siglec-9 not only reduces the production of pro-inflammatory cytokines, but also enhances IL-10 production through ITIM signaling [21]. Together, these studies demonstrate that inhibitory receptors can potently suppress TLR-induced inflammatory cytokine production, either by direct inhibition of the TLR signaling or indirectly by increased production of anti-inflammatory cytokines. In contrast, some inhibitory receptors may enhance inflammatory cytokine production. Finally, some inhibitory receptors do not seem involved in regulating pathogen-associated cell activation, but specifically modulate DAMP signaling. The distinct capacities of various inhibitory receptors will therefore contribute to an orchestrated immune response during successive stages of infection.

Differential effect of inhibitory receptor signaling on migration of phagocytes

Tissue-infiltration by phagocytes requires tight regulation to limit tissue damage by release of inflammatory mediators. Infiltration may be reduced directly through modulation of G protein-coupled receptor (GPCR)-mediated chemotaxis, adherence or transmigration, or indirectly by desensitization of phagocytes to these processes. Intriguingly, specific inhibitory receptors seem to have opposite effects on granulocyte migration. Mouse neutrophils deficient in paired Ig-like receptor-B (PIR-B) (the mouse ortholog of ILT2-5) have enhanced chemotactic responses *in vitro* after stimulation with macrophage inflammatory proteins (MIP)-1α, MIP-2, CCL19 and CCL21 [22], indicative of a suppressive function for this receptor. In contrast, Ly49Q is indispensable for neutrophil polarization and migration after N-*formylated* methionyl-leucyl-phenylalanine (fMLP) or cytokine-induced neutrophil chemoattractant (KC) stimulation, although it inhibits neutrophil adhesion in steady-state conditions [23]. Neutrophil polarization and infiltration into inflamed air-pouches is also impaired in Ly49Q knock-out mice [23]. PECAM-1 was originally characterized as an adhesion molecule and promotes leukocyte adherence to the endothelium and traversing through endothelial junctions and the perivascular basement membrane through homophilic PECAM-1/PECAM-1 adhesive interactions between leukocytes and endothelial cells [24]. Although adhesion in itself may be independent of signaling, it was demonstrated that PECAM-1/PECAM-1 interactions increase expression of the integrin $\alpha_6\beta_1$, involved in the migration process, on transmigrated neutrophils [25], and that PECAM-1 is essential for neutrophil

chemotaxis [26]. While the suppressive effect on migration exerted by PIR-B is in accordance with the anticipated function of an inhibitory receptor, the enhanced migration induced by Ly49Q and PECAM-1 activation is perhaps unexpected. This raises the question whether these inhibitory receptors specifically enhance migration and suppress other effector functions. Indeed, PECAM-1 has opposing effects on inflammatory cytokine production and cell migration, illustrating that not all cellular functions are suppressed. Since non-functional inhibitory receptors may predispose to excess phagocyte activation, one could speculate that a reduced migratory capacity for cells with deficient inhibitory receptor signaling would be a mechanism to prevent tissue damage by infiltrated leukocytes. This perspective shows some similarity with the licensing theory in NK cells (which states that NK cells are 'licensed' for functional competence by prior signaling through an inhibitory receptor [27]), in that immune cells that cannot be properly controlled are prevented from becoming activated.

Inhibitory receptor signaling can induce immune cell apoptosis

An ongoing immune response must be appropriately terminated to restore immune homeostasis. This process includes clearing of excess immune cells by apoptosis. Several inhibitory receptors may be involved in this process. CD33-related Siglec-8 and Siglec-9 are inhibitory receptors that have frequently been associated with increased apoptosis in myeloid cells [28]. *In vitro*, cross-linking of Siglec-9 results in increased apoptosis in resting neutrophils [29]. Moreover, inflammatory neutrophils obtained from patients with acute septic shock or rheumatoid arthritis demonstrated enhanced Siglec-9-mediated neutrophil death compared with healthy controls [29]. The increased Siglec-9-mediated cell death could be reproduced by priming of neutrophils with pro-inflammatory cytokines, such as granulocyte macrophage-colony stimulating factor (GM-CSF), IFN-α or IFN-γ *in vitro* [29]. This indicates that Siglec-9 may indeed have a role in regulating apoptosis of activated neutrophils to balance the immune response. While it has been reported that Siglecs mediate apoptosis via generation of ROS [29], others have shown that signaling via Siglec-9 reduces ROS production rather than enhancing it [30]. Thus, the mechanism leading to enhanced immune cell apoptosis remains inconclusive, but may be explained by the experimental set-up. An increase in ROS production is demonstrated after using anti-Siglec antibodies for cross-linking [29], whereas decreased ROS production was found after ligation through a bacterial ligand [30]. Besides Siglecs, death receptors of the TNF or nerve growth factor family, such as TNF-R, Fas or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

may also be important regulators of apoptosis in neutrophils, with the ITIM-like sequence in these receptors being crucial for their function [31]. Stimulation of these receptors disrupts anti-apoptotic pathways initiated by survival factors in neutrophils [31]. Conversely, carcinoembryonic antigen-related cell adhesion molecule (CEACAM)1 signaling was shown to promote survival of rat neutrophils by a delay in spontaneous and Fas ligand-induced apoptosis, which depended on CEACAM1 tyrosine phosphorylation and activation of ERK1/2 and caspase-3 [32]. CEACAM1 also protects human monocytes from spontaneous apoptosis by activating Protein Kinase B (PKB/c-akt) via phosphoinositide 3-kinase (PI3K) [33]. Thus, although signaling through a commonly shared motif, inhibitory receptors can have opposing effects on phagocyte survival.

Are inhibitory receptors involved in phagocytosis and ROS production?

Pathogen elimination is the key function of phagocytes and is achieved by phagocytosis, followed by fusion of the phagosome with lysosomal granules and elimination of trapped bacteria by degrading enzymes and ROS production [34]. The importance of ROS production in microbial killing is most apparent by the recurrent bacterial infections typical of chronic granulomatous disease (CGD), in which patients have defective ROS production due to mutations in the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex [35]. Antibody-opsonization of pathogens leads to triggering of Fc receptors, which mediate phagocytosis and ROS production. Excess ROS generation can lead to tissue damage and therefore production requires tight regulation. However, few studies have reported on the influence of inhibitory receptor signaling on ROS production, perhaps due to the paucity of studies investigating inhibitory receptor signaling in neutrophils. Signal inhibitory receptor on leukocytes (SIRL)-1, which we recently characterized as a functional inhibitory receptor on human neutrophils and monocytes [36], inhibits Fc receptor-induced ROS production in human phagocytes, leading to reduced microbial killing (Steevels et al., unpublished data).

Compared to the oxidative burst, the effect on phagocytosis by inhibitory receptors has been better studied, which is for a large part attributable to extensive studies on the role of SIRP-α. CD47 is a broadly expressed functional ligand for SIRP-α [37;38] and is present on platelets and red blood cells (RBC), inhibiting phagocytosis by binding macrophage SIRP-α [39-41]. CD47 knock-out mice have normal RBC parameters, but administration of CD47-knock-out RBC to wild type mice leads to rapid RBC clearance [39]. Expression of CD47 by healthy cells will prevent their elimination or uptake by SIRP-α expressing macrophages,

while cells that become infected or undergo apoptosis may down-regulate CD47 to facilitate phagocytosis of damaged cells by macrophages. Importantly, leukemic cells may use this to their advantage and up-regulate CD47 expression to evade immune detection and subsequent elimination [42]. It was demonstrated that the AML cell line MOLM-13 can be rescued from its *in vivo* growth defect by CD47 expression and that CD47 expression levels on MOLM-13 cells determine its tumorigenic potential [42].

Recognition and phagocytosis of apoptotic cells is critical for resolution of inflammation or maintenance of immune homeostasis, and macrophages play an important role herein. Inflammation often accompanying phagocytosis may be suppressed by recognition of phosphatidylserine and calreticulin on the surface of apoptotic cells, although the receptors responsible for this anti-inflammatory effect remain to be identified [43]. However, proteases from lysed neutrophils stimulate inflammatory cytokine production [44], suggesting that anti-inflammatory signals induced by phosphatidylserine expression can be overcome by proteases released during lysis, in which case the outcome will be determined by the predominating signal [44]. It is therefore interesting that CD200 is a p53-target gene, and CD200 mRNA and protein expression is increased in apoptotic cells [45]. While the CD200-CD200R interaction may not inhibit phagocytosis in itself, it may reduce inflammatory responses in macrophages upon phagocytosis of CD200-expressing apoptotic bodies, hence contributing to apoptotic cell-induced immune suppression. To conclude, inhibitory receptors may inhibit Fc receptor-induced ROS production, affect phagocytosis of (Ig-opsonized) particles, or possibly modulate the inflammatory response that may accompany phagocytosis.

What effector molecules mediate inhibition?

As discussed, inhibitory receptors can perform opposing roles in regulating phagocyte activation (Figure 1), but why do ITIM-bearing receptors differ in their functional outcome, while signaling through a commonly shared motif? A phosphorylated ITIM will often recruit the SH2 domain-containing tyrosine phosphatases SHP-1 and/or SHP-2 [46], which dephosphorylate upstream molecules in the activating pathway, including the receptor itself, recruited Src family kinases (SFK), and Syk family kinases [46]. SHP-1 and SHP-2 both have distinct functions in cell signaling. The importance of SHP-1 in suppressing myeloid cell activation has been demonstrated by the severe inflammatory disease, including lung inflammation, hair loss, inflamed paws, and splenomegaly, in RAG-1- and SHP-1-double

deficient mice [47]. In contrast, SHP-2 has a dual role in immune cell regulation. While SHP-2 recruited by ITIM-bearing receptors can inhibit immune cell activation [48;49], SHP-2 is also a positive regulator of cytokine and growth factor receptor signaling, mediating Ras and ERK activation by controlling C-terminal Src kinase (Csk) recruitment [50]. ITIMs differ in their affinity for SHP-1 and SHP-2, and specific recruitment may contribute to inhibitory capacity. For example, CD300a interacts only with SHP-1 [51], while Ly49Q and PECAM-1 bind both SHP-1 and SHP-2 [23;52]. This may partly explain the positive regulation in neutrophil migration for the latter two inhibitory receptors.

Furthermore, inhibitory receptors may recruit alternative molecules to inhibit cell activation. CD200R for example does not contain ITIMs, but is capable of recruiting Dok-1 and Dok-2 adapter proteins to its phosphorylated tyrosines [53]. Dok-1 binds to the SH2 domain-containing inositol 5-phosphatase (SHIP) and both Dok-1 and Dok-2 recruit RasGAP, which mediates the inhibition of the Ras/MAPK pathways [53-55]. IL-3- or FcεRI-induced activation of ERK and p38 MAPK is inhibited by CD200R engagement [53]. Recruitment of alternative molecules has also been demonstrated for various ITIM-bearing receptors. Besides recruiting SHP-1 and SHP-2, FcγRIIb and PECAM-1 can also recruit SHIP [56;57], which negatively regulates PKB recruitment [58;59] and inhibits ERK activation [60]. LAIR-1, SIRP-α and Ig-like transcript (ILT)-2 can recruit Csk [61-63] in addition to SHP-1 and SHP-2. Csk functions by phosphorylation of SFK at the C-terminal tyrosine residue, resulting in SFK inactivation [64]. Finally, CD33 and Siglec-7 can recruit suppressor of cytokine signaling 3 (SOCS3) [65]. SOCS3 acts as a pseudosubstrate inhibitor for Janus kinase (JAK) and blocks the interaction of JAK with signal transducer and activator of transcription (STAT), leading to termination of signal propagation. Hence, SOCS3 negatively regulates cytokine receptor signaling. The specific function of Siglecs in apoptosis may therefore be explained by recruitment of SOCS3. It is likely that further alternatively recruited molecules will be identified, contributing to our understanding on the mechanism of inhibitory receptor specificity.

Can inhibitory receptors only regulate activation signals that signal though Src family kinases?

As illustrated, intracellular signaling by various receptors, such as TLRs, chemokine GPCRs, and Fc receptors can be modulated by inhibitory receptors. Are inhibitory receptors limited in the range of activation signals they can regulate? The inhibitory signaling of ITIM-bearing receptors is classically studied in the context of activation signals relayed by immunorecep-

tor tyrosine-based activation motifs (ITAMs), which are phosphorylated by SFK upon receptor ligation [66]. SFKs are also implicated in the signaling of other activating receptors, such as TLR signaling [67], cytokine and growth factor receptors and integrin signaling [68]. It has been postulated that phosphorylation of ITIMs by SFK is dependent on *in trans* co-engagement of inhibitory and activating receptors. Alternatively, clustering of inhibitory receptors may be sufficient to recruit SFK that phosphorylate the ITIMs [66]. In the latter case, activation of ITIM-bearing receptors would not involve clustering with an activating receptor, and would be independent of SFK recruited by the activating receptor, thus broadening the quantity of activating signals that can be inhibited.

The role of PIR-B in chemotaxis is supportive of SFK-independent recruitment by inhibitory receptors. Neutrophils deficient in the granulocyte SFK members Hck and Fgr migrate normally through transwell filters and even show enhanced migration in response to chemoattractants [22], indicating that chemokine-induced migration does not require SFK. Nevertheless, PIR-B can negatively regulate chemokine signaling, since PIR-B deficient neutrophils show increased migration in response to chemo-attractants [22]. The fact that PIR-B phosphorylation is impaired in Hck and Fgr deficient cells [22] suggests that PIR-B is phosphorylated by SFK. Thus, enhanced migration in Hck and Fgr deficient cells may be due to lack of signaling by PIR-B and possibly other inhibitory receptors. This illustrates that the inhibitory capacity of ITIM-bearing receptors is not dependent on SFK recruited by activating receptors and broadens the range of activating signals that are possibly modulated.

How do SHP-1 and SHP-2 mediate inhibition or activation?

As already discussed, inhibitory receptors may recruit alternative molecules to modulate activation pathways. Nevertheless, SHP-1 and SHP-2 are generally engaged by ITIM-bearing receptors, and their inhibitory capacity is often impaired in SHP-1/2 deficient cells [69-71]. Classically these phosphatases are considered to dephosphorylate upstream molecules in the activating pathway [46] and consequently inhibit all downstream events [46]. However, the observation that some inhibitory receptors show selective inhibition of specific signal transduction pathways may argue against the dogma of upstream inhibition. CD300a for example, inhibits Eotaxin-induced transmigration and cytokine production, but not Eotaxin-induced Ca^{2+} mobilization [72]. This could be explained by kinetics or degree of phosphorylation. CD300a may reduce phosphorylation of an activating molecule to a certain degree, which could be permissive for Ca^{2+} mobilization, while hampering transmigration

and cytokine production. Alternatively, it may suggest that CD300a does not induce dephosphorylation of an upstream signaling molecule. This raises the question whether ITIM-recruited SHP-1 and SHP-2 exclusively inhibit cellular activation through dephosphorylation of upstream events.

Two major signaling pathways can be used by TLRs [73]. TLR signaling can activate Myd88, which in turn activates IL-1 receptor associated kinase1 (IRAK1), through IκB kinases (IKK) complex formation leading to production of inflammatory cytokines such as TNF, IL-1 and IL-6 [73]. An alternative pathway involves activation of Toll-IL-1R domain-containing adaptor-inducing IFN-β (TRIF), which induces activation and nuclear translocation of IFN-regulatory factors (IRFs), leading to type I IFN production [73]. SHP-1 has been shown to inhibit TLR-mediated IRAK1 phosphorylation, hence reducing inflammatory cytokine production, but promoting type I IFN production [74]. SHP-2 has a dual role in TLR regulation, it can negatively regulate both IRAK1 and TRIF activation, which leads to reduced type I IFN and pro-inflammatory cytokine production [75]. Conversely, SHP-2 is required for IKK complex formation [76] and thus also essential for pro-inflammatory cytokine production.

Interestingly, Kong and co-workers postulated that SIRP-α negatively regulates cytokine production by sequestration of SHP-2 away from IKKs [77], providing a novel mechanism by which an inhibitory receptor may exert its function. Indeed, phosphatase recruitment by inhibitory receptors may generally influence signaling pathways by affecting cellular location rather than by the phosphatase activity itself. Sasawatari and colleagues have reported that Ly49Q is constitutively associated with SHP-1 and associates with SHP-2 only upon cell stimulation. Sustained Src kinase activation by fMLP and integrins is dependent on Ly49Q with an intact ITIM and it was postulated that Ly49Q recruitment of SHP-2 to the lipid raft compartment enables neutrophil polarization and migration [23]. On the other hand, Ly49Q-associated SHP-1 would prevent neutrophil adhesion in steady-state conditions [23]. A similar role for LY49Q cellular location was demonstrated in TLR signaling. Ly49Q augments TLR9 signaling by co-localizing with CpG DNA in endosomes and lysosomes [18], although the exact mechanism leading to enhance production remains to be elucidated. Ly49Q binds MHCI and is functionally analogous to human killer cell Ig-like receptors (KIRs) [78]. Intriguingly, it was recently demonstrated that in addition to binding HLA, KIR3DL2 can directly bind CpG DNA, which leads to enhanced cytokine production [79]. It would be interesting to examine whether Ly49Q has similar binding capacities.

The importance of cellular localization of inhibitory receptors is also evident from studies in NK cells. Inhibitory receptor-mediated inhibition of NK cell activity is known to act locally,

as NK cells contacting both resistant and susceptible target cells are capable of selective killing of susceptible target cells [80;81]. Inhibitory receptors present in the immunological synapse between target cell and effector cell mediate the localized inhibition of activating receptor cytotoxicity [80]. Thus, SHP-1 and SHP-2 play an important role in ITIM-mediated inhibition of various activation pathways (Figure 2). Their mode of action does not *per se* involve dephosphorylation of upstream molecules. Instead, controlled cellular localization of the receptor itself or associated molecules may lead to inhibition of cell activation by sequestration or, conversely, be essential in cellular activity. Possibly, the capacity to co-localize with activating receptors may determine whether the inhibitory receptor is selective in its action or has broad capacity. Few groups have thoroughly addressed this issue, expansion of these studies would further improve our understanding on the mechanism behind inhibitory receptor function.

Bacterially encoded ligands of inhibitory receptors

Co-evolution of interacting species drives molecular evolution through continual natural selection for adaptation and counter-adaptation. Hence, pathogens co-evolving with humans have developed multiple mechanisms to evade immune recognition. A pathogen that encodes a functional ligand for a phagocyte inhibitory receptor could enhance survival by suppressing effector functions such as phagocytosis, ROS and cytokine production. It has been shown that *S. aureus* binds specifically to PIR-B, a suppressor of TLR-mediated inflammatory responses, and PIR-B-deficient macrophages display enhanced inflammatory responses to *S. aureus* [82]. The specific bacterial protein that binds to PIR-B remains to be determined. Bacterially encoded ligands have also been found for Siglec-5 and Siglec-9 [30;83]. The group B *streptococcus* cell wall-anchored β protein specifically binds Siglec-5, and it was shown that Siglec-5 activation through β protein results in less phagocytosis, less oxidative burst, less neutrophil extracellular traps (NETs [84]) and reduced IL-8 production in neutrophils [83]. Other examples of bacterially encoded proteins that act as a functional ligand for inhibitory receptors include interaction of surface protein A1 on *M. catarrhalis* or opacity-associated proteins on *N. meningitidis* with CEACAM1 [85].

Evolutionary selection of pathogens that produce ligands for inhibitory receptors indicates it can lead to an evolutionary advantage, which in turn underlines the importance of inhibitory receptors as regulators of phagocyte cell function. Considering the number of inhibitory receptors on phagocytes, it is likely that many more bacterially encoded ligands for inhibitory receptors will be discovered.

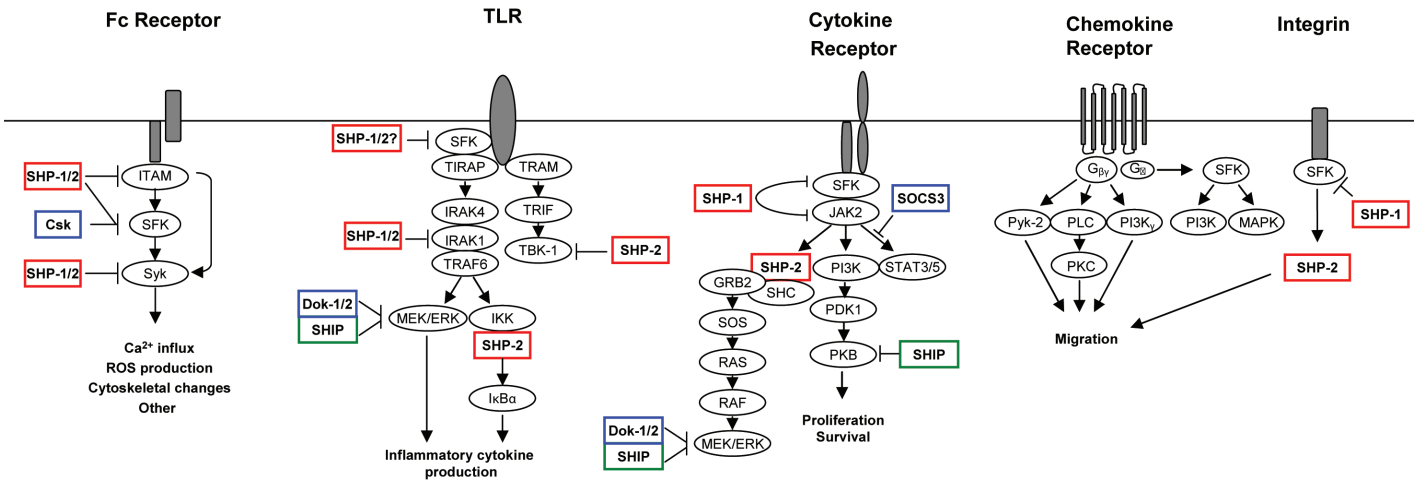

Figure 2. Molecules recruited by inhibitory receptors can deactivate several stages of the signal transduction pathways that are initiated by Fc receptor, Toll-like receptor (TLR), cytokine receptor, chemokine receptor and integrin signaling. For ITAM-mediated signaling, the role of SHP-1 and SHP-2 (red-rimmed) in dephosphorylating upstream signaling molecules, such as the ITAM, Src family kinases (SFK) or Syk has been amply demonstrated. In addition, some inhibitory receptors recruit Csk (blue-rimmed), which can deactivate SFK. The mechanism by ITIM-bearing receptors can modulate TLR signaling is less clear. SFK may play a role in initial TLR phosphorylation, which then could be inhibited by SHP-1 or SHP-2. Alternatively, SHP-1 and SHP-2 may affect TLR signaling by deactivation of IRAK1 and TBK-1, or sequestration of SHP-2 may prevent IκBα activation. Other ITIM-recruited molecules possibly involved are Dok-1/2 (blue-rimmed) and SHIP (green-rimmed), which can deactivate the MEK/ERK pathway. SHP-1 may also dephosphorylate upstream signaling molecules activated by cytokine receptor signaling, such as SFK and JAK2. JAK2 activates the MEK/ERK pathway through SHP-2 and it is possible that sequestration of SHP-2 will abrogate this activation. SOCS3 (blue-rimmed) can prevent JAK2-induced STAT3/5 activation, and SHIP inhibits protein kinase B (PKB), leading to impaired proliferation and survival. The mechanism by which inhibitory receptors can suppress chemokine receptor signaling is unclear. Migration usually involves integrin signaling, and this signals through SFK. SHP-1 possibly dephosphorylates the SFK, whereas SHP-2 may be positively involved in migration. Again, sequestration of SHP-2 may lead to reduced migration.

Concluding remarks

Inhibitory receptors play a pivotal role in diverse aspects of phagocyte function and can provide an activation threshold, regulate or terminate immune cell activation, hence contributing to immune homeostasis. Inhibitory receptors thus play an important regulatory role during various stages of the immune response.

Bacteria may encode ligands for inhibitory receptors that lead to reduced immune cell activation, hence providing them evolutionary advantage. An intriguing possibility is that besides acknowledged ligands for inhibitory receptors, some inhibitory receptors may bind additional molecules, as demonstrated for Siglec-10 with CD24 and KIR3DL2 with CpG DNA, these interactions could contribute to inhibitory receptor specificity. Indeed, it is intriguing that while signaling through a commonly shared motif, each inhibitory receptor has specific functionality, most inhibiting, but some enhancing immune cell function. The affinity with which SHP-1 and/or SHP-2 are recruited, regulated receptor and ligand expression may add to the non-redundant roles of inhibitory receptors in immune regulation. In addition, alternative molecules recruited to the phosphorylated ITIMs may contribute to specific function, and it is likely that more alternative molecules will be recognized. Finally, cellular localization of inhibitory receptors and associated SHP-1/2 may be a major determinant of inhibitory receptor capacity.

To conclude, the general view of inhibitory receptors as global inhibitors of immune cell activation does not fully represent their functional repertoire. Further research is necessary to elucidate the molecular mechanisms behind inhibitory receptor function that lead to divergent or even opposing roles in phagocytic cell regulation.

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Co-expression of collagen receptors Leukocyte-Associated Ig-like Receptor-1 and Glycoprotein VI on a subset of megakaryoblasts

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CHAPTER 5

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Abstract

Background

The collagen receptor Glycoprotein VI generates activating signals through an Immunoreceptor Tyrosine-based Activation Motif on the co-associated Fc receptor gamma chain. Leukocyte-Associated Ig-like Receptor-1 also ligates collagen but generates inhibitory signals through Immunoreceptor Tyrosine-based Inhibitory Motifs. Thus far, the cellular expression of Glycoprotein VI and Leukocyte-Associated Ig-like Receptor-1 appears mutually exclusive.

Design and methods

Using flow cytometry, we studied expression of collagen receptors on differentiating human megakaryocytes. CD34+ cells were isolated from umbilical cord blood and matured to megakaryocytes *in vitro*. Freshly isolated bone marrow cells were used to study primary megakaryocytes. Upon cell sorting, cytospins were made to examine cytologic characteristics of differentiation.

Results

Megakaryocyte maturation is accompanied by up-regulation of Glycoprotein VI and down-regulation of Leukocyte-Associated Ig-like Receptor-1. Interestingly, both in cultures from hematopoietic stem cells and primary cells obtained directly from bone marrow, we identified a subset of morphologically distinct megakaryocytes which co-express Glycoprotein VI and Leukocyte-Associated Ig-like Receptor-1.

Conclusions

This is the first report of a primary cell that co-expresses these collagen receptors with opposite signaling properties. Since megakaryocytes mature in the collagen-rich environment of the bone marrow, these findings may point to a role for Leukocyte-Associated Ig-like Receptor-1 in the control of megakaryocyte maturation/migration.

Introduction

Glycoprotein VI (GPVI) and $\alpha_2\beta_1$ play a crucial role in the platelet response to collagen.[1] Both receptors are expressed when hematopoietic stem cells differentiate into megakaryocytes and are abundantly present on platelets. Unlike GPVI, which can bind collagen directly, $\alpha_2\beta_1$ needs affinity modulation by inside-out signaling through ligated GPVI or other receptors before effectively binding collagen. GPVI stimulation on platelets initiates Ca^{2+} mobilization through a mechanism dependent on the tyrosine-kinase Syk, which initiates a downstream signaling cascade leading via LAT and SLP-76 to activation of multiple effector molecules such as $PLC\gamma_2$, small G-proteins, and phosphoinositide-3 kinase.[2,3] Also in megakaryocytes GPVI is capable of signal transduction. In these cells, cross-linking via GPVI-specific agonists such as convulxin and collagen-related peptide (CRP) results in tyrosine phosphorylation of Syk and $PLC\gamma_2$, and Ca^{2+} mobilization.[4-6] Megakaryocytes mature in the collagen-rich environment of the bone marrow and platelet formation is preceded by migration from osteoblastic stem cell niches to sinusoids where the platelets are shed in the circulation. The role of collagen receptors in these processes is poorly understood.

A molecule structurally related to GPVI is the inhibitory receptor leukocyte-associated Ig-like receptor (LAIR)-1.[7,8] The genes encoding LAIR-1 and GPVI are both located on the leukocyte receptor complex (LRC) on human chromosome 19. The genomic proximity and structural homology between the two receptors suggest that LAIR-1 and GPVI have a common origin. The intracellular tail of GPVI signals via calmodulin[9] and associated Src kinases Fyn and Lyn.[10,11] Furthermore, GPVI has a charged arginine in its transmembrane domain that mediates association with the immunoreceptor tyrosine-based activation motif (ITAM)-containing Fc receptor gamma chain (FcRγ).[12-14] GPVI-associated Fyn and Lyn are crucial for the phosphorylation of the FcRγ ITAM.[10] LAIR-1 contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic tail to impart its inhibitory effect through the phosphatases SHP-1, SHP-2 and the C-terminal Src kinase Csk.[15]

In leukocytes, LAIR-1 plays an important role in dampening immune responses and hence in the maintenance of a balanced immune system.[16,17] We have previously demonstrated that besides effector immune cells, hematopoietic stem cells (HSC) also express LAIR-1.[18] We have recently shown that collagens are high-affinity ligands for LAIR-1, and that binding of collagen to LAIR-1 results in inhibition of immune cell activation.[8] This is the only inhibitory receptor described so far that binds collagen and the collagen-binding site in LAIR-1 and GPVI overlaps between the two receptors.[19-21] In collaboration with our group, Tomlinson et al. showed that when both receptors are ectopically expressed on the same

cell, LAIR-1 cross-linking abrogates collagen-induced GPVI-signaling.[22] Co-expression of both receptor types on primary cells would therefore potentially affect their responsiveness to collagen. However, at present, GPVI and LAIR-1 expression appear mutually exclusive, with GPVI being regarded as a platelet-specific receptor and LAIR-1 being broadly expressed on leukocytes.

Megakaryocytes differentiate from HSC in the bone marrow, primarily under control of thrombopoietin (TPO).[23] HSC initially develop into megakaryocyte progenitors (CFU-MEG). Further transition from progenitor cell to mature megakaryocyte is divided into four stages. The first stage of megakaryocytopoiesis is represented by megakaryoblasts, which have a low cytoplasmic/nuclear ratio, compact nucleus, basophilic cytoplasmic staining and small cell size. Successive stages are represented by promegakaryocytes, granular megakaryocytes and finally mature megakaryocytes. During differentiation the nucleus becomes highly lobulated, cell size and cytoplasmic mass increases, and the cytoplasmic staining becomes eosinophilic.[24] These cells form proplatelet projections shedding several thousands of platelets per cell.[23] In addition to cytologic characteristics, the expression of surface receptors can be used as markers for differentiation. Expression of CD34 decreases, and CD41/CD61 expression is induced, followed by expression of CD42b.[25] Upon further maturation, GPVI and $\alpha_2\beta_1$ are induced[4] making these proteins markers for the late maturation stages.

In the present report we identify a subset of megakaryoblasts co-expressing an activating and inhibiting collagen receptor. This property may mark a separate stage in human megakaryocytopoiesis with possibly important consequences for the maturation/differentiation of megakaryocytes.

Design and methods

Materials (Antibodies and reagents)

Fetal calf serum (FCS) was from Bodinco (Alkmaar, The Netherlands). Horse serum, L-glutamine, RPMI 1640, IMDM and Fischer's medium pH 7.0 were from Gibco (Breda, The Netherlands). Bovine serum Albumin (BSA) was from Sigma. Hy101 anti-GPVI monoclonal antibody (mAb) was kindly provided by Prof. Kahn, University of Pennsylvania. Anti-human FcγRI and FcγRIII mAbs (clone 10.1 and 3G8) were from Biolegend, Anti-human FcγRII (clone 6C4) was from eBiosciences. CLB-MB15 anti-CD42b-biotin (mIgG1) mAb was purchased from Sanquin (Amsterdam, the Netherlands). Hy101 anti-GPVI (mIgG1) mAb was labeled with FITC (Molecular Probes). Y2/51 anti-CD61 FITC (mIgG1) mAb was from Dako. AK-7 anti-CD49b FITC (mIgG1) (to stain the α subunit of $\alpha_2\beta_1$) was from Biolegend. anti-CD11b FITC was from Immunotech. Goat anti-mouse APC was from Southern Biotech. 8A8 anti-LAIR biotin (mIgG1) was from own production. DX26 anti-LAIR PE (mIgG1), RUU-PL7F12 anti-CD61 PerCP (mIgG1), Streptavidin (SA)-PerCP, MphiP9 anti-CD14 APC Cy7 (mIgG2b), RPA2.10 anti-CD2 FITC (mIgG1), UCTH1 anti-CD3 FITC (mIgG1), RPA-T4 anti-CD4 FITC (mIgG1), M-T701 anti-CD7 FITC (mIgG1), RPA-T8 anti-CD8 FITC (mIgG1), M5E2 anti-CD14 FITC (mIgG2a), HIB19 anti-CD19 FITC (mIgG1), 2H7 anti-CD20 FITC (mIgG2b), GA-R2 anti-CD235a FITC (mIgG2b), 8G12 anti-CD34 PE-Cy7 (mIgG1), HIT2 anti-CD38 APC (mIgG1), 7G3 anti-CD123 PE (mIgG2a), HI100 anti-CD45RA PE Cy5 (mIgG2b), mouse isotype control mAbs IgG1 biotin, IgG1 FITC, IgG2a FITC, IgG2b FITC, IgG1 PE-Cy7, IgG1 APC, IgG2a PE, IgG2b PE-Cy5 and SA-APC-Cy7 were purchased from BD Biosciences. A CD34 progenitor cell isolation kit based on magnetic-activated cell sorting was from Miltenyi Biotech (Bergisch Gladbach, Germany). Stem cell factor (SCF) and thrombopoietin (TPO) were from Peprotech (Rocky Hill, NJ, USA). Giemsa stain was from Sigma, May Grünwald was from Merck Chemicals.

Cell Lines

Three megakaryoblastic cell lines were analyzed. MEG-01 cells were cultured in RPMI 1640 supplemented with 20% FCS. DAMI cells were cultured in IMDM containing HEPES, supplemented with 10% horse serum. CHRF-288-11 (further referred to as CHRF) cells were cultured in Fischer's medium pH 7.0 supplemented with 20% horse serum. Cell lines were analyzed by flow cytometry using DX26 anti-LAIR-1 PE, anti-GPVI FITC and anti-CD49b FITC. Dead cells were excluded by gating on base of forward and side scatter.

Platelet isolation

Freshly drawn venous blood was collected with informed consent from healthy donors into 0.1 volume 130 mmol/L trisodium citrate 3. Blood was centrifuged (15 min, 200 * g, 22 °C), platelets were resuspended in Hepes-Tyrode buffer (145 mmol/l NaCl, 5 mmol/l KCl, 0.5 mmol/l Na2HPO4, 1 mmol/l MgSO4, 10 mmol/l Hepes, 5 mmol/l D-glucose, pH 6.5). Prostaglandin I2 was added to a final concentration of 10 ng/ml and after centrifugation cells were resuspended in Hepes-Tyrode buffer (pH 7.2). Platelet count was adjusted to 2.25 * 10^{11} cells/l and suspensions were left at room temperature for 30 min to ensure a resting state. Fc receptors on platelets were blocked using anti-human FcγRI, anti-human FcγRII and anti-human FcγRIII mAbs, and cells were analyzed by flow cytometry using DX26 anti-LAIR-1 PE, anti-GPVI FITC and anti-CD49b FITC.

Analysis of hematopoietic progenitor cells

Umbilical cord blood was collected during normal full-term deliveries and used within 48 hours. All mothers gave written informed consent before labor and delivery. CD34+ progenitor cells were isolated from umbilical cord blood by Ficoll-paque density gradient centrifugation followed by MACS purification of CD34-positive cells. Samples were stained and washed in PBS supplemented with 5% FCS. Live cells were gated on base of forward and side scatter. Lineage positive cells were detected with a mixture of anti-CD2 FITC, anti-CD3 FITC, anti-CD4 FITC, anti-CD7 FITC, anti-CD8 FITC, anti-CD11b FITC, anti-CD14 FITC, anti-CD19 FITC, anti-CD20 FITC and anti-CD235a FITC. A mixture of FITC-labeled mouse isotype control mAbs (IgG1, IgG2a and IgG2b) was used to make the distinction between lineage positive and negative cells. Lineage negative (LIN^-) cells were gated and expression of the progenitor markers was detected using anti-CD34 PE-Cy7, anti-CD38 APC, anti-CD123 PE and anti-CD45RA PE-Cy5 by flow cytometry. To ensure specificity of the progenitor staining, a separate staining was performed in which the panel of lineage markers and CD34 and CD38 was combined with isotype control mAbs for CD123 (IgG2a PE) and CD45RA (IgG2b PE-Cy5), LIN- cells were gated, and quadrants were set based on isotype controls. To examine LAIR-1 expression on HSC and progenitor cells, stainings with both the lineage markers and the progenitor markers CD34, CD38, CD123 and CD45RA were combined with either a mouse IgG1 biotin isotype control mAb or with 8A8 anti-LAIR biotin. SA-APC-Cy7 was used as a secondary antibody to detect LAIR-1 expression. Protocols were approved by the ethics committee of the University Medical Center Utrecht.

Analysis of megakaryocytic cells in bone marrow samples

Bone marrow cells were obtained from healthy donors. All donors gave written informed consent. Erythrocytes were lysed from the population using pH7.4 Ammonium Chloride shock buffer. Cells were incubated and washed in PBS supplemented with 1% BSA and 5 mM EDTA. Cells were first stained with anti-GPVI, followed by secondary step goat anti-mouse APC. Third step anti-CD42b biotin, followed by fourth step SA-PerCP, anti-CD14 APC-Cy7, anti-CD61 FITC and DX26 anti-LAIR-1 PE. Samples were analyzed using the BD LSRII flow cytometer, or anti-CD14 APC Cy7, anti-CD61 PerCP and DX26 anti-LAIR-1 PE were used to sort megakaryocytic cells with the BD FACSAria flow cytometer to obtain isolated megakaryocyte subsets. Live cells were gated on base of forward and side scatter, CD14-negative cells were gated for CD61 and CD42b. CD61+ CD42b+ cells were analyzed for expression of GPVI and LAIR-1. Isotype control stainings for CD61, CD42b, LAIR-1 and GPVI (mIgG1 FITC, mIgG1 biotin followed by SA-PerCP, mIgG1 PE and mIgG1 followed by goat anti-mouse APC respectively) were used to set quadrants depicting positive and negative stainings. Protocols were approved by the ethics committee of the University Medical Center Utrecht.

***In vitro* culture of megakaryocytes from CD34+ cells**

CD34+ cells were isolated from umbilical cord blood by Ficoll-paque density gradient centrifugation followed by MACS purification of CD34-positive cells. The purity of the isolated population, based on CD34 expression, was determined by flow cytometry and always exceeded 90%. Isolated CD34-positive cells were seeded at $3*10^5$ cells/ ml. To induce megakaryocyte development, 50 ng/ ml SCF and 20 ng/ ml TPO were added to the culture media on day 0 and 3 of subculture. From day 7 on, cells were seeded at $5*10^5$ cells/ ml and only TPO was added to the culture. Culture media consisted of IMDM supplemented with 1% L-glutamine, 0.1 mM BSA-absorbed cholesterol, 0.5% BSA, 10 µg/ml insulin, 200 µg/ml iron-saturated transferrin, 50 µM β-mercaptoethanol and antibiotics (adapted from Den Dekker et al.[26]). On day 0; 3; 7; 10 and 14, cells were analyzed by flow cytometry after staining with anti-CD34 PE-cy7, anti-CD61 PerCP, CD42b biotin, GPVI FITC, and DX26 anti-LAIR PE, or the same staining with a PE isotype control instead of anti-LAIR. SA -APC was used as a second step to detect CD42b expression. Another staining was performed with only anti-CD49b FITC. Live cells were gated on base of forward and side scatter. Quadrants

depicting positive and negative stainings were set based on isotype control stainings for CD61, CD42b, GPVI, CD49b and LAIR-1.

Preparation of cytospins

Cytospins were made from sorted bone marrow samples and from cells obtained from *in vitro* megakaryocytopoiesis. 10.000 to 100.000 cells were collected and centrifuged on glass coverslips. Samples were fixed in 100% methanol, and stained with Giemsa and May Grünwald.

Results

Expression of collagen receptors on platelets and megakaryocytic cell lines

Expression of the collagen receptors LAIR-1, GPVI and $\alpha_2\beta_1$ was studied in the megakaryocytic cell lines MEG-01,[27] DAMI[28] and CHRF[29] by flow cytometric analysis and compared with platelets as a reference to end-stage receptor expression on mature megakaryocytes (Figure 1). These cell lines are thought to show similarities with megakaryocytes at different developmental stages, with MEG-01 representing an early stage, DAMI an intermediate stage and CHRF a late stage of normal megakaryocytopoiesis.[30] In line with this arbitrary classification, the expression of GPVI and $\alpha_2\beta_1$ was low in MEG-01, increased in DAMI cells, and high in CHRF. Conversely, LAIR-1 expression was absent in MEG-01, high in DAMI and intermediate in CHRF. Thus, the DAMI and CHRF cell lines co-express GPVI and LAIR-1. This is the first identification of a cell type that co-expresses an activating and an inhibitory receptor for collagen. Importantly, GPVI and $\alpha_2\beta_1$ were highly expressed by platelets but LAIR-1 expression was absent.

LAIR-1 is expressed on hematopoietic stem cells and on multipotent progenitor cells

We have previously reported that LAIR-1 is highly expressed by HSC.[18] To investigate its expression during further differentiation, we determined LAIR-1 expression on subsets of hematopoietic multipotent progenitor cells. To this end, we gated LIN^- cells and analyzed these cells for expression of progenitor markers (Figure 2). Quadrants were defined based

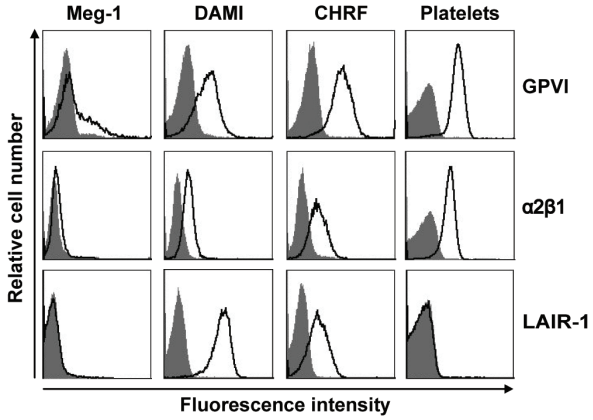

Figure 1. Megakaryoblastic cell lines show differential expression of GPVI, $\alpha_2\beta_1$ and LAIR-1. The expression of the collagen receptors LAIR-1, GPVI and $\alpha_2\beta_1$ was studied on platelets and on the megakaryocytic cell lines MEG-01, DAMI and CHRF. Freshly drawn venous blood was centrifuged and platelets were resuspended in Hepes-Tyrode buffer pH 6.5. Prostaglandin I2 was added and after centrifugation cells were resuspended in HepesTyrode buffer (pH 7.2). Platelet count was adjusted to 2.25 * 10^{11} cells/L and suspensions were left at room temperature for 30 min to ensure a resting state. Platelets and cell lines were stained with anti-LAIR, anti-GPVI, anti-CD49b (for $\alpha_2\beta_1$) or isotype control mAbs and analyzed by flow cytometry. For the cell lines, live cells were gated on base of forward and side scatter. The grey histograms represent isotype control stainings; the open histograms represent LAIR-1, GPVI and $\alpha_2\beta_1$ stainings. The results are representative of three independent experiments (in total 3 donors).

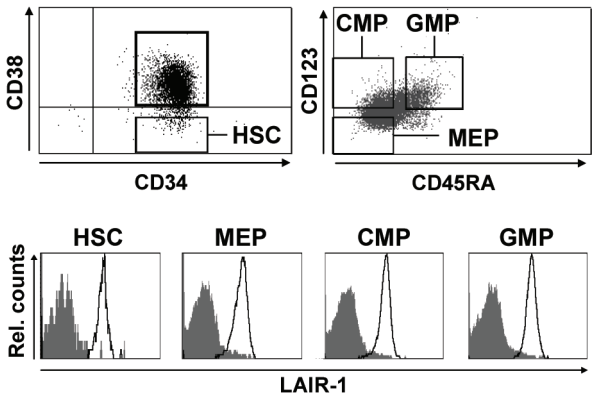

Figure 2. LAIR-1 is expressed on HSC and on all lineages of multipotent progenitor cells. Hematopoietic stem cells (HSC) and progenitor cells were isolated from umbilical cord blood by Ficoll-paque density gradient centrifugation. Samples were stained and washed in PBS supplemented with 5% FCS. Live cells were gated on base of forward and side scatter. Cells negative for lineage markers CD2, CD3, CD4, CD7, CD8, CD11b, CD14, CD19, CD20 and CD235a were analyzed for expression of progenitor cell markers (top panels). Quadrants were set on base of isotype control stainings. LAIR-1 was analyzed in progenitor subsets (bottom panels). The grey histograms represent isotype control staining; the open histograms represent LAIR-1 staining. MEP stands for megakaryocyte/erythrocyte progenitor; CMP for common myeloid progenitor and GMP for granulocyte/ macrophage progenitor. The results are representative of two independent experiments (in total 4 donors).

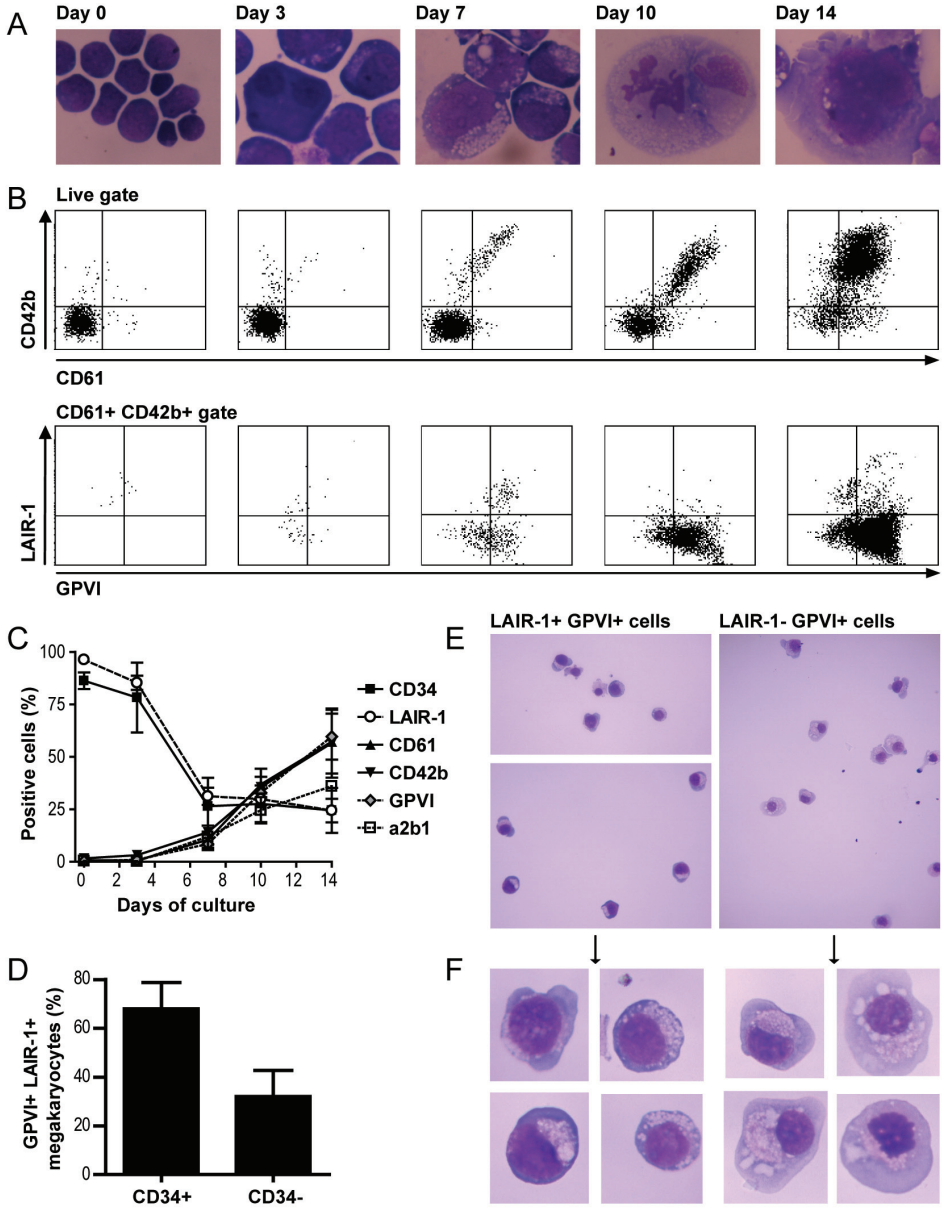

Figure 3. Expression of LAIR-1 and GPVI is differentially regulated during *in vitro* megakaryocytopoiesis. Umbilical cord blood was collected during normal full-term deliveries. Hematopoietic stem and progenitor cells were isolated from umbilical cord blood by Ficoll-paque density gradient centrifugation followed by magnetic-activated cell sorter (MACS, Milteney Biotech) purification of CD34-positive cells. (A) Cytospins were made on day 0; 3; 7; 10 and 14 of *in vitro* megakaryocytopoiesis, fixed in methanol and stained with Giemsa and May-Grünwald. Representative cytospins are shown. Pictures were taken with 1000x magnification. (B) On day 0; 3; 7; 10 and 14 of *in vitro* megakaryocytopoiesis, live cells were gated on base of forward and side scatter and analyzed for expression of

CD42b and CD61 by flow cytometry. In the lower panels, cells co-expressing CD42b and CD61 were gated to specifically examine GPVI and LAIR-1 expression on megakaryoblasts. Quadrants were set on base of isotype stainings. (C) as in *(B)*, live cells were gated on base of forward and side scatter and analyzed for expression of CD34, GPVI, CD42b, CD61, $\alpha_2\beta_1$ and LAIR-1 by flow cytometry. The percentages of receptor-positive cells are averaged for three donors. Error bars represent SEM. (D) On day 7 of *in vitro* megakaryocytopoiesis, live cells were gated on base of forward and side scatter and CD42b+ CD61+ GPVI+ LAIR-1+ cells were analyzed for expression of CD34. The specificity of the staining was confirmed by the use of isotype control mAbs. The percentage of CD34-positive cells is averaged for three donors. Error bars represent SEM. (E) LAIR-1+ GPVI+ and LAIR-1- GPVI+ cells were sorted on day 7 of *in vitro* culture and cytospins were made as described in *(A).* Representative pictures from one out of 4 donors are shown. Pictures were taken with 400x magnification. (F) As in *(E)*, the same pictures were taken with 1000x magnification to show individual cells in greater detail.

on isotype control stainings. True HSC were defined as CD34+ CD38- cells, megakaryocyte/erythrocyte progenitors (MEP) as CD34+ CD38+ CD123- CD45RA- cells, common myeloid progenitors (CMP) as CD34+ CD38+ CD123+ CD45RA- cells and granulocyte/ macrophage progenitors (GMP) as CD34+ CD38+ CD123+ CD45RA+ cells.[31] Detailed analysis revealed that all subsets express LAIR-1. Thus, HSC as well as hematopoietic progenitor cell subsets have a considerable expression of LAIR-1.

Expression of LAIR-1 and GPVI is differentially regulated during *in vitro* megakaryocytopoiesis

Since we observed co-expression of GPVI and LAIR-1 in DAMI and CHRF megakaryocytic cell lines, we investigated whether *ex vivo* generated megakaryocytes also showed this property. CD34+ cells were cultured with TPO and SCF and cells were collected after 0, 3, 7, 10 and 14 days culture. Cytospins were stained with May-Grünwald Giemsa reagent and lobulation of the nucleus, nuclear/cytoplasmic ratio, cytoplasmic staining and cell size were examined. Cytospins showed the transition of progenitor cells through stage I, II and III of normal megakaryocytopoiesis during the 14-day culture (Figure 3A). Cells and nuclei increased significantly in size and quantity of cytoplasm, however lobulation of the nucleus was less pronounced. Flow cytometric analysis showed that LAIR-1 was expressed on all progenitor cells, whereas only a small proportion of cells expressed LAIR-1 later in differentiation (Figure 3B-C). Similarly, CD34 expression was expressed on all progenitor populations and lost during differentiation. Concomitantly, expression of CD61, CD42b and GPVI, absent in progenitor cells, gradually increased during culture with CD61 and CD42b expression induced after three days on a small population of cells. GPVI and $\alpha_2\beta_1$ expression was detected from day seven onwards. In line with the observations in the cell

lines, we observed a subset of megakaryocytes which co-expressed LAIR-1 and GPVI. This population amounted to approximately 20% of all GPVI+ cells at day 7 of culture (Figure 3B bottom panel). LAIR-1 expression on GPVI+ cells was completely lost during further maturation (Figure 3B). Since the progenitor marker CD34 is down-regulated concurrently with LAIR-1 in the total cell population (Figure 3C), we determined whether both markers were co-expressed on megakaryocytes, as expression of CD34 is indicative of the differentiation state of the cells. We analyzed CD34 expression on CD42b+CD61+GPVI+LAIR-1+ cells from day 7 of *in vitro* culture and found that about one-third of the LAIR-1+ megakaryocytes had lost CD34 expression, whereas 65% of the population was positive for both markers (Figure 3D). To further characterize the maturation status of LAIR-1 expressing cells, LAIR-1+ GPVI+ and LAIR-1- GPVI+ cells were sorted on day 7 of culture and cytospins were made and stained for morphologic analyses. With regard to nuclear/cytoplasmic ratio and cytoplasmic staining, LAIR-1+ GPVI+ cells consisted of CFU-MEG and megakaryoblasts and were consequently more immature than LAIR-1- GPVI+ cells, which consisted predominantly of promegakaryocytes (Figure 3E-F). Thus, a subset of GPVI+ megakaryoblasts expressed LAIR-1 in all donors during an early stage of *in vitro* culture.

LAIR-1 and GPVI are co-expressed by megakaryoblasts *in vivo*

Since *in vitro* maturation of megakaryocytes may differ from *in vivo* maturation, we investigated whether megakaryocytes freshly isolated from bone marrow also contained a subpopulation which co-expressed LAIR-1 and GPVI. Expression of these receptors was determined in the CD14- CD42b+ CD61+ population, with quadrants set based on isotype controls. Nearly all cells positive for CD61 were also positive for CD42b and GPVI. Notably, all donors examined showed a large population of LAIR-1 and GPVI co-expressing megakaryocytes amounting to about 50% of GPVI-expressing cells (Figure 4A-B). In addition, two out of four donors examined showed besides the population of GPVI+ LAIR-1+ cells also a population of GPVI+ LAIR-1^{high} cells (Figure 4A). This population amounted to approximately 6% of total GPVI+ cells. We next determined whether LAIR-1 expressing cells co-express CD34, as seen in the *in vitro* culture. CD14-CD61+CD42b+GPVI+LAIR-1+ cells were analyzed for the expression of CD34. Surprisingly, the percentage of CD34+ cells was much lower in bone marrow than during *in vitro* culture (Figure 4C). To further determine the maturation stage of these megakaryocytes, CD61+ cells were sorted on high, intermediate or absent LAIR-1 expression (Figure 4D) and cytospins were made and stained

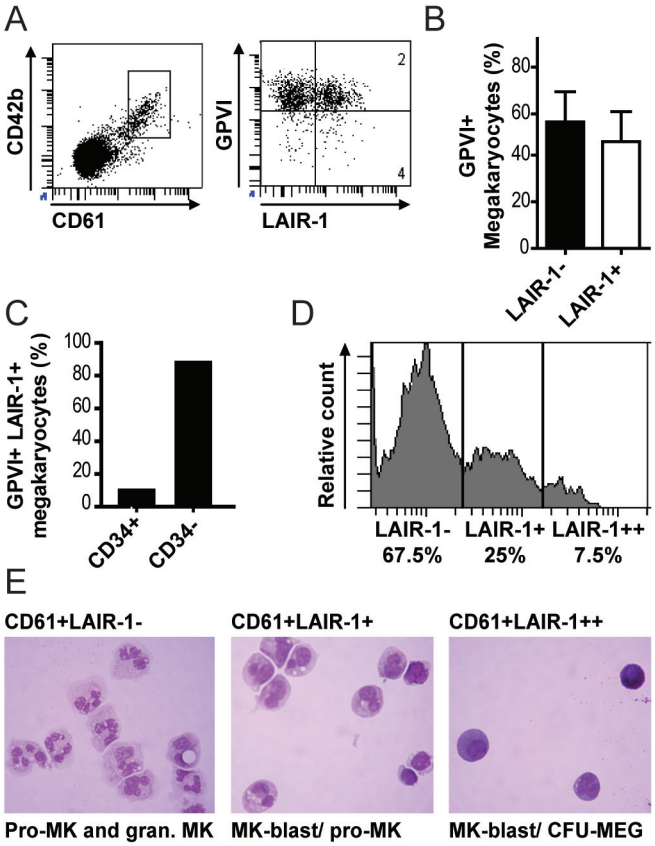

Figure 4. A subset of megakaryoblasts co-expresses GPVI and LAIR-1 *in vivo*. Bone marrow cells were obtained from healthy donors. Erythrocytes were lysed from the population using pH7.4 Ammonium Chloride shock buffer. PBS supplemented with 1% BSA and 5 mM EDTA was used for staining and washing of bone marrow cells. (A) Primary live megakaryocytes in human bone marrow were gated on base of forward and side scatter, and CD14- CD42b+ CD61+ megakaryocytes were gated (left panel). All gated cells were GPVI-positive, and ~50% of cells co-expressed LAIR-1 (right panel). Quadrants were set on base of isotype stainings. (B) Cells were analyzed as described in *(A)*. The percentages of LAIR-1+ cells are averaged for three donors. Quadrants were set on base of isotype stainings. (C) Live bone marrow cells were gated on base of forward and side scatter and CD14- CD42b+ CD61+ GPVI+ LAIR-1+ cells were analyzed for expression of CD34. The specificity of the staining was confirmed by the use of isotype control mAbs. The percentage of CD34-positive cells is shown for a representative donor (n=2). (D) CD14- CD61+ megakaryocytes were sorted on base of LAIR-1 expression. (E) Cytospins were made of sorted cells from *(D)*, fixed in methanol and stained with Giemsa and May-Grünwald. LAIR-1^{neg} cells had the most mature phenotype and consisted of promegakaryocytes (pro-MK) and granular megakaryocytes (MK), whereas the LAIR-1^{high} cells were most immature and consisted of megakaryoblasts (MK-blasts) and CFU-MEG. Pictures were taken with 400x magnification. Data shown are representative of at least three different donors analyzed in independent experiments.

for morphologic analyses. LAIR-1^{high} cells were the most immature population, consisting of megakaryoblasts and CFU-MEG. LAIR-1^{dim} were intermediate-stage, consisting of megakaryoblasts and a few promegakaryocytes. Cells that did not express LAIR-1 were the most mature cells, consisting of promegakaryocytes and granular megakaryocytes (Figure 4E). These findings demonstrate that LAIR-1 expression is down-regulated during *in vivo* differentiation of CD61-positive megakaryocytes.

Discussion

Here, we have examined the expression of collagen receptors (LAIR-1, GPVI, $\alpha_2\beta_1$) at different stages of megakaryocyte maturation using a combination of cytologic characteristics and surface-marker expression of CD34, CD61 and CD42b. During megakaryocyte maturation, the inhibitory collagen receptor LAIR-I is down-regulated while the activating collagen receptor GPVI is up-regulated. An intermediate stage isolated from bone marrow shows cells that co-express these collagen receptors with opposite functions (Figure 5).[25]

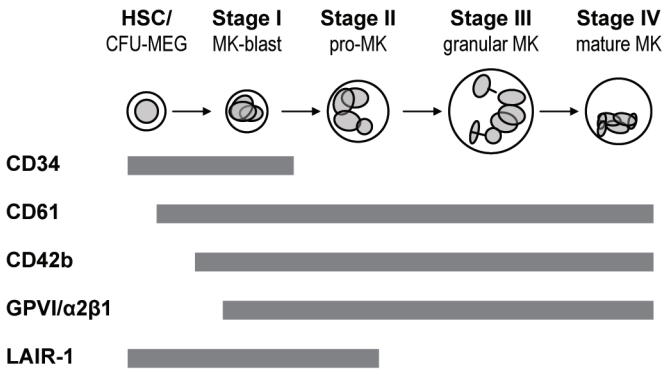

Figure 5. Expression of cell surface markers during megakaryocytopoiesis. The transition from hematopoietic stem cell (HSC) to mature megakaryocyte is divided into four distinct stages. Cells develop into multi-potent megakaryocyte progenitors (CFU-MEG), before differentiating into megakaryoblasts (MK-blast, stage I) with a low cytoplasmic/nuclear ratio, compact nucleus and small cell size. Successive stages are represented by promegakaryocytes (pro-MK), granular megakaryocytes (MK) and finally mature megakaryocytes. During differentiation the nucleus becomes highly lobulated and cell size and cytoplasmic mass increases. Expression of the progenitor cell marker CD34 is lost in an early phase of differentiation. CD61 expression is induced first in CFU-MEG. CD42b is a later marker for differentiation, preceded by CD61. Upon further maturation, GPVI and $\alpha_2\beta_1$ are induced. LAIR-1 is expressed early in megakaryocytopoiesis and on HSC and progenitor cells. A population of stage I and stage II megakaryocytes co-expresses GPVI and LAIR-1.

Analysis of cytospins of sorted CD61+ LAIR-1^{high}, CD61+ LAIR-1^{dim} and CD61+ LAIR-1^{neg} megakaryocytes revealed that LAIR-1^{high} cells have the most immature phenotype and consisted of CFU-MEG and megakaryoblasts. LAIR-1^{dim} cells consisted of megakaryoblasts and promegakaryocytes. During *in vitro* megakaryocytopoiesis, LAIR-1 expression was only found in an early phase of culture and disappeared after ten days from the CD61+ CD42b+ GPVI+ population. Part of the GPVI+ LAIR-1+ population had lost CD34 expression both *in vivo* and *in vitro*, indicating that LAIR-1 expression is maintained for a longer period during differentiation. Thus, LAIR-1 is a novel marker for megakaryocytopoiesis and is expressed by megakaryoblasts and promegakaryocytes.

Cells co-expressing both activating and inhibitory collagen-receptors might represent an important intermediate in megakaryocyte maturation since they are present in a significant number. About 50% of the megakaryocytes isolated from bone marrow co-express GPVI and LAIR-1, and 20 - 40% of cells are GPVI+ LAIR+ after 7 days of *in vitro* culture. Discrepancies between percentages of GPVI+ LAIR-1+ cells may be partly explained by the fact that we start with isolated stem and progenitor cells and differentiate cells in phase for the *in vitro* culture, whereas during *in vivo* differentiation cells are not synchronized. In addition, the presence and dose of thrombopoietin and other cytokines may differ between *in vitro* and *in vivo* maturation. These factors may also be an explanation for the difference in percentages of LAIR-1-CD34 double-positive cells in bone marrow and *in vitro* culture.

Alternatively, the difference in number of GPVI+ LAIR-1+ cells might be caused by differences in distribution. In bone marrow, mature megakaryocytes migrate to the capillary-rich vascular niche where they shed platelets. Collection of bone marrow samples might favor sampling of cells from the osteoblastic environment. Indeed, cytospin analysis from bone marrow megakaryocytes revealed the presence of stage II and stage III cells, but not mature megakaryocytes (Figure 4).

Some megakaryocytes derived from *in vitro* culture seem to develop from LAIR-1- GPVI- to LAIR-1- GPVI+ instead of from LAIR-1+ GPVI- via LAIR-1+ GPVI+ to LAIR-1- GPVI+ (Figure 3). Most likely this is due to the fact that HSC differentiate along multiple, partially asynchronous routes.[32,33] It remains to be determined whether this alternative differentiation route is also followed *in vivo*.

The concept that MEG-01, DAMI and CHRF cells represent megakaryocytes at increasing stages of maturation would predict that MEG-01 cells express more LAIR-1 than DAMI cells and this is clearly not observed. Initially, the classification was based on expression of GPIIb-IIIa and GPIb[30,34] and the expression of GPVI and $\alpha_2\beta_1$ reported here supports this

early definition. Morphologic criteria such as relative absence of α-granules and demarcation membranes, suggest that both MEG-01 and DAMI represent early megakaryoblasts[27,28] and this property together with LAIR-I expression would define DAMI cells as a less mature stage than MEG-01. Megakaryocytic cell lines obtained from monoclonal leukemic progenitor cells that have differentiated via partially asynchronous routes may differ in the onset of megakaryocyte protein expression. How cells that co-express LAIR-I and GPVI respond to collagen in terms of Ca^{2+} mobilization or secretion of granule contents remains a subject for further studies.

The importance of collagen receptors in megakaryocyte maturation, motility and platelet shedding is poorly understood. Differentiating megakaryocytes reside in the bone marrow niche, which abundantly expresses collagen. Unlike LAIR-1 and GPVI, which can bind collagen directly, $\alpha_2\beta_1$ needs affinity modulation by inside-out signaling through ligated GPVI or other receptors before effectively binding collagen. Sabri et al. demonstrated that primary megakaryocytes depend on both GPVI and $\alpha_2\beta_1$ ligation for optimal formation of actin stress fibers[35] and therefore migration.[36] The latter, however, was not affected by expression of constitutively active $\alpha_2\beta_1$.[37] Alternatively, collagen signaling might lead to inhibition of platelet formation. Megakaryocytes adhering to collagen by $\alpha_2\beta_1$ ligation produce fewer proplatelets than control cells.[35]

One could speculate that GPVI and $\alpha_2\beta_1$ signaling induce migration of megakaryocytes, which is inhibited by LAIR-1 signaling on immature cells. Upon maturation, LAIR-1 expression is lost, and megakaryocytes migrate to the capillary-rich vascular niche. In this collagen-low environment, GPVI and $\alpha_2\beta_1$ signaling ceases and proplatelet formation and platelet release occurs.

In conclusion, LAIR-1 is differentially expressed during megakaryocytopoiesis and a novel marker to classify different stages of megakaryocyte development. The activating and inhibitory collagen receptors GPVI and LAIR-1 are simultaneously expressed on a subset of megakaryoblasts and promegakaryocytes. This property might reveal a role for LAIR-1 in increasing the threshold of collagen-activation through GPVI and $\alpha_2\beta_1$ in developing megakaryoblasts.

Acknowledgements

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Distinct collagen binding properties of LAIR-1a and LAIR-1b isoforms

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CHAPTER 6

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Abstract

Leukocyte-associated immunoglobulin receptor-1 (LAIR-1) is an inhibitory receptor expressed on the majority of human peripheral blood mononuclear cells. The most prominent splice variants of LAIR-1 are LAIR-1a and LAIR-1b, with LAIR-1b lacking 17 amino acids in the stalk region of the extracellular domain. Here, we investigated the expression pattern of LAIR-1a and LAIR-1b by Western blot and show that while B and T cells express the LAIR-1b isoform, monocytes express both isoforms and granulocytes solely express LAIR-1a. We next determined whether the isoforms have distinct capacities with regard to collagen binding or signaling. Indeed, using flow cytometry, we show that FITC-labeled collagen has enhanced adherence to LAIR-1a compared to LAIR-1b. To investigate whether homo-dimerization may affect the LAIR-1-collagen interaction, bioluminescence resonance energy transfer (BRET) was utilized. We could not demonstrate dimerization for LAIR-1a or -1b, with or without collagen incubation. We next investigated whether differences in collagen binding could lead to differences in signaling capacities between the two LAIR-1 isoforms. Utilizing a mouse reporter T cell line, both isoforms were found to inhibit T cell receptor-induced NFAT activation to a similar extent after incubation with collagen. In contrast, K562 cells ectopically expressing LAIR-1a display increased adherence to collagen-coated plates compared to LAIR-1b, while both isoforms bind equally well to anti-LAIR mAb coated plates. Concluding, LAIR-1a and LAIR-1b differ in their collagen-binding capacity, with potential consequences for cell adherence and migration.

Introduction

Leukocyte migration to inflammatory loci is a fundamental process typically required to resolve infection.[1] Endothelial cells play an important role in the recruitment of leukocytes to inflammatory sites through regulated expression of cell surface adhesion molecules that mediate interactions with leukocytes in the bloodstream. Inflammatory signals induce expression of molecules involved in leukocyte rolling and adhesion on endothelial cells, including E- selectin, P-selectin, VCAM-1 and ICAM-1, and in addition induce the release of chemoattractants such as IL-8. These molecules stimulate leukocyte emigration from the vasculature.[2;3]

Certain inhibitory receptors such as platelet endothelial cell adhesion molecule (PECAM)-1 and carcinoembryonic antigen-related cell adhesion molecule1 (CEACAM1) have been demonstrated to play an important role in regulating leukocyte migration.[4;5] Similar to other inhibitory receptors, PECAM-1 and CEACAM1 contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail that can relay inhibition of cell activation. Both PECAM-1 and CEACAM1 are expressed by a variety of cell types, including endothelial cells and various lymphoid and myeloid cells, and are primarily involved in homophilic interactions.[4;5] PECAM-1 promotes leukocyte adherence to the endothelium and transmigration through endothelial junctions and the perivascular basement membrane to enter the inflamed tissue. One of the well-established mechanisms by which PECAM-1 promotes these processes is through homophilic PECAM-1/ PECAM-1 adhesive interactions between leukocytes and endothelial cells.[5]

Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is constitutively expressed on the majority of human peripheral blood mononuclear cells, contains two ITIMs and can recruit the phosphatases SHP-1, SHP-2 and the C-terminal Src kinase Csk to mediate its inhibitory effect.[6] *In vitro*, LAIR-1 strongly inhibits NK cell and T cell activity.[7;8] Recently, we and others reported collagens as functional, high affinity ligands for LAIR-1.[9-11] Collagens are the most abundant proteins in the human body, being important in maintenance of tissue structure and hemostasis. Although collagens are not normally exposed in the vasculature, they may become so after tissue damage or mechanical injury. LAIR-1 is the only inhibitory immune receptor described so far that binds collagen, and it is tempting to speculate that LAIR-1, similar to PECAM-1 and CEACAM1, may also function in regulating cell adhesion.

Two biochemically distinct forms of LAIR-1 have been identified. The protein indicated as LAIR-1a has an estimated size of 40 kDa, the smaller protein, LAIR-1b, is approximately

32 kDa.[12] The difference between the two isoforms is that LAIR-1b lacks 17 amino acids located at the stalk region between the transmembrane and Ig-like domain. The relatively large difference in size between the two proteins could indicate that these 17 amino acids are heavily glycosylated. The isoforms LAIR-1a and -1b are formed by alternative splicing of exon 4 and are differentially expressed by NK and T cells.[8] However, it is unclear whether LAIR-1a and LAIR-1b are functionally distinct. We previously addressed this question using cross-linking antibodies,[8] which may not truly reflect physiological conditions, where LAIR-1 will be activated by binding to collagen. Since the natural ligand of LAIR-1 has now been identified, we here investigated whether splice variants LAIR-1a and LAIR-1b are distinct with regard to collagen binding and collagen-induced signaling. We demonstrate that both isoforms have similar inhibitory capacity in response to collagen. However, LAIR-1a shows enhanced collagen binding compared to LAIR-1b, and cells expressing LAIR-1a show enhanced adherence to a collagen-coated surface compared to cells expressing LAIR-1b. This may have important implications for the adhesive and tissue infiltrating capacity of leukocytes expressing predominantly LAIR-1a or LAIR-1b.

Materials and Methods

Antibodies and reagents

Monoclonal antibodies (mAb) against LAIR-PE (DX26), CD3ε and CD3-APC, and isotype control mIgG1-PE were from BD Pharmingen, anti-mouse IgG-APC was from Southern Biotech and isotype control mIgG1 was from eBioscience. HRP-linked anti-mouse antibodies were from DakoCytomation (Fort Collins, CO). Collagen I and collagen III were from Sigma Aldrich, and were FITC conjugated (Molecular Probes). Calceine AM was from Invitrogen (Breda, the Netherlands). Bovine serum albumin (BSA) and fugene were from Roche Diagnostics (Mannheim, Germany). Immobilon-P membranes were from Millipore (Bedford, MA). Enhanced chemiluminescence Supersignal was from Pierce (Rockford, IL, USA). RPMI 1640 was from GIBCO® Invitrogen Cell Culture (Merelbeke, Belgium). Fetal calf serum was from Bodinco (Alkmaar, the Netherlands).

Analysis of LAIR-1a and LAIR-1b expression on primary leukocyte cell types

Peripheral blood was obtained from healthy volunteers. All donors gave written informed consent and protocols were approved by the institutional review board. Mononuclear cells

and granulocytes were isolated by Ficoll-Histopaque density gradient centrifugation. Isolated mononuclear cells were sorted on base of forward and side scatter and on expression of CD8 (T lymphocytes), CD4 (T lymphocytes), CD19 (B cells) or CD14 (monocytes) on a FACSaria to obtain isolated cell fractions. Isolated granulocytes were sorted on base of forward and side scatter and on CD11b expression. Cells were lysed in Laemmli buffer [0.12 mol/L Tris-HCL (pH 6.8), 4% SDS, 20% glycerol, 0.05 µg/µL bromphenol blue, 35 mmol/L β-mercaptoethanol] and boiled for 5 minutes. Equal cell numbers were loaded and analyzed by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes. Western blots were incubated with anti-LAIR-1 mAb, followed by HRP-linked anti-mouse antibodies. Enhanced chemiluminescence Supersignal was used for detection.

cDNA constructs

Constructs containing LAIR-1a and LAIR-1b were described before.[8] LAIR-1 sequences without stop codons for BRET were amplified by PCR from cDNA and cloned in-frame into either pGFP2-N1 or pRLuc-N1 (PerkinElmer), resulting in an end product with GFP or Luciferase C-terminal of LAIR-1. The forward primer (5'-CGGATATCCACCATGTCTCCCCACCCCACC) and the reverse primer (5'-CGGGATCCCCGTGTCTGGCAACGGCTGC) were supplemented with the restriction sites EcoRV and BamHI respectively. This resulted in the following constructs: LAIR-1a-Luc, LAIR-1a-GFP, LAIR-1b-Luc, and LAIR-1b-GFP. Control BRET constructs containing CTLA-4 and CD86 were described before.[13]

Cell lines

Human Embryonic Kidney (HEK) 293T cells were used for transfections with Fugene (Roche Diagnostics, Mannheim, Germany) to produce virus particles for retroviral transduction of the myeloid leukemia cell line K562 and the mouse 2B4 NFAT-GFP reporter T cells (further referred to as NFAT reporter T cells), which was kindly provided by Prof. L. Lanier (UCSF, USA). Retroviral pMX vectors containing LAIR-1a and 1b constructs were packaged with the pcl ampho system[14] and virus was used to infect K562 and NFAT reporter T cells. Cells were sorted for high expression on day three of transduction by flow cytometry (FACSaria, BD Biosciences). K562 cells stably expressing LAIR-1a were described before.[9] All cells were cultured in RPMI 1640 with addition of 10% fetal calf serum, β-mercaptoethanol, 175 µl/10 ml phosphate buffered saline (PBS), and antibiotics.

NFAT-GFP reporter assay

96-well MAXIsorp (Nunc) flat-bottom plates were coated overnight at 4˚C with 1-4 µg/ml collagen I (Sigma Aldrich) or 1-4 µg/ml homotrimeric peptides derived from collagen II and III (Collagen II and III Toolkit)[15] and co-coated with anti-CD3ε mAb (0.33 µg/ml). Collagens and peptides were coated in PBS containing 2mM acetic acid. After washings, $2.25*10^5$ cells were added per well and incubated at 37˚C for 20-24 h, after which GFP expression was analyzed by flow cytometry. The percentage inhibition for NFAT reporter T cells containing LAIR-1a/LAIR-1b was calculated as follows: percentage inhibition = 100 * (% GFP+ cells after anti-CD3ε stimulation - % GFP+ cells stimulated with anti-CD3ε and peptide/collagen/antibody) / (% GFP+ cells after anti-CD3ε stimulation).

Collagen binding assays

K562 cells, containing LAIR-1a and -1b were stained with four different concentrations of collagen I and III-FITC, diluted in Facsbuffer (PBS supplemented with 1% BSA, 0.1% sodium azide and 50mM EDTA), and anti-LAIR-PE.

To determine the adhesive capacity of cells expressing LAIR-1a or LAIR-1b, 96-well MAXI-sorp (Nunc) flat-bottom plates were coated overnight at 4˚C with 1.25-10 µg/ml of collagen I, collagen III, anti-LAIR mAb or as a control with 10 µg/ml BSA (100 µl/ well, collagens and peptides were coated in PBS containing 2 mM acetic acid). Coated plates were washed 3 times with PBS and blocked with 1% BSA in PBS for at least 1 hour at RT. K562 cells or K562 transfectants ($5*10^6$) were labeled with 5 µM calceine AM in PBS for 30 min at 37˚C. Cells were washed 5 times with RPMI 1640 1% FCS and $1.5*10^5$ cells were added per well to the coated 96-wells plate and cells were incubated at 37˚C for 3.5 h. Plates were measured before washing to determine input fluorescence, and washed up to 20 times with 1% RPMI. The fluorescence was determined by a Fluoroskan Ascent reader (Thermo Labsystems) for washing step 1, 2, 3, 4, 5, 10, 15, and 20 as a percentage of input fluorescence.

Bioluminescence resonance energy transfer

Bioluminescence resonance energy transfer (BRET) was performed as described.[13] In short: HEK293T cells were collected 24h post-transfection and resuspended at $1.5*10^6$ cells/ml. Light emission was collected in the 410 ± 40 nm (BRET-A) and 515 ± 15 nm (BRET-B) wavelength ranges on a Fusion microplate analyzer (PerkinElmer). To determine GFP and Luc ex-

pression, 100 ml of cells were dispensed in a separate well, excited at 420 nm and emission was measured at 515 ± 15 nm three times over 1 sec, to obtain the total fluorescence units (FU). Then cells were incubated in the same well with 10 mM coelenterazine-h for 2 min before reading total emission three times integrated over 1 sec, to obtain the total luminescence units (LU). BRET values were calculated after background subtraction, as BRET-B/BRET-A, corrected for luciferase expression alone (typically 7% of BRET-A luminescence). As the concentration of tagged molecules is proportional to the signal detected, the acceptor/donor ratio can be calculated as [GFP]/[Luc] = (kGFU)/(k LLU) = K(FU/LU), where kG and kL are constants specific to GFP and Luc, respectively, and K=kG/kL. For type-2 BRET analysis, cells were transfected with a specific acceptor/donor ratio (12:1). Thus, the expression level could be varied systematically when samples were collected at regular intervals, before being assessed as above for BRET. The level of protein expression is determined by using total Luc expression.

Statistical analysis

The indicated data were analyzed using SPSS 15.0 software (SPSS, Chigaco, Illinois, USA). A p-value of ≤ 0.05 was considered statistically significant.

Results

Differential expression of LAIR-1a and LAIR-1b

Previously we demonstrated that Jurkat T cells exclusively express LAIR-1b, whereas two NK cell clones expressed both LAIR-1a and -1b.[8] We here aimed to further substantiate the expression pattern of the two isoforms. Since it is impossible to distinguish the two different isoforms by quantitative RT-PCR, we analyzed LAIR-1 expression in cell lysates from isolated leukocyte cell types by Western blot. We found that both CD4+ and CD8+ T lymphocytes express predominantly LAIR-1b (Figure 1), indicating that both T cell lines and primary T cells express the LAIR-1b isoform. Similarly, LAIR-1b is the principal isoform detected in CD19+ B lymphocytes. In CD14+ monocytes expression of both LAIR-1a and LAIR-1b could be demonstrated, with the LAIR-1b isoform being more prevalent. In contrast, only LAIR-1a expression was found in CD11b+ granulocytes (Figure 1). So far, granulocytes are the only cell type for which exclusive expression of the LAIR-1a isoform has been demonstrated.

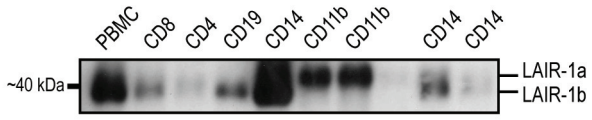

Figure 1. LAIR-1a and LAIR-1b isoforms are differentially expressed by primary cells. Peripheral blood was obtained from healthy volunteers. Mononuclear cells and granulocytes were isolated by Ficoll-Histopaque density gradient centrifugation. Isolated peripheral blood mononuclear cells (PBMC) were sorted on base of forward and side scatter and on expression of CD8 (T lymphocytes), CD4 (T lymphocytes), CD19 (B cells) or CD14 (monocytes). Isolated granulocytes were sorted on base of forward and side scatter and on CD11b expression. Total PBMC and sorted cells fractions were from 2 separate donors. Each lane containing CD14+ monocytes or CD11b granulocytes was obtained from a separate donor. Cells were lysed in Laemmli buffer and equal cell numbers were loaded and analyzed by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes. Western blots were incubated with anti-LAIR-1 mAb, followed by HRP-linked anti-mouse antibodies.

LAIR-1a shows enhanced binding to FITC-labeled collagen compared to LAIR-1b

To elucidate whether LAIR-1 isoform expression could have biological relevance, we investigated whether the difference in the extracellular domain of LAIR-1a and LAIR-1b could result in differential collagen-binding capacity. K562 cells were stably transfected to ectopically express LAIR-1a and LAIR-1b, and were analyzed utilizing DX26 anti-LAIR-1 PE mAb and various concentrations of FITC-conjugated collagen. To be able to compare their collagen binding properties, transfected K562 cells were first gated on comparable LAIR-1 expression (Figure 2A), after which the mean fluorescence intensity (MFI) of collagen-binding was determined. Both collagen I-FITC and collagen III-FITC bound to LAIR-1-expressing cells in a dose-dependent manner. (Figure 2B-C). Notably, LAIR-1a bound more collagen than LAIR-1b, as determined by MFI after collagen-FITC incubation, indicating that both isoforms have distinct binding capacities.

LAIR-1a and LAIR-1b do not dimerize

Glycoprotein (GP) VI is structurally related to LAIR-1[7;9] and requires dimerization in order to bind collagen.[16] Thus, it is possible that dimerization is also a prerequisite for LAIR-1 binding, and that absence of dimerization for LAIR-1b would explain decreased collagen binding. To determine whether LAIR-1a and LAIR-1b were capable of dimerization, we generated LAIR-1a and -1b BRET pairs (GFP and luciferase) and tested whether these proteins dimerized. CD86 and CTLA4, a known monomer and dimer were included in the experiment as a negative and positive control respectively. BRET efficiency (BRETeff) is the ratio

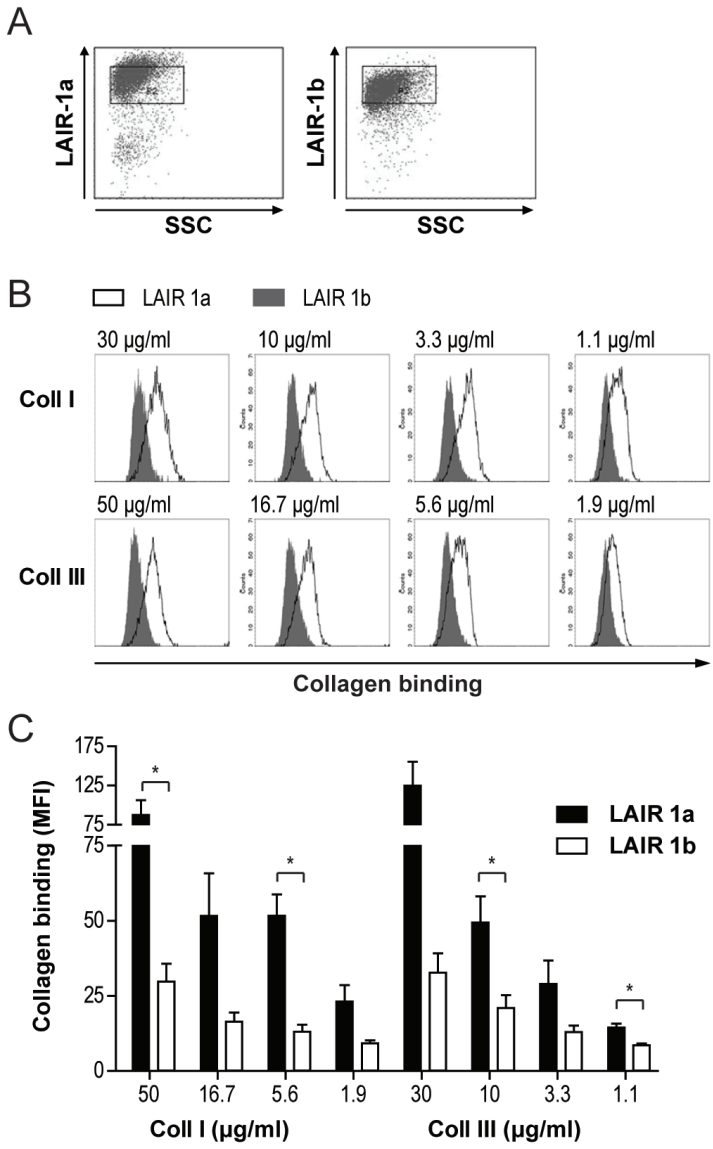

Figure 2. LAIR-1a binds collagen more efficiently than LAIR-1b. (A) K562 cells were stably transduced with LAIR-1a or LAIR-1b and gated for comparable LAIR-1 expression. (B) K562 cells, gated as indicated in *(A)* were incubated with the indicated concentrations of collagen I-FITC (top panels) and collagen III-FITC (bottom panels). Grey histograms represent cells expressing LAIR-1b, open histograms represent cells expressing LAIR-1a. A representative experiment is shown. (C) schematic representation of *(B)*. The Y-axis represents the mean fluorescence intensity (MFI) of the collagen-FITC binding. The collagen incubations using LAIR-1a and LAIR-1b were compared using a paired samples T test (* = p-value (P) ≤ 0.05). Error bars represent SEM (n=3).

of GFP emission to that of Luc emission. Dimerization is indicated by an increased BRETeff when increasing the ratio of GFP to Luc. If the BRETeff remains low when increasing the ratio of GFP to Luc, there is no indication of dimerization.[13] We initially performed experiments in which the GFP/Luc ratio was variable. First, receptor dimerization was determined in unstimulated cells. Both LAIR-1a and LAIR-1b had the same BRETeff as the negative control CD86 (Figure 3A), indicating that neither isoform can dimerize without stimulation. In contrast, the BRETeff of CTLA4 pairs increased at higher GFP/Luc ratio. Subsequently, cells were stimulated with soluble and plate-bound collagen. Again the BRETeff levels remained low (Figure 3B), indicating that collagen binding did not induce dimerization.

We next performed experiments in which the acceptor/donor ratio was kept constant and dimerization was followed in time after addition of collagen (Figure 3C). Two different ratios of GFP to Luc were used for each construct. Again, no increase in the BRETeff was found at any time point, although the LAIR-1a pairs show a somewhat increased signal compared to LAIR-1b. This can be explained if only a few LAIR-1 molecules dimerize, and the remaining monomers decrease the signal. In order to determine the optimal concentration of soluble collagen, collagen was titrated (Figure 3D). Once again, no difference in BRETeff was found over a large range of concentrations. These data demonstrate that no dimerization of LAIR-1a or LAIR-1b occurred as detected by BRET. However, an important limitation of BRET is that it can only detect dimers of molecules within 100Å of each other.[13] Thus, it is still possible that LAIR-1 forms dimers after collagen incubation, with the individual receptors insufficiently close to be detected by BRET. In summary, LAIR-1 dimerization, with or without collagen incubation, could not be demonstrated by BRET and does not explain differences in collagen binding between LAIR-1a and LAIR-1b.

LAIR-1a and LAIR-1b inhibit T cell receptor activation after collagen incubation

To determine whether differences in collagen binding resulted in differential inhibitory capacity after collagen stimulation, we stably transfected NFAT reporter T cells[9] with LAIR-1a or LAIR-1b constructs. Both isoforms were comparably expressed at the cell surface (Figure 4A). NFAT is activated through stimulation of the endogenous mouse T cell receptor (TCR) with anti-CD3 antibodies, resulting in GFP expression that is analyzed by flow cytometry. We investigated whether collagen-mediated cross-linking of LAIR-1a or LAIR-1b could inhibit this activation, and found that both isoforms inhibited TCR signaling after incubation with collagen I in a dose-dependent manner (Figure 4B). However, no significant difference in inhibitory capacity was observed between cells expressing LAIR-1a and LAIR-1b.

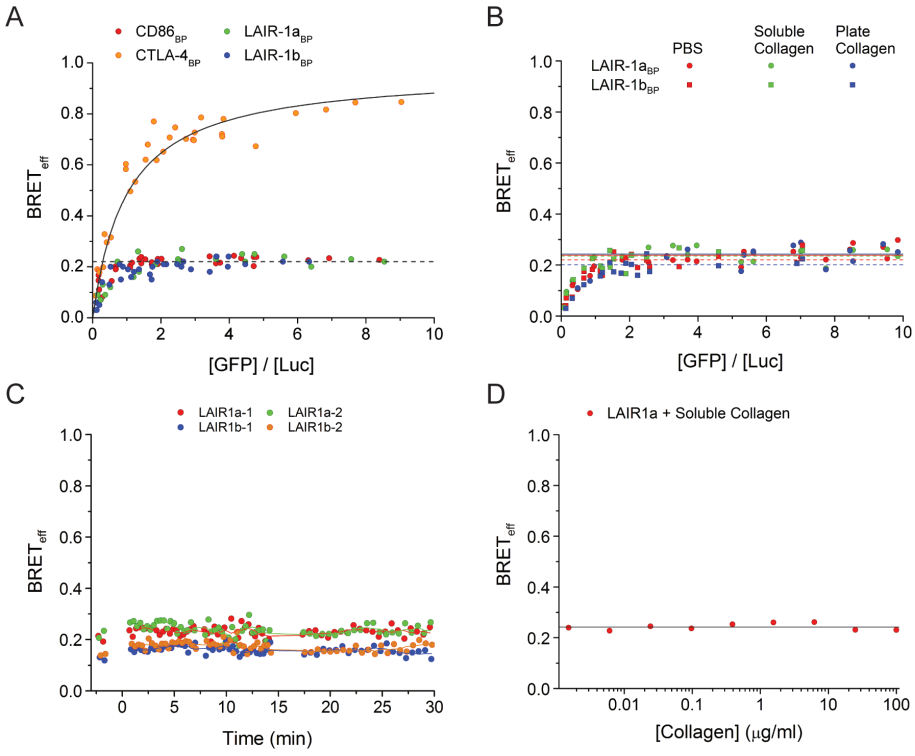

Figure 3. No dimerization of LAIR-1a and LAIR-1b was detected using Bioluminescence resonance energy transfer (BRET). (A) BRET pairs were made for LAIR-1a, LAIR-1b, CTLA-4, and CD86. (B) Both isoforms of LAIR-1 were stimulated with PBS, soluble collagen, or plate-bound collagen (20µg/ml for 15 min at 37°C). (C) Collagen (20µg/ml) was added to two different ratios of GFP and Luc for each isoform, depicted as 1 and 2 in the figure. (D) No dimerization could be demonstrated for LAIR-1a when adding an increasing amount of collagen.

Next, we stimulated LAIR-1 utilizing specific synthetic trimeric peptides derived from collagen II and III that have been demonstrated to be potent inhibitors of immune cell activation.[15] Activation of LAIR-1a and LAIR-1b through collagen peptide II-56 and collagen peptide III-30 resulted in inhibition of TCR signaling (Figure 3C-D), but once again we did not observe any significant differences between cells expressing LAIR-1a and LAIR-1b. In conclusion, despite differences in collagen-binding potential, LAIR-1a and LAIR-1b did not significantly differ in inhibitory capacity after collagen incubation.

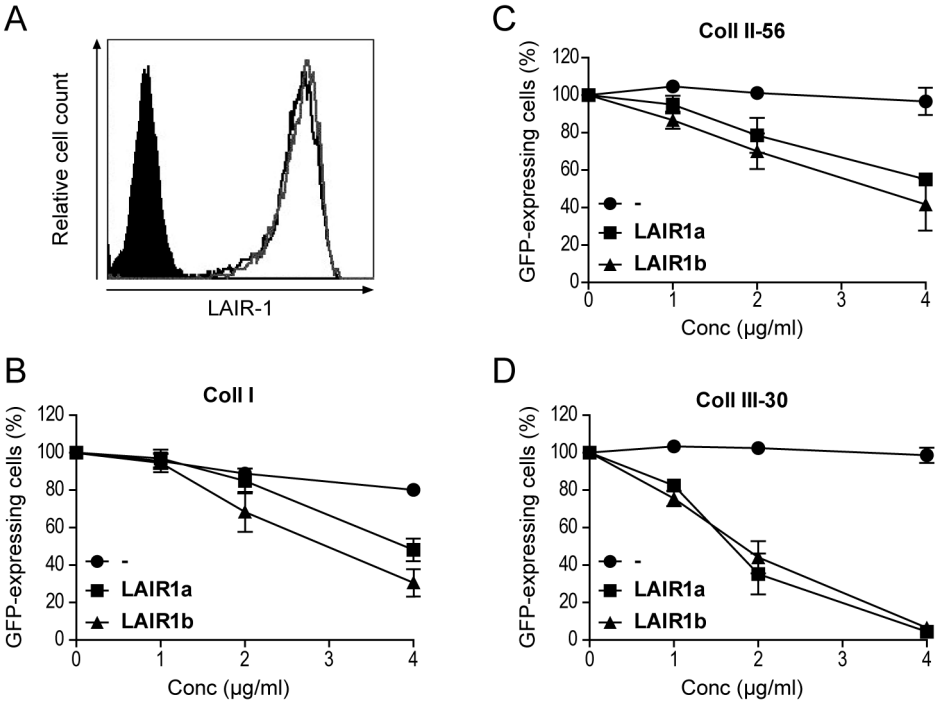

Figure 4. Comparable inhibition of T cell receptor activation by LAIR-1a and LAIR-1b after collagen incubation. (A) LAIR-1 expression was analyzed in non-transfected NFAT reporter T cells (black filled line), cells stably transduced with LAIR-1a (black line) or with LAIR-1b (grey line). Non-transfected NFAT reporter T cells and cells stably transfected with LAIR-1a or LAIR-1b were stimulated overnight with anti-CD3 (0.33 µg/ml) and the indicated concentrations of collagen I (B) or collagen peptides II-56 (C) and III-30 (D). GFP expression was analyzed by flow cytometry. Stimulation with anti-CD3 only is set on 100%. Graphs represent means from three independent experiments. Error bars represent SEM (n=3).

LAIR-1a shows enhanced adherence to collagen compared to LAIR-1b

Collagens are abundantly expressed in tissues, and it is conceivable that transmigrating leukocytes expressing LAIR-1 will adhere to these collagens. To investigate whether the differences in collagen binding capacity between LAIR-1a and LAIR-1b are reflected in differences in collagen-mediated cell adhesion, we performed an adhesion assay in which calcein-labeled K562 cells transfected with either LAIR-1a or LAIR-1b were allowed to adhere to various concentrations of immobilized collagen. As previously determined, both LAIR-1a and LAIR-1b were comparably expressed on the cell surface (Figure 2A). Notably, K562 cells expressing LAIR-1a showed enhanced adherence to collagen I-coated plates compared to K562 cells expressing LAIR-1b (Figure 5A). The difference in collagen binding was much

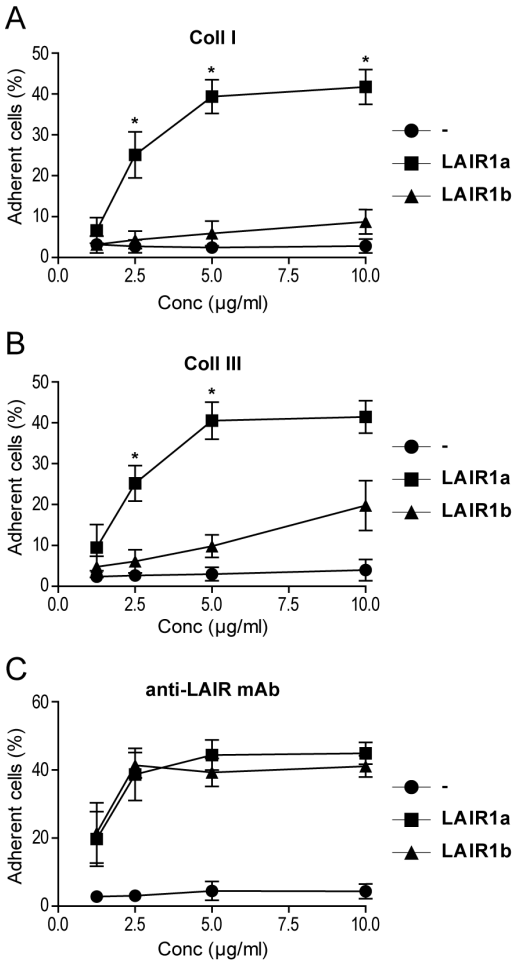

Figure 5. LAIR-1a-expressing cells have enhanced adherence to collagen-coated plates compared to LAIR-1b-expressing cells. Non-transfected K562 cells or K562 transfected with LAIR-1a or LAIR-1b ($5*10^6$) were labeled with 5 µM calceine AM in PBS for 30 min. Cells were washed 5 times in RPMI 1640 1% FCS and $1.5*10^5$ cells were added per well to plates coated with the indicated concentrations of collagen I (A), collagen III (B) and anti-LAIR mAb (C) and incubated at 37°C for 3.5 h. Fluorescence of the plates was determined before washing to determine input signal, and this measurement was repeated after washing the plates 5 times. The fluorescent signal after washings is shown as a percentage of the input fluorescence. The collagen incubations using K562 transfected with LAIR-1a and LAIR-1b were compared using a paired samples T test (* = p-value (P) ≤ 0.05). Error bars represent SEM (n=3).

more pronounced than expected on base of flow cytometry data (Figure 2). Cells expressing LAIR-1b demonstrated little adherence to collagen coated plates. When plates were coated with collagen III, cells expressing LAIR-1a also showed increased binding compared to cells expressing LAIR-1b (Figure 5B), although the adherence of LAIR-1b expressing cells to the collagen III-coated plates was somewhat higher than to the collagen I-coated plates. In contrast, both cell lines bound equally well to plates coated with anti-LAIR-1 mAb (Figure 5C), demonstrating that the difference in adhesion between the cell lines is mediated specifically through differences in natural ligand binding. No binding was observed for LAIR-1a or LAIR-1b transfected K562 cells to BSA-coated plates (data not shown). Furthermore, non-

transfected K562 cells did not bind to collagen-coated plates (Figure 5A-B), indicating that binding depended on the LAIR-1-collagen interaction. In conclusion, K562 cells expressing LAIR-1a show much more efficient adhesion to collagen I- and collagen III-coated plates than K562 cells expressing LAIR-1b.

Discussion

Here, we demonstrate that LAIR-1a and LAIR-1b isoforms are differentially expressed by leukocyte subsets, B and T cells express the LAIR-1b isoform, monocytes express both isoforms and granulocytes solely express LAIR-1a. We show that LAIR-1a has enhanced adherence to collagen compared to LAIR-1b, and K562 cells ectopically expressing LAIR-1a display increased adherence to collagen-coated plates compared to LAIR-1b, while both isoforms bind equally well to anti-LAIR mAb coated plates. The observed differences in collagen binding do not lead to differences in signaling capacities between the two LAIR-1 isoforms. In addition, dimerization for LAIR-1a or LAIR-1b, with or without collagen incubation, could not be demonstrated.

How can the observed differential collagen binding capacity between the LAIR-1 isoforms be explained? Dimerization could not be demonstrated for either isoform and thus cannot explain increased collagen-LAIR-1a binding. On the other hand, the additional LAIR-1a 17 amino acids ETSGGPDSPDTEPGSSA, containing many negatively charged aspartic acid (D) and glutamic acid (E) residues and rich of serine/threonine (S/T) and proline (P), may explain the distinct collagen binding capacity. The large number of extracellular negatively charged residues could induce the formation of a stretched stalk region, in which residues repelling each other by negative charge are maximally apart. In addition, mucin-type O-glycosylation can form O-glycans on serine and threonine, which could stabilize the formed stalk region. Indeed, the presence of O-glycosylation was expected on base of the large difference in protein size between LAIR-1a and LAIR-1b. Thus, LAIR-1a could contain a stretched stalk region, resulting in an increased distance of the receptor from the cell surface compared to LAIR-1b. Since LAIR-1 is a relatively small receptor, with only a single extracellular Ig domain, the presence of such a stalk region may make a considerable difference for the exposure of LAIR-1 above the cell surface, and may contribute to a maximal exposure of the collagen binding site. A similar role for glycosylation has been described for GPIbα, which is part of the GPIb-IX-V complex that bind von Willebrand factor on platelets.[17] This molecule contains a heavily O-glycosylated central stalk region rich

in serine, threonine, and proline residues.[17] The von Willebrand factor-binding site of GPIbα is exposed well above the platelet surface by the presence of this 45-nm-long highly O-glycosylated stalk.[18] In contrast, LAIR-1b may lie adjacent to the cell surface and may thus have more difficulty binding collagen. Antibodies against LAIR-1 on the other hand may bind equally well to both molecules, since these are relatively small compared to collagen and may hence have easier access to the cell surface.

Surprisingly, few studies have investigated the function of splice variants of immune inhibitory receptors. For PECAM-1 several isoforms have been described that are expressed by distinct cell types.[19] Interestingly, specific splice variants of PECAM-1 may also differ in ligand binding properties.[20;21] These isoforms differ in the intracellular domain, and effects on adhesive properties probably result from the deletion of a domain that mediates calcium-dependent, heterophilic adhesion.[20] We showed that LAIR-1a can adhere to collagen-coated plates, whereas this was almost undetectable for LAIR-1b. Thus, LAIR-1a expressing leukocytes could bind to collagen in tissue or to collagen exposed in the vasculature after damage, and this could stimulate migration into the tissue to resolve potential infection. We demonstrate that LAIR-1a, but not LAIR-1b, is present in neutrophils, although it is not expressed at the surface of unstimulated neutrophils.[22] Interestingly, LAIR-1 surface expression is induced by incubation of neutrophils with the inflammatory cytokine G-CSF, both *in vitro* and *in vivo*.[22] It is tempting to speculate that upon immune activation, surface LAIR-1a expression is induced on neutrophils and influences neutrophil migration to the inflammatory locus. Would LAIR-1 binding to collagen lead to enhanced migration or impaired migration? When considering the role of other inhibitory receptors with adhesive properties, PECAM-1 and CEACAM1, both positively affect leukocyte migration[4;5] and we propose that LAIR-1a may also enhance neutrophil migration. LAIR-1, PECAM-1 and CEACAM1 may perform equivalent roles on neutrophils. Similar to PECAM-1, LAIR-1 expression is down-regulated during neutrophil differentiation,[22;23] for CEACAM1 it was demonstrated that expression is up-regulated on activated neutrophils, comparable to what was observed for LAIR-1 expression.[24]

Although important in resolving infection, leukocyte adhesion and migration may also contribute to pathophysiological conditions. For example, atherosclerotic plaques are covered by a cap rich in fibrillar collagens.[25] It has been suggested that $\alpha_1\beta_1$ or other collagen receptors expressed on leukocytes may be involved in leukocyte invasion during atherogenesis.[25] LAIR-1a expression may similarly be involved in the inflammatory process in atherosclerotic plaques.

Besides immune effector cells, we have recently demonstrated that hematopoietic stem cells (HSC) and multipotent progenitor lineages also express LAIR-1, and in addition LAIR-1 is expressed on a subset of developing megakaryocytes.[26] HSC and progenitor cells reside in the collagen-rich bone marrow microenvironment, megakaryocytes mature in this milieu and platelet formation is preceded by migration from osteoblastic stem cell niches to sinusoids where the platelets are shed in the circulation.[27] Adhesion through collagen receptors may therefore be an important regulatory element in megakaryocyte migration.

The role of LAIR-1 on HSC remains unclear since no effect of collagen incubation on proliferation, differentiation or apoptosis of LAIR-1-expressing HSC could be demonstrated *in vitro* (our unpublished results). The possible role of LAIR-1 as an adhesion molecule sheds new light on this issue. It has been postulated that the HSC niche is essential for the long-term maintenance of the HSC pool and is involved in the regulation of normal HSC numbers and maintenance of the quiescent long-term HSC pool.[28;29] Possibly, LAIR-1 expression contributes to the adhesive capacity of HSC, resulting in enhanced adherence to the bone marrow niche, hence preventing differentiation. Determining whether LAIR-1a or LAIR-1b are expressed by HSC and megakaryocytes will the first step in this investigation.

In conclusion, LAIR-1a and LAIR-1b are both capable of inhibiting immune cell activation *in vitro*. Our present data suggest that besides inhibitory capacities, specific LAIR-1 isoforms may also affect leukocyte adhesion, suggesting a dual role for this inhibitory receptor in immune cell regulation.

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General Discussion

CHAPTER 7

Immune inhibitory receptors are differentially expressed by immune cells and negatively regulate the immune response.[1] So far, more than 60 inhibitory receptors have been characterized,[2] and multiple inhibitory receptors can be expressed on a single immune cell. Most inhibitory receptors contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) with the consensus sequence V/L/I/SxYxxV/L/I,[3] and can recruit SH2 domain-containing phosphatases SHP-1, SHP-2 or SHIP.[2] In spite of their abundance and the overlap in recruited phosphatases, inhibitory receptors play non-redundant roles in the regulation of the immune response.[1] Specificity is partly achieved through regulated receptor expression, expression of the ligand, recruitment of phosphatases and the affinity with which these are recruited. However, other mechanisms may play a role, such as recruitment of alternative cell-type specific accessory molecules to mediate inhibitory effects.[4-7] In addition, while inhibitory receptors are generally considered to globally inhibit all effector functions, they can also selectively suppress specific cellular activity. Further research is required to fully understand the molecular mechanisms underlying inhibitory receptor function. Since only approximately one fifth of the 300 potential inhibitory receptors have been characterized,[2] further identification and characterization of inhibitory receptors will contribute to a more thorough understanding of the biological role of this group of immune regulators.

In this thesis, we have characterized signal inhibitory receptor on leukocytes-1 (SIRL-1, encoded by *VSTM1*), as a novel, functional inhibitory receptor expressed on human phagocytes. The closest homolog of SIRL-1 is leukocyte-associated Ig-like receptor-1b (LAIR-1b), although the homology is not extensive. LAIR-1 forms the second focus of this thesis. We further analyzed the expression pattern of LAIR-1 on hematopoietic stem cells (HSC), progenitor cells, and megakaryocytes, investigating the apparently mutually exclusive expression patterns of LAIR-1 and the activating collagen receptor GPVI. Finally, we analyzed expression of splice variants LAIR-1a and LAIR-1b in leukocyte subtypes. This general discussion addresses the implications of this work and further directions following the data presented in this thesis. Initially the focus is on the role of SIRL-1 in immune regulation: what intracellular molecules realize the inhibitory effect and is there an immunological advantage of limited inhibitory capacity for inhibitory receptors? Thereafter, the role of LAIR-1 on HSC and differentiating leukocytes, and possible function as an adhesion molecule will be discussed. Finally, we will discuss general implications of our findings for the field of inhibitory receptors.

SIRL-1 is a phagocyte specific receptor

Individual immune inhibitory receptors have a specific expression pattern and leukocyte subtypes can express multiple inhibitory receptors simultaneously. Some inhibitory receptors are broadly expressed, whereas others are expressed only by a specific cell type. For example, LAIR-1 is generally expressed by both differentiating and mature immune cells, whereas Ig-like transcript 3 is expressed exclusively by cells of the monocytic lineage and dendritic cells. In Ctulture of cord blood-derived HSC to neutrophils, SIRL-1 expression was found only during the final stage of differentiation (unpublished results). Thus, SIRL-1 is specifically expressed on mature phagocytes. The specific expression pattern of an inhibitory receptor, together with the expression pattern of its ligand will dictate in which situation the receptor will be functional. So far, the ligand for SIRL-1 has not been identified. Because of the moderate homology with LAIR-1 we investigated whether SIRL-1 could also bind collagen, but this was not detected (data not shown).

In Chapter 3 we demonstrate that expression of SIRL-1 may be regulated during infection, as suggested by cytokine-induced or pattern recognition receptor (PRR)-induced phagocyte activation resulting in down-regulation of SIRL-1 expression on phagocytes. As discussed in Chapter 3 and Chapter 4, many immune inhibitory receptors show specific up- or down-regulation upon cell activation. For example, LAIR-1 is down-regulated on activated T cells,[8] whereas LAIR-1 expression on neutrophils is induced upon activation[9], being absent from resting neutrophils. Expression of CD200R is also up-regulated after cell activation,[10] while SIRP-α expression on macrophages is down-regulated after LPS stimulation.[11] Regulated expression may serve to tune the inflammatory response. Inhibitory receptors that are initially highly expressed may create an immune activation threshold, while receptors that are up-regulated after cell stimulation may function in the termination of the immune response.

SIRL-1 can inhibit production of reactive oxygen species

In Chapter 3 we demonstrate that SIRL-1 can inhibit Fc Receptor (FcR)-induced reactive oxygen species (ROS) production in phagocytes. The nicotinamide adenine dinucleotide phosphate (NADPH) complex is responsible for ROS production in phagocytes, resulting in generation of hydrogen peroxide and hypochlorous acid.[12] ROS produced by phagocytes is crucial in microbial killing, and reduced ROS production through SIRL-1 cross-linking correspondingly leads to reduced killing of internalized bacteria. This function, combined with

the regulated expression indicates that SIRL-1 prevents inappropriate ROS production in steady-state conditions. When infection is sensed, SIRL-1 expression is down-regulated on phagocytes, enabling bacterial killing for instance in response to Ig-opsonized pathogens (Figure 1).

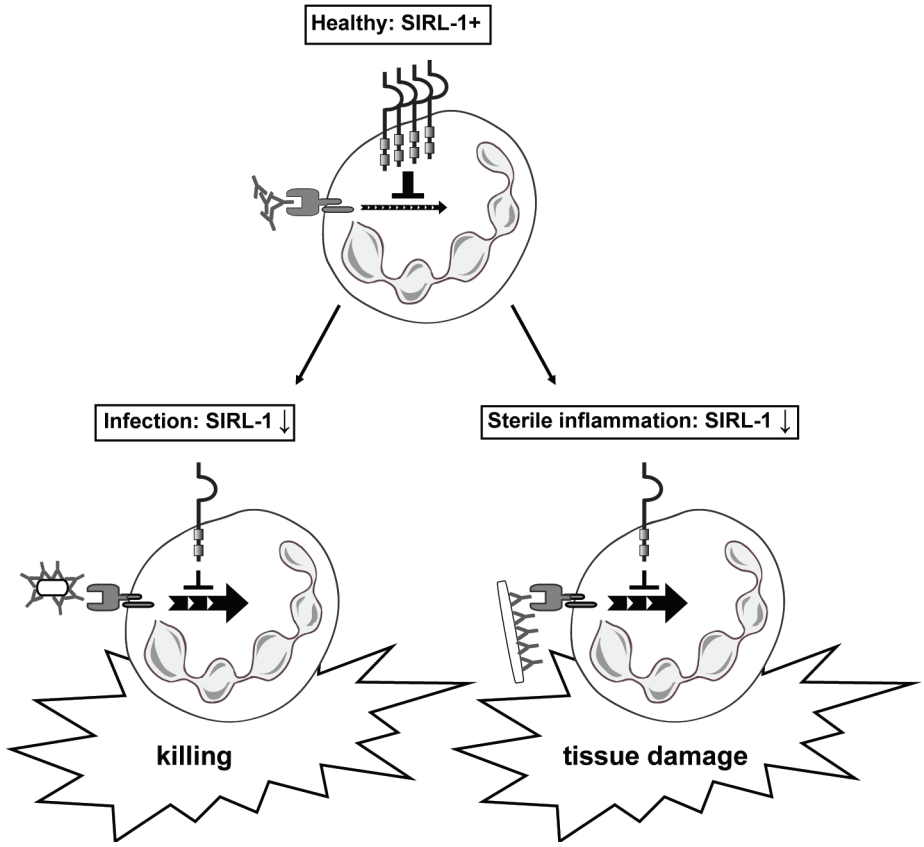

Figure 1. Model for SIRL-1 biological function. In steady state conditions, SIRL-1 expression on phagocytes prevents inappropriate ROS production, for example induced by non-specific antibody clustering (top panel). Upon infection, SIRL-1 expression on phagocytes is down-regulated through stimulation of pattern recognition receptors. This will lead to enhanced reactive oxygen species (ROS) production upon stimulation of Fc receptors, which will lead to optimal microbial killing (bottom panel, left). SIRL-1 expression on phagocytes is also down-regulated by inflammatory cytokines, thus decreasing the threshold for ROS production. In conditions of sterile inflammation, this could enhance disease pathology by ROS-induced tissue damage (bottom panel, right).

Phagocytes have additional effector functions, such as phagocytosis and cytokine production. We show in Chapter 3 that SIRL-1 does not modulate these functions, suggesting that SIRL-1 selectively regulates the oxidative burst. What mechanism leads to a specific reduction of FcR-induced ROS production? Ligand-mediated activation of FcRs results in ITAM phosphorylation by Src family kinases, which will also phosphorylate and activate Syk.[13] Syk is a central player in FcR signaling, and activation eventually results in calcium influx, ROS production and phagocytosis, as well as cytokine production.[14] Through Syk, ITAM-mediated signals activate the adaptor protein CARD9, which signals via Bcl10 and Malt1[15] and is essential for cytokine production by myeloid cells through activation of the transcription factor NF-κB.[16] It is not clear whether CARD9 is also involved in ROS production, as conflicting results have been reported.[17;18] Also MAPK signaling can lead to the production of inflammatory cytokines.[19] El Benna and workers showed that $p47^{phox}$ can be phosphorylated by MAPK p38 and ERK,[20] however, others have shown that ROS production is independent of MAPK signaling.[21] Thus, although the data are not unambiguous, intracellular pathways leading to cytokine production may be quite distinct from pathways leading to the oxidative burst. In contrast, the intracellular pathways leading to ROS production and phagocytosis are closely related, as discussed below.

The NADPH oxidase complex of phagocytes is composed of membrane-integrated cytochrome b_{558} (comprising $gp91^{phox}$ and $p22^{phox}$) and cytosolic components (Rac, $P67^{phox}$, $p47^{phox}$ and $p40^{phox}$). For activation it is essential that cytosolic factors translocate to the plasma membrane to form an active complex, $p47^{phox}$ plays a central role herein. FcγR-mediated signaling will via Syk activate the guanine nucleotide exchange factor Vav, which in turn leads to activation of Rac, PLC-γ2 and PI3K.[22;23] PLC activation results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-trisphosphate (IP3) and diacylglycerol, leading to the release of Ca^{2+} and PKC activation. Through PKC,[24;25] FcγR activation induces $p47^{phox}$ phosphorylation, which then binds $p22^{phox}$.[26] Concomitantly, activated Rac recruits $p67^{phox}$, which associates with $p47^{phox}$ and cytochrome b_{558} to form an active enzyme complex,[26-28] and activated phosphoinositide 3-kinase (PI3K) generates 3'-phosphoinositides, which bind $p40^{phox}$ and facilitate translocation of the phox-proteins to the plasma membrane.[29;30] Both Rac and PKC are also essential for FcR-induced phagocytosis.[31;32] In contrast, PI3K is not required for phagocytosis of IgG-opsonized particles smaller than 2 μm,[33] and will therefore probably not be involved in the phagocytosis of serum-opsonized bacteria. Thus, it is possible that SIRL-1 specifically inhibits PI3K, leading to impaired $p40^{phox}$ activation and reduced active NADPH oxidase complex formation. Al-

ternatively, SIRL-1 may be capable of directly dephosphorylating other phox-proteins, such as p47^{phox} (Figure 2).

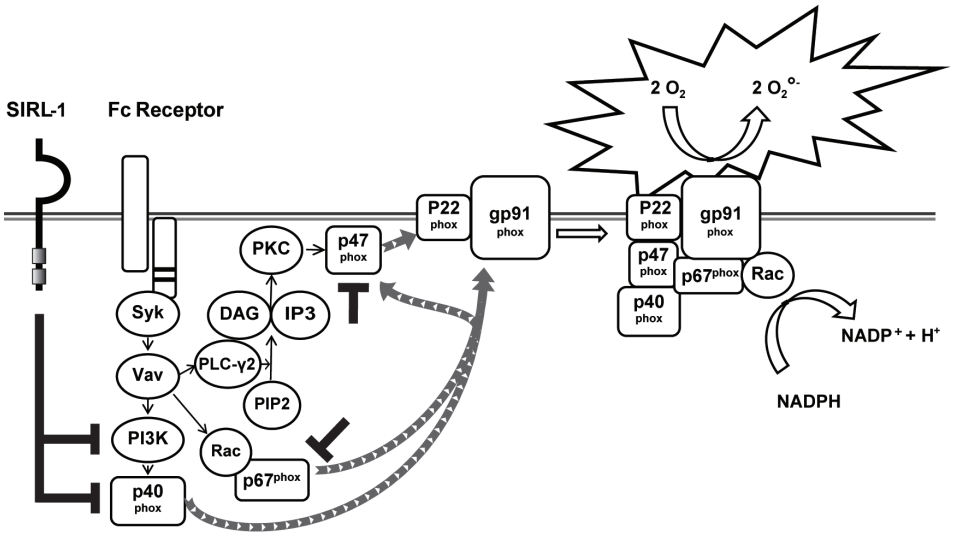

Figure 2. Model for selective regulation of ROS production by SIRL-1. The NADPH oxidase complex of phagocytes is composed of membrane-integrated cytochrome b558 (comprising gp91^{phox} and p22^{phox}) and cytosolic components (Rac, P67^{phox}, p47^{phox} and p40^{phox}). For activation it is essential that cytosolic factors translocate to the plasma membrane to form an active complex (right side of Figure). Fc receptor stimulation will via Syk activate the guanine nucleotide exchange factor Vav, which in turn leads to activation of Rac, PLC-γ2 and PI3K. PLC activation results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-trisphosphate (IP3) and diacylglycerol, leading to the release of Ca^{2+} and PKC activation. Through PKC, FcγR activation induces p47^{phox} phosphorylation, which then binds p22^{phox}. Activated Rac recruits p67^{phox}, which associates with p47^{phox} and cytochrome b558 to form an active enzyme complex, and phosphoinositide 3-kinase (PI3K) will activate p40^{phox} and facilitate translocation of the phox-proteins to the plasma membrane. In contrast to PI3K, Rac and PKC are also essential for FcR-induced phagocytosis (not depicted). Thick black lines indicate effector molecules (PI3K, p40^{phox}, p47^{phox} or p67^{phox}) possibly inhibited by SIRL-1.

The specific inhibition of ROS production demonstrated in Chapter 3 conflicts with the classical view of inhibitory receptors that dephosphorylate upstream signaling molecules through recruitment of SHP-1 and/or SHP-2 and consequently inhibit all subsequent events.[34] However, global inhibition of all effector functions, presumably through SHP-1 and SHP-2, has been predominantly demonstrated for a number of killer cell Ig-like receptors (KIRs) in natural killer (NK) cells.[35-37] A model for the general mechanism leading to

inhibitory receptor-mediated inhibition may have been inappropriately extrapolated from these early studies. If SIRL-1 does function by upstream inhibition, the differential inhibition may be explained by kinetics or degree of phosphorylation: SIRL-1 may reduce phosphorylation of the FcR or Src kinase up to a certain degree, which could be permissive for cytokine production while hampering ROS production. However, as reviewed in Chapter 4, SHP-1 and SHP-2 can also mediate inhibition of cellular activation through alternative pathways. Cellular localization of both inhibitory receptor and recruited molecules may be directly responsible for determining which signal transduction pathways are regulated. For example, Sasawatari and colleagues present a model in which Ly49Q, accompanied by raft components, is endocytosed after chemokine stimulation. SHP-2 recruited by the Ly49Q ITIM in endosomes is crucial for sustained activation of endosomal raft-associated signaling, which contributes to neutrophil polarization and migration.[38] In addition, inhibitory receptor-mediated inhibition of NK cell activity is known to act locally, as NK cells contacting both resistant and susceptible target cells are capable of selective killing of susceptible target cells.[39;40] Inhibitory receptors present in the immunological synapse between target cell and effector cell mediate the localized inhibition of activating receptor cytotoxicity.[39] Possibly, SIRL-1 does not co-localize with FcR or other activating receptors, and therefore cannot dephosphorylate upstream events. It would be interesting to investigate the cellular localization of SIRL-1, SHP-1, FcR and the NADPH oxidase complex during SIRL-1-mediated inhibition of FcR-induced ROS production by confocal microscopy.

Is SIRL-1 strictly dependent on SHP-1 or SHP-2 for signaling? In Chapter 2, we showed that SIRL-1 can recruit SHP-1 in a myeloid cell line and in primary monocytes. We were also able to demonstrate SHP-1 recruitment by primary neutrophils (data not shown). Although many ITIM-bearing receptors have impaired inhibitory capacity in SHP-1/2 deficient cells,[41-43] recruitment is not proof of requirement. For example, LAIR-1 strongly recruits both SHP-1 and SHP-2, but can fully inhibit B cell receptor-induced calcium mobilization in SHP-1/2 deficient cells, possibly through recruitment of C-terminal Src kinase (Csk).[6] Indeed, recruitment of alternative molecules has been demonstrated for other inhibitory receptors, such as suppressor of cytokine signaling 3 (SOCS3) for CD33 and Siglec-7,[4] Csk for SIRP-α and Ig-like transcript (ILT)-2[5-7] and Dok1/2 for CD200R. Furthermore, we demonstrated that SHP-1 recruitment by SIRL-1 single tyr-phe mutants was abrogated, while these mutants can still partially inhibit degranulation in a basophilic cell line, and suppress ROS production in human myeloid PLB cells (Chapter 2 and Chapter 3 respectively). Therefore, it

is likely that SIRL-1 recruits alternative molecules besides SHP-1 and it would be of interest to investigate if SIRL-1 inhibits ROS production in SHP-1 knock-down PLB cells.

For other inhibitory receptors, inhibition of specific signal transduction pathways has also been reported. For example, the immune inhibitory receptor CD300a can inhibit Eotaxin-induced migration, but not Eotaxin-induced calcium influx in eosinophils.[44] The authors demonstrate that CD300a signaling inhibits Eotaxin-induced JAK2, ERK and p38 phosphorylation, which could be responsible for the effects on migration. Thus, although not frequently reported, additional studies have demonstrated that inhibitory receptors regulate specific activation signals.

The dogma that inhibitory receptors are broad inhibitors of the immune response may not necessarily reflect the capacity of many inhibitory receptors. Since negative data are less likely to be reported, a bias may develop towards the variety of signals that can be regulated, while the unaffected pathways are not discussed. In addition, early documentation of prominent inhibitors may have biased the field. Inhibitory receptors with broad suppressive capacity may be easier identified than selective inhibitors. Thus far, approximately one fifth of the 300 potential type I and type II ITIM-containing molecules in the human genome have been characterized.[2] Provided that transcriptional expression is confirmed, it is important to further characterize these potential inhibitory receptors. If hitherto primarily broad inhibitors are recognized, the characterization of novel inhibitory receptors with restricted inhibitory capacity will lead to a more accurate view of the role of these receptors in orchestrating the immune response.

Is it advantageous for the immune system to express a receptor that specifically inhibits ROS production? ROS production is crucial in bacterial killing, as is evident from the recurrent bacterial infections suffered by patients with chronic granulomatous disease (CGD).[45] These patients have defective ROS production due to mutations in the NADPH oxidase complex. However, excessive ROS production can damage host tissue and contribute to diseases characterized by persistent inflammation, such as atherosclerosis and chronic obstructive pulmonary disease (COPD). It will be interesting to investigate whether SIRL-1 expression is altered in these diseases. In addition, ROS production can induce apoptosis.[46] Siglec-9 signaling in neutrophils induces ROS production by NADPH oxidase, leading to death signals.[47] Hence, SIRL-1 signaling in neutrophils during the immune response may increase the life span of these cells and this may be a mechanism to limit excessive neutrophil cell death upon trauma.

Evolutionary conservation of SIRL

LAIR-1, KIRs, leukocyte Ig-like receptors (*LILRs*, also known as *Ig-like transcripts (ILTs*) and *VSTM1*, encoding SIRL-1, are all localized to the leukocyte receptor complex (LRC) on human chromosome 19q13.4, suggesting that these molecules have evolved from a common ancestral gene.[48;49] The coding regions of SIRL-1 are conserved in chimpanzee and marmosets (Figure 3) and functional SIRL-1 transcripts have been found in these species.

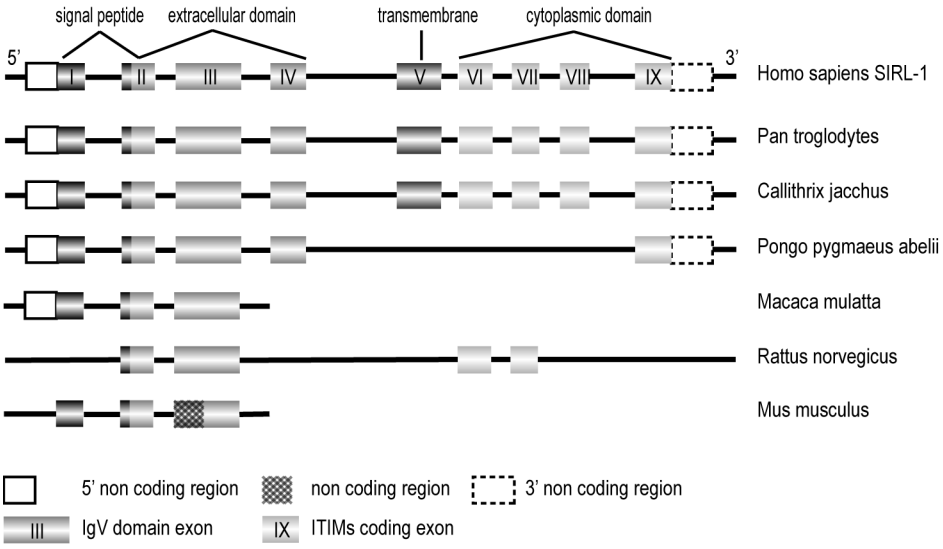

Figure 3. *VSTM1*, the gene encoding SIRL-1, is not broadly conserved. Coding regions of *VSTM1* are conserved in chimpanzee (Pan troglodytes) and marmosets (Callithrix jacchus). In contrast, in orangutan (Pongo pygmaeus abelii), rhesus macaque (Macaca mulatta), rat (Rattus norvegicus) and mouse (Mus musculus) certain coding regions of *VSTM1* are lost, and no functional SIRL-1 transcripts are found.

In orangutan and rhesus macaque coding regions are lost, and no functional SIRL-1 transcripts are found. In mice, *VSTM1* is localized on chromosome 7, the syntenic chromosome of human chromosome 19q13.4, but again, this gene is rudimentary -lacking essential coding regions- and no evidence of an mRNA transcript has been found, as is the case for rat *VSTM1*. One may wonder why these species would not need a receptor that specifically regulates ROS production, but perhaps they encode a functionally analogous protein, as has been demonstrated for other inhibitory receptors. For example, the KIR family of inhibi-

tory receptors plays a very important role in regulating human NK cells, but these receptors do not have a functional mouse homolog. Instead, the structurally divergent, c-type lectin Ly49 family of inhibitory receptors performs a similar role in regulating mouse NK cell activity, and has comparable ligands.[50] It remains to be determined whether a functionally analogous protein for SIRL-1 is encoded in animal genomes.

The role of LAIR-1 during hematopoiesis

As discussed above, LAIR-1 is broadly expressed by immune cells, being expressed on NK cells, T cells, B cells, monocytes, eosinophils and dendritic cells.[9;51] Activation of T cells results in a down-regulation of cell surface LAIR-1.[8] Conversely cell surface LAIR-1 expression on neutrophils is induced upon activation[9] being absent from resting neutrophils. It is thus likely that LAIR-1 serves a distinct role on T cells compared to neutrophils. Besides being expressed on effector cells, LAIR-1 is also expressed on thymocytes, HSC and differentiating neutrophils.[9;51] In addition, we demonstrate in Chapter 5 that LAIR-1 is expressed on all subsets of multipotent progenitor cells and by a subset of megakaryocytes. *In vitro*, a strong LAIR-1-mediated inhibition of cytotoxicity and calcium mobilization has been demonstrated in NK cells, T cells and B cells respectively.[51-53] However, the role of LAIR-1 on HSC, progenitor cells and megakaryocytes is less clear.

As discussed in Chapter 5, GPVI is structurally related to LAIR-1[54] and the genes encoding LAIR-1 and GPVI are both localized to the LRC region. The genomic proximity and structural homology between the two receptors suggest that LAIR-1 and GPVI have a common origin and the collagen-binding site in LAIR-1 and GPVI overlaps between the two receptors.[55-57] The intracellular tail of GPVI associates with the ITAM-containing FcRγ.[58-60] Furthermore, LAIR-1 cross-linking abrogates collagen-induced GPVI signaling when both receptors are ectopically expressed on the same cell.[61] Thus, expression of both receptors on megakaryocytes could modulate megakaryocyte function. So far, we have not been able to elucidate the biological role of LAIR-1 in regulating GPVI signaling. Megakaryocytes are scarce, fragile cells, which impedes studies that investigate functionality of megakaryocyte receptors. The role of GPVI on megakaryocytes is also unclear, although it has been shown to be capable of signaling in these cells.[62-64] Studies investigating the effect of GPVI signaling after collagen stimulation on megakaryocytes may have been hindered by the fact that the presence of an inhibitory collagen receptor on these cells was not acknowledged. Therefore, our study may contribute to determining the biological role of GPVI on megakaryocytes.

The function of high and stable LAIR-1 expression on HSC has so far remained elusive. HSC can either proliferate, differentiate or die, processes that are predominantly regulated by cytokine signaling. Inhibitory receptors can affect signals relayed through various types of activating receptors (reviewed in Chapter 4), and we hypothesize that LAIR-1 on HSC would affect cytokine or growth factor-induced proliferation, differentiation or apoptosis. Several observations support these hypotheses: first, multiple inhibitory receptors can affect cytokine-induced survival signals,[47;65;66] second, LAIR-1 signaling induces apoptosis in dendritic cells and myeloid tumor cells.[67;68] Moreover, SHP-1, which is recruited by LAIR-1, negatively regulates cytokine receptor-induced signaling.[69] Finally, HSC reside in a collagen-rich bone marrow niche and collagens are high affinity ligands for LAIR-1, suggesting that LAIR-1 could be constitutively active on these cells. Despite these indications, we were unable to demonstrate any effect of collagen incubation on cytokine-induced proliferation, differentiation or apoptosis of LAIR-1-expressing HSC *in vitro* (unpublished results), nor did we find an effect of LAIR-1 cross-linking on cytokine-induced signal transduction, proliferation or apoptosis in the IL-3-dependent human pro-B cell line Ba/f3 (unpublished results). Possibly HSC lack expression of signal transduction molecules required for LAIR-1-mediated inhibitory function. However, for other inhibitory receptors functionality on these cells could be demonstrated. For example, expression of platelet endothelial cell adhesion molecule-1 (PECAM-1) on HSC and progenitor cells protects from starvation-induced apoptosis by activation of PKB.[70] Obviously, the *ex vivo* hematopoiesis models utilized do not fully recapitulate the bone marrow niche. Perhaps the quantity of cytokines added *in vitro* provides a strong activation signal that cannot be inhibited by LAIR-1. Alternatively, LAIR-1 may be differently involved in the regulation of HSC, regulating other signals than those transmitted through cytokine receptors.

Is LAIR-1 an adhesion molecule?

In Chapter 6, we investigated whether splice variants LAIR-1a and LAIR-1b are distinct with regard to adhesion and signaling capacities after collagen binding. We demonstrate that LAIR-1a shows enhanced adherence to collagen-coated plates compared to LAIR-1b. Since hematopoiesis takes place in the collagen-rich bone marrow microenvironment, it would be interesting to investigate whether HSC and megakaryocytes express the LAIR-1a or the LAIR-1b isoform. It has been postulated that the HSC niche is essential for the long-term maintenance of the HSC pool and is involved in the regulation of normal HSC numbers and

maintenance of the quiescent long-term HSC pool.[71;72] Possibly, LAIR-1 expression contributes to the adhesive capacity of HSC, resulting in enhanced adherence to the bone marrow niche, hence preventing differentiation.

Megakaryocytes also mature in the bone marrow microenvironment and platelet formation is preceded by migration from osteoblastic stem cell niches to sinusoids where the platelets are shed in the circulation. Adhesion through collagen receptors may be an important regulatory element in megakaryocyte migration, and LAIR-1a expression could contribute to this process. Since LAIR-1 is only expressed during an early stage of megakaryocytopoiesis, expression may be important to retain immature cells in the bone marrow microenvironment. Interestingly, the inhibitory receptor PECAM-1 also functions as an adhesion molecule and is also expressed on both HSC and megakaryocytes.[73;74] PECAM-1 knock-out mice show excessive megakaryocytopoiesis and altered megakaryocyte localization, possibly through disturbed adhesive capacity.[74] The role of PECAM-1 may be indicative of LAIR-1 function on these cells. Further research is necessary to determine during HSC and megakaryocyte differentiation if LAIR-1 would function as either an adhesion molecule or a signaling molecule, or as both.

In Chapter 6, we show that T cells and B cells express the splice variant LAIR-1b, monocytes express both isoforms, and neutrophils express exclusively LAIR-1a. Since collagens are not expressed in the vasculature, mature leukocytes will interact with collagen only after migration into the tissue or after tissue damage, with tissue collagens becoming exposed. It is not certain whether the LAIR-1a-collagen interaction affects leukocyte migration, and if so, whether it enhances or impairs migration properties. The LAIR-1a-collagen interaction could lead to cell retention, preventing further movement, or may improve the contact between the migratory leukocyte and the tissue, thus facilitating binding of the leukocyte to other receptors involved in migration. When leukocytes infiltrate tissue, this requires initial leukocyte adhesion through activated integrins, suggesting that adhesion positively regulates migration. A positive role in leukocyte migration has been demonstrated for other inhibitory receptors such as PECAM-1 and carcinoembryonic antigen-related cell adhesion molecule1 (CEACAM1).[75;76] PECAM-1 promotes leukocyte adherence to the endothelium and transmigration through endothelial junctions and the perivascular basement membrane through homophilic PECAM-1/ PECAM-1 adhesive interactions between leukocytes and endothelial cells.[76] In analogy with these receptors, collagen in the tissue, or collagen exposed in the vasculature after damage could enhance binding of LAIR-1a expressing leukocytes, which could then migrate into the tissue to resolve a possible infec-

tion. It is therefore of interest that activated neutrophils exclusively express LAIR-1a (Chapter 6). It is interesting to speculate that LAIR-1 expression is induced upon infection and serves to facilitate neutrophil migration into tissue that is damaged by infection. If indeed positively regulating migration, this would indicate that LAIR-1 serves a dual role in inflammation, both suppressing immune activation and enhancing tissue infiltration, as has been demonstrated for PECAM-1.[76]

Regulated splicing is also seen for other immune receptors and can have important functional consequences.[77] For example, both soluble and transmembrane splice variants of CTLA4 and CD95 exist. T cell stimulation results in increased expression of transmembrane variants, thus increasing sensitivity to inhibition and apoptosis respectively.[78;79] It would be interesting to investigate whether stimulation of for example monocytes, NK cells or T cells would result in different expression of LAIR-1 splice variants.

In Chapter 6, we show that although LAIR-1a has enhanced binding to collagen compared to LAIR-1b, the inhibitory capacity of both isoforms is comparable. This suggests that the signaling capacity is independent of the strength of receptor binding. Possibly, the LAIR-1-collagen binding site has multiple contact points, of which some are crucial for signaling and others serve to enhance binding. Alternatively, the LAIR-1b-collagen interaction may be unstable due to the position of LAIR-1b on the cell surface, albeit sufficient for activation. Nevertheless, the similar inhibitory capacity of these two isoforms is surprising, as one would expect that an increased interaction time by enhanced binding would result in stronger signaling. Possibly, our results may be explained by the experimental set-up (Figure 4, Chapter 6). Plates were coated with both anti-CD3, to stimulate the T cell receptor (TCR), and with collagen. The high affinity interaction between the TCR and directed antibodies may bind cells to the collagen-coated plate, thus relieving the need of a firm collagen-LAIR-1 interaction for signaling. The independent effect of collagen adherence and signaling could be investigated in a set-up that does not involve co-coating of collagen with an activating receptor. However, the mechanism of binding and holding a cell through various receptors may also be relevant *in vivo*. It would also be of interest to investigate whether simultaneous expression of LAIR-1a and LAIR-1b, as is observed in NK cells[51] and in monocytes (Chapter 6), would have further effects on signaling and adherence.

Concluding remarks

We investigated LAIR-1 expression on HSC and progenitor cells and on megakaryocytes and showed that LAIR-1 is expressed on a subset of megakaryocytes. So far, the function of LAIR-1 on these cells remains unclear. The splice variants LAIR-1a and LAIR-1b are differentially expressed by various leukocyte subtypes. We found that LAIR-1a has enhanced binding to its ligand collagen compared to LAIR-1b. Through its adhesive properties, LAIR-1a could affect neutrophil transmigration and HSC and progenitor cell retention in the bone marrow niche. These hypotheses are subject of further investigation.

In addition, we have characterized SIRL-1, a novel immune inhibitory receptor that regulates the oxidative burst in human phagocytes. ROS production by phagocytes is crucial for bacterial killing; hence SIRL-1 may play an important role during bacterial infections. The identification of the natural ligand for SIRL-1 will greatly contribute to our understanding of this receptor function *in vivo*. SIRL-1 does not inhibit other related phagocyte functions such as phagocytosis and cytokine production, indicating that its function is specific. Intracellular location and alternatively recruited molecules may be important in the inhibition of specific signal transduction pathways. Our identification of an inhibitory receptor that regulates a specific cellular function may represent only the tip of the iceberg, challenging the dogma of upstream inhibition by ITIM-bearing receptors.

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Samenvatting in het Nederlands

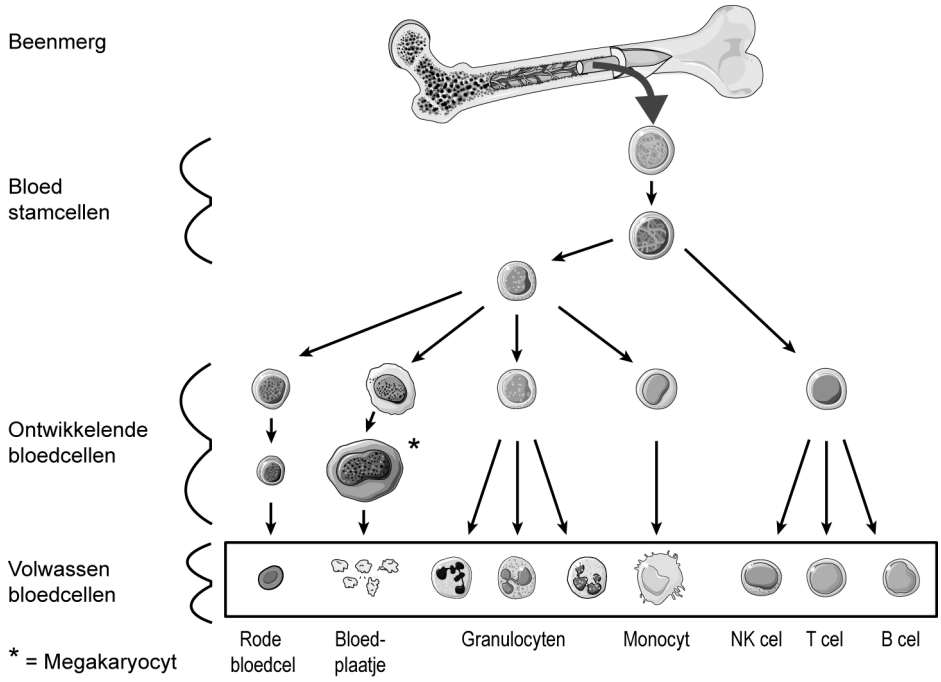
Inleiding

Een goed functionerend immuunsysteem houdt ons lichaam gezond door ziekteverwekkers (pathogenen) en geïnfecteerde of kwaadaardige cellen op te ruimen. Wanneer cellen of eiwitten in het lichaam als lichaamsvreemd of geïnfecteerd herkend worden, treedt het immuunsysteem krachtig op om de bedreiging te elimineren. Hoe belangrijk het immuunsysteem is in het tegenhouden van pathogenen is vooral duidelijk bij mensen met afwijkingen aan het immuunsysteem. Kinderen die geboren worden met SCID (Severe Combined Immune Deficiency Syndrome) overlijden bij uitblijven van behandeling aan ernstige, terugkomende infecties, zoals longontstekingen. Ook mensen met AIDS (Acquired Immune Deficiency Syndrome) kunnen bezwijken aan infecties doordat een gedeelte van hun immuunsysteem niet meer goed werkt.

Hoewel de activiteit van het immuunsysteem ons beschermt tegen pathogenen, is het van groot belang dat de immuunreactie niet krachtiger is dan nodig om binnengedrongen pathogenen te elimineren. In een ongunstig geval kan het immuunsysteem door het herkennen van een pathogeen zo sterk geactiveerd raken dat de immuunreactie het lichaam schade toebrengt. Ontstekingsfactoren en signaalstoffen (cytokines) worden uitgescheiden om het lichaam te waarschuwen dat er een infectie is, maar een overmaat aan deze stoffen kan leiden tot orgaanfalen en uiteindelijk de dood. Aan de Spaanse griep in 1918-1919, die aan 20 tot 100 miljoen mensen wereldwijd het leven kostte, zijn vooral sterke, jonge mensen tussen de 20 en 40 jaar bezweken. Deze groep heeft normaal gesproken de beste weerstand tegen infecties. Tegenwoordig wordt gedacht dat deze mensen slachtoffer zijn geworden van een overreactie van hun eigen immuunsysteem. Behalve het reguleren van de sterkte van de immuunreactie is het ook van belang dat er onderscheid wordt gemaakt tussen pathogenen en gezonde lichaamscellen. Bij onder meer auto-immuunziekten gebeurt het toch dat het immuunsysteem de eigen lichaamscellen aanvalt. Het immuunsysteem kan bijvoorbeeld de eilandjes van Langerhans vernietigen; dit leidt tot type I diabetes omdat deze eilandjes nodig zijn voor de insulineproductie. Het immuunsysteem is dus krachtig genoeg om zowel pathogenen als ons eigen lichaamscellen te vernietigen. Een goede regulatie van de immuunreactie is daarom van levensbelang, en dit onderwerp staat centraal in dit proefschrift. Om beter uit te leggen wat in dit proefschrift onderzocht is, zal ik eerst de opbouw van het immuunsysteem toelichten.

Hoe werkt het immuunsysteem?

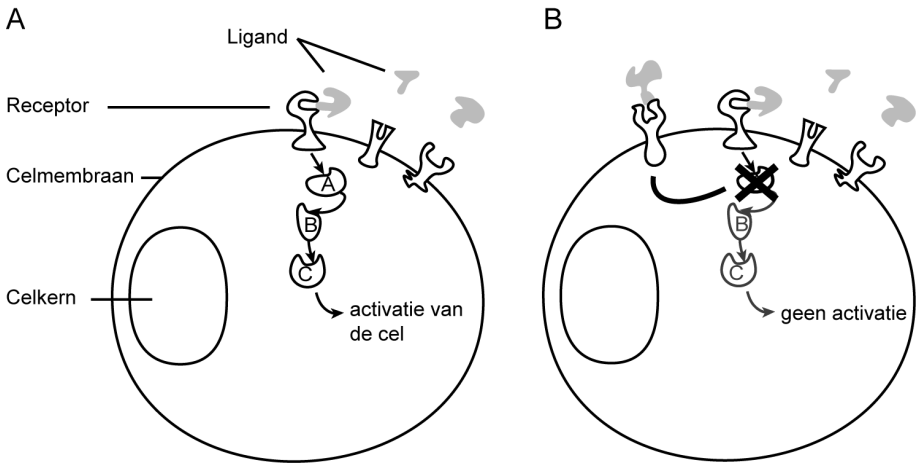
Het immuunsysteem bestaat uit een aantal eiwitten en verschillende typen cellen, waarvan de meeste in het bloed voorkomen (de zogenaamde witte bloedcellen). In het bloed vinden we daarnaast bloedplaatjes, van belang bij bloedstolling, en rode bloedcellen, die ervoor zorgen dat zuurstof via het bloed naar de weefsels wordt vervoerd. Alle witte bloedcellen, bloedplaatjes en rode bloedcellen ontwikkelen uit dezelfde voorloper- of moedercel, die we de hematopoietische stamcel noemen (Figuur 1).



Figuur 1. Tijdens de hematopoiese kan een bloedstamcel zich vermenigvuldigen en ontwikkelen tot verschillende soorten uitgerijpte bloedcellen. De uitrijping van bloedstamcellen vindt plaats in het beenmerg. De uitgerijpte bloedcellen zijn rode bloedcellen, bloedplaatjes of witte bloedcellen. De laatste categorie omvat granulocyten, monocyten, NK-cellen, T-cellen en B-cellen.

Hematopoietische stamcellen zitten in het beenmerg, en het grootste deel van het ontwikkelingsproces van stamcel naar uitgerijpte bloedcel vindt ook plaats in het beenmerg. Na het uitrijpen verspreiden de cellen zich in het bloed, in het lymfe systeem en in de weefsels.

Als er een infectie is in een bepaald weefsel of orgaan, zullen witte bloedcellen daarheen trekken. Bij witte bloedcellen onderscheiden we cellen van het adaptieve en het aangeboren immuunsysteem. T- en B-cellen behoren tot het adaptieve deel; deze cellen herkennen een specifiek stukje eiwit van een bepaalde pathogeen. Eenmaal ontwikkeld kunnen deze cellen soms wel levenslang bescherming bieden tegen die pathogeen. Een nadeel is dat ze wel een dag of 4-5 nodig hebben om zich te ontwikkelen en in actie te komen tijdens de infectie (dit is lang, want een bacteriepopulatie kan zich elke paar uur verdubbelen). NK-cellen, monocyten, macrofagen en granulocyten (granulocyten kunnen neutrofielen, eosinofielen of basofielen zijn) behoren tot het aangeboren deel van het immuunsysteem en zijn direct beschikbaar om een binnengedrongen pathogeen onschadelijk te maken. Deze cellen herkennen bepaalde structuren die pathogenen met elkaar gemeen hebben. Tijdens de eerste fase van een infectie komt dus vooral het aangeboren immuunsysteem in actie. Veel cellen van het aangeboren immuunsysteem, zoals neutrofielen, monocyten en macrofagen, kunnen binnengedrongen bacteriën opeten (fagocyteren) en doden deze vervolgens binnen in de cel door zuurstofradicalen te produceren, de zogenaamde oxidatieve burst. Deze cellen scheiden ook signaalstoffen (cytokines) uit om meer witte bloedcellen naar de plaats van infectie te krijgen en te waarschuwen dat er mogelijk gevaar is. Hoe merken witte bloedcellen dat er pathogenen in het lichaam zijn? Het celmembraan, een structuur die de cel als een soort vliesje omgeeft, bevat een groot aantal eiwitten dat als 'receptor' fungeert. Als immuunreceptoren binden aan bepaalde (bacteriële) structuren of eiwitten geven ze een signaal door aan de cel (Figuur 2A) dat er bijvoorbeeld een bacterie opgegeten (gefagocyteerd) moet worden, cytokines geproduceerd of dat het nodig is om vanuit de bloedbaan het weefsel in te trekken om een infectie op te ruimen. Er zijn verschillende soorten immuunreceptoren, die elk binden en geactiveerd raken door andere moleculen (Figuur 2A). Een molecuul dat bindt aan een receptor en deze activeert wordt ook wel "ligand" genoemd. Liganden kunnen door het lichaam geproduceerd worden (een cytokine is bijvoorbeeld een ligand voor een cytokine-receptor), maar ook door pathogenen (bacteriestructuren kunnen herkend worden door de pathogeenreceptoren op onze witte bloedcellen). Over het algemeen kan een receptor zijn functie pas uitoefenen nadat hij geactiveerd is door zijn ligand. Witte bloedcellen hebben receptoren nodig om met elkaar te communiceren, om infecties te detecteren en om informatie over de omgeving binnen te krijgen. Receptoren zijn in dat opzicht de ogen en de oren van de cel.



Figuur 2. Receptoren op het celmembraan van witte bloedcellen geven signalen door aan de cel. A, verschillende receptoren hebben elk een specifiek ligand, dat aan de receptor kan binden en deze daardoor activeert. Een geactiveerde receptor geeft, via moleculen binnen de cel (hier weergegeven als A, B en C), signalen door aan de cel die uiteindelijk leiden tot een specifieke effectorfunctie. B, wanneer een remmende receptor gebonden wordt door zijn ligand (hier de linkerreceptor), kan de remmende receptor signalen van activerende receptoren remmen. Dit verhindert of vermindert de effectorfunctie van de witte bloedcel.

Immuun inhibitoire receptoren

Immuunreceptoren zijn verantwoordelijk voor het activeren van witte bloedcellen als er een infectie is, maar niet alle soorten immuunreceptoren activeren de witte bloedcel. Er is ook een klasse receptoren die de witte bloedcel juist remt in zijn activatie. Dit is de groep van de immuun inhibitoire, ofwel remmende, receptoren, die centraal staan in het onderzoek beschreven in dit proefschrift. Net als activerende immuunreceptoren zitten remmende receptoren vaak op het celmembraan. Als ze een bepaalde structuur herkennen worden ze 'aangezet', en remmen dan activerende signalen die door andere activerende immuun-receptoren aan de cel worden doorgegeven (Figuur 2B). Door deze eigenschap spelen remmende receptoren een grote rol bij het in toom houden van de immuunreactie. Er is zowel een groot aantal activerende als een groot aantal remmende receptoren aanwezig op het celmembraan van elke witte bloedcel. De sterkte van alle remmende en activerende signalen samen bepalen of een immuunreactie plaats vindt, en zo ja, in welke mate.

Er zijn op dit moment ongeveer 60 verschillende remmende receptoren bekend, met elk hun specifieke expressiepatroon. Dat wil zeggen dat de ene remmende receptor bijvoorbeeld alleen op T-cellen voorkomt, een andere alleen op monocyten, en weer een andere op bijna alle witte bloedcellen. Op elke witte bloedcel komen meerdere remmende receptoren voor. Elke remmende receptor heeft een specifieke en unieke rol in het reguleren van de immuunreactie. Sommige receptoren zijn bijvoorbeeld van belang bij het voorkomen van immuunactivatie in afwezigheid van infectie. Andere receptoren zijn meer betrokken bij het beëindigen van de immuunreactie als de pathogeen verslagen is. Het missen van een remmende receptor kan leiden tot ontwikkeling van bijvoorbeeld auto-immuunziekten of chronische ontstekingen.

Dit proefschrift

Op dit moment is onze kennis van remmende receptoren nog beperkt. Van de 300 potentiële remmende receptoren in het humane genoom zijn er pas ongeveer 60 uitgebreider beschreven. Meer kennis over deze receptoren draagt bij aan ons inzicht in het functioneren van het immuunsysteem, en kan uiteindelijk leiden tot een betere behandeling van bijvoorbeeld auto-immuunziekten. Daarom wilden we in dit proefschrift de remmende receptoren SIRL-1 (Signal Inhibitory Receptor on Leukocytes-1) en LAIR-1 (Leukocyte-Associated Ig-like Receptor-1) bestuderen. Van de remmende receptor SIRL-1 was voorheen niets bekend. In dit proefschrift hebben wij onderzocht op welke witte bloedcellen SIRL-1 tot expressie komt, of SIRL-1 op deze cellen een remmend effect op celstimulatie kan hebben en wat de rol van SIRL-1 in het lichaam is. LAIR-1 is een receptor die al langer bestudeerd wordt en die op veel soorten witte bloedcellen voorkomt, zoals T-cellen, B-cellen, NK-cellen en monocyten. Een paar jaar geleden hebben we ontdekt dat collagenen LAIR-1 kunnen binden en activeren. LAIR-1 is tot nu toe de enige remmende receptor die aan collagenen bindt. Er zijn wel meerdere activerende receptoren beschreven die aan collagenen binden. Collagenen behoren tot de meest voorkomende eiwitten in het lichaam en zijn nodig om het weefsel structuur en sterkte te geven. Wij wilden in dit proefschrift onderzoeken of LAIR-1 misschien een functie heeft in het remmen van activerende collageen receptoren zoals glycoprotein VI (GPVI). Hiervoor bepalen we eerst of er cellen zijn waarop zowel LAIR-1 als GPVI voorkomt.

Verder is het een raadsel waarom er meerdere varianten van LAIR-1 zijn, die bovendien erg op elkaar lijken. De bekendste varianten zijn LAIR-1a en LAIR-1b. Er is nooit onderzocht

of deze varianten van elkaar verschillen in hun binding aan collageen en in hoe sterk ze kunnen remmen. Het is ook niet volledig bekend op welke celtypen de 1a variant zit en op welke cellen de 1b variant. Wij wilden daarom onderzoeken of deze varianten mogelijk een totaal andere rol in de immuunregulatie hebben. De hier beschreven doelstellingen van het proefschrift zijn samengevat in Hoofdstuk 1.

In het eerste deel van het proefschrift onderzoeken we de remmende receptor SIRL-1. In Hoofdstuk 2 tonen we aan dat SIRL-1 specifiek tot expressie komt op neutrofielen, eosinofielen en monocyten. Dit betekent dus dat SIRL-1 alleen zit op cellen die betrokken zijn bij de eerste verdediging tegen pathogenen en niet op cellen van het adaptieve immuunsysteem. Om te onderzoeken of SIRL-1 een remmende functie heeft maken we in eerste instantie gebruik van cellijnen. Dit zijn cellen die onsterfelijk gemaakt zijn en buiten het lichaam in leven worden gehouden. We laten in Hoofdstuk 2 zien dat als we SIRL-1 kunstmatig in een cellijn stoppen, dat SIRL-1 dan de immuunfunctie van deze cellijn kan remmen. Samengevat is Hoofdstuk 2 een belangrijke studie om te laten zien dat SIRL-1 inderdaad functioneert als een remmende receptor en dat hij specifiek door bepaalde cellen van het aangeboren immuunsysteem tot expressie wordt gebracht.

Het feit dat SIRL-1 alleen op cellen van het aangeboren immuunsysteem zit en niet op de andere witte bloedcellen zou er op kunnen wijzen dat SIRL-1 een functie remt die specifiek is voor het aangeboren immuunsysteem. Dit blijkt inderdaad zo te zijn, zoals onderzocht in Hoofdstuk 3. In Hoofdstuk 3 tonen we aan dat SIRL-1 de oxidatieve burst remt. De oxidatieve burst, gekenmerkt door de productie van zuurstof radicalen door cellen van het aangeboren immuunsysteem, zorgt ervoor dat opgegeten bacteriën gedood worden. De bedoeling van SIRL-1 is waarschijnlijk niet om schadelijke bacteriën te laten overleven, maar om schadelijke effecten door zuurstofradicalen aan het eigen weefsel te voorkomen. Om te zorgen dat in geval van infectie wel zuurstofradicalen geproduceerd worden, maar niet in afwezigheid van infectie, heeft het lichaam een handig trucje bedacht. In Hoofdstuk 3 beschrijven we dat de SIRL-1 expressie op witte bloedcellen direct een stuk lager wordt als witte bloedcellen pathogenen in de buurt waarnemen (bijvoorbeeld doordat bacteriële producten of cytokines binden aan activerende receptoren). Hierdoor remt SIRL-1 de immuunreactie niet als er een pathogeen in het lichaam is en kan de pathogeen dus efficiënt gedood worden. Bij afwezigheid van infectie voorkomt de (hoge) SIRL-1 expressie juist wel de productie van schadelijke zuurstofradicalen. SIRL-1 zorgt er dus voor dat er alleen zuurstofradicalen geproduceerd worden tijdens infectie en beschermt zo het lichaam tegen weefselschade in afwezigheid van infectie.

In Hoofdstuk 4 bespreken we in een review het huidige inzicht in de rol van remmende receptoren op cellen van het aangeboren immuunsysteem (om precies te zijn op neutrofielen, monocyten en macrofagen). Een belangrijke conclusie van Hoofdstuk 4 is dat de verschillende soorten remmende receptoren op deze cellen een totaal andere, soms zelfs tegengestelde functie hebben in de regulatie van deze cellen. Dit verrassend omdat remmende receptoren in structuur erg op elkaar lijken.

Het tweede deel van dit proefschrift richt zich op de remmende receptor LAIR-1. In Hoofdstuk 5 kijken we naar de expressie van LAIR-1 op megakaryocyten (zie Figuur 1), dit zijn de voorlopercellen van bloedplaatjes. Zoals gezegd wordt LAIR-1 aangezet door te binden aan collagenen. Sommige activerende receptoren worden ook aangezet door collagenen, zoals GPVI. In dit Hoofdstuk beschrijven we dat LAIR-1 en GPVI beide op ontwikkelende megakaryocyten voorkomen. We denken dat LAIR-1 de functie van GPVI kan remmen op deze cellen. De ontwikkeling van je bloedcellen vindt plaats in het beenmerg en deze omgeving is rijk aan collagenen. Als de megakaryocyt uitgerijpt is zal hij het collageenrijke beenmerg verlaten voordat bloedplaatjes worden afgescheiden. Mogelijk zorgt het specifieke expressiepatroon van activerende (GPVI) en remmende (LAIR-1) collageenreceptoren ervoor dat de megakaryocyt op het juiste rijpingsmoment het beenmerg verlaat.

In Hoofdstuk 6 gaan we in op de vraag waarom er verschillende 'splice varianten' van LAIR zijn. Deze splice varianten, LAIR-1a en LAIR-1b zijn vrijwel geheel identiek, alleen is LAIR-1b een pietsje kleiner dan LAIR-1a (17 aminozuren om precies te zijn). In eerste instantie bekijken we op welke witte bloedcellen LAIR-1a voorkomt en op welke witte bloedcellen LAIR-1b. Het blijkt dat T-cellen en B-cellen vooral LAIR-1b hebben, monocyten hebben beide vormen, terwijl neutrofielen alleen LAIR-1a hebben. We laten zien dat LAIR-1a en LAIR-1b niet verschillen in hun remmende capaciteit: beide varianten kunnen even goed celactivatie remmen na blootstelling aan collageen. Het ontbreken van dat kleine stukje eiwit zorgt er echter voor dat LAIR-1b veel minder goed aan collageen blijft plakken dan LAIR-1a. In het lichaam zou dit wel eens belangrijke consequenties kunnen hebben. Mogelijk kunnen witte bloedcellen met LAIR-1a veel beter door het weefsel bewegen dan witte bloedcellen met LAIR-1b. Een andere mogelijkheid is dat hematopoietische stamcellen door LAIR-1a expressie goed op hun plek blijven in de collageenrijke beenmergomgeving. Dit moet blijken uit verder onderzoek.

De resultaten van het proefschrift worden bediscussieerd in Hoofdstuk 7. We bespreken hier de verschillen tussen remmende receptoren, het belang van de nieuwe remmende receptor SIRL-1 en zijn waarschijnlijke biologische functie. Daarnaast bespreken we

de mogelijkheid dat LAIR-1 niet alleen als een remmende receptor werkt maar ook als een bindingsmolecuul (adhesiemolecuul), en wat de consequenties hiervan zouden zijn in het lichaam. Onze bevindingen maken duidelijk dat remmende receptoren complexere functies hebben dan vaak wordt aangenomen in de literatuur, en dragen daardoor bij aan het inzicht in de rol van remmende receptoren in de immuunregulatie.

Dankwoord

Dankwoord

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Over de auteur

Tessa Steevels werd op 3 augustus 1981 geboren in Amsterdam. Na het behalen van haar VWO diploma aan het Hervormd Lyceum Zuid in Amsterdam is zij in september 1999 begonnen met de studie biologie aan de Wageningen Universiteit. Afstudeervakken werden uitgevoerd aan de Wageningen Universiteit bij de afdeling Human and Animal Physiology onder begeleiding van Dr. B. van de Heijning en de afdeling Nutrition, Metabolism and Genomics onder begeleiding van Dr. F. Zandbergen en Dr. S. Kersten. Een onderzoeksstage werd gelopen op de universiteit van Guelph, Canada, bij de afdeling Human Health and Nutritional Sciences onder begeleiding van Dr. W.D. Woodward. In februari 2006 begon zij als promovendus bij de afdeling Immunologie van het Universitair Medisch Centrum Utrecht, onder begeleiding van Prof. Dr. L. Meyaard en Prof. Dr. P.J. Coffer. De resultaten van haar onderzoek staan beschreven in dit proefschrift.

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Abbreviations

BRET	bioluminescence resonance energy transfer
BSA	bovine serum albumin
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1
CFU	colony-forming units
CFU-MEG	megakaryocyte progenitors
CGD	chronic granulomatous disease
CMP	common myeloid progenitors
COPD	chronic obstructive pulmonary disease
CRP	collagen-related peptide
Csk	C-terminal Src kinase
DAMP	danger associated molecular pattern
FcR	Fc Receptor
FcRγ	Fc receptor gamma chain
FCS	fetal calf serum
fMLP	N-*formylated* methionyl-leucyl-phenylalanine
GM-CSF	granulocyte macrophage-colony stimulating factor
GMP	granulocyte/ macrophage progenitors
GPVI	Glycoprotein VI
GPCR	G-protein coupled receptor
HSC	hematopoietic stem cells
IFN	interferon
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IgV	variable-like immunoglobulin
IL	interleukin
ILT	Ig-like transcript
IP3	inositol-1,4,5-trisphosphate
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITAM	immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
KC	cytokine-induced neutrophil chemoattractant
KIRs	killer cell Ig-like receptors
LILRs	Leukocyte Ig-like receptors
LRC	Leukocyte Receptor Complex

mAb	monoclonal antibody
MEP	megakaryocyte/erythrocyte progenitors
MHC I	major histocompatibility complex I
MIP-1α	macrophage inflammatory protein
NADPH	nicotinamide adenine dinucleotide phosphate
NETs	neutrophil extracellular traps
NF-κB	nuclear factor kappa B
NK	natural killer
NRC	natural cytotoxicity receptor
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PECAM-1	platelet endothelial cell adhesion molecule-1
PI3K	phosphoinositide 3-kinase
PIP2	phosphatidylinositol-4,5-bisphosphate
PIR	paired Ig-like receptor
PKB	protein kinase B
PRR	pattern recognition receptor
RBC	red blood cells
ROS	Reactive Oxygen Species
SFK	Src family kinases
SHP	SH2 domain-containing phosphatase
SHIP	SH2 domain-containing inositol 5-phosphatase
Siglec	Sialic-acid-binding immunoglobulin-like lectin
SIRL-1	Signal Inhibitory Receptor on Leukocytes-1
SIRP-α	Signal Regulatory Protein alpha
SLE	systemic lupus erythematosus
SOCS3	suppressor of cytokine signaling 3
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	transforming growth factor-β
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
TPO	thrombopoietin
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Tyr-phe	tyrosine-to-phenylalanine