Signal Inhibitory Receptor on Leukocytes-1 Is a Novel Functional Inhibitory Immune Receptor Expressed on Human Phagocytes

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Signal Inhibitory Receptor on Leukocytes-1 Is a Novel Functional Inhibitory Immune Receptor Expressed on Human Phagocytes

Tessa A. M. Steevels,* Robert Jan Lebbink,* Geertje H. A. Westerlaken,* Paul J. Coffer,*+ and Linde Meyaard*

Myeloid cells play a crucial role in controlling infection. Activation of these cells needs to be tightly regulated, because their potent effector functions can damage host tissue. Inhibitory receptors expressed by immune cells play an important role in restricting immune cell activation. In this study, 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 ITIMs in its cytoplasmic tail. As a classical ITIM-bearing receptor, SIRL-1 is capable of inhibiting FcγRI-mediated signaling and can recruit the Src homology 2 domain-containing phosphatases SHP-1 and 2. To investigate the specific involvement of the individual ITIMs in this study, 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-1high monocytes produce less TNF-α than SIRL-1low 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. The Journal of Immunology, 2010, 184: 000–000.

Neutrophils and cells of the monocytic lineage are the most important effector cells of the innate immune response. Postinfection, 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, because 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.

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Abbreviations used in this paper: CP, cytoplasmic domain; Csk, C-terminal Src kinase; cys, cysteine; EC, extracellular domain; HEK, human embryonic kidney; IgSF, Ig superfamily; IP, immunoprecipitation; LAIR-1, leukocyte-associated Ig-like receptor; LP, leader peptide; LRC, leukocyte receptor complex; NCBI, National Center for Biotechnology Information; PV, perovskate; SH, Src homology; SHP, Src homology region 2 domain-containing phosphatase; SIRL-1, signal inhibitory receptor on leukocytes-1; SIRPs, signal regulatory protein α; TM, transmembrane domain; TNF, tumor necrosis factor; Tyr-phe, tyrosine-to-phenylalanine; WB, Western blotting; WCL, whole-cell lysate.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/09/10S16/00 upon ligand binding, these receptors relay inhibitory signals that increase the threshold for cellular activation. Their suppression of cell function is usually mediated via ITIMs in the intracellular tail with the consensus sequence V/L/SuYxxV/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 Src homology (SH)2-domain containing tyrosine phosphatases SH region 2 domain-containing phosphatase (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. Although some inhibitory receptors are expressed on multiple cell types, the expression pattern of others is more restricted. For example, leukocyte-associated Ig-like receptor (LAIR)-1 is expressed on most immune cells (8), whereas Ig-like transcript 3 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 downstream molecules and the affinity with which these are recruited. The family of immune inhibitory receptors is still expanding: only approximately 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 Ig superfamily (IgSF) members, is particularly rich in putative ITIM-containing molecules (11).

In this study, 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

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 (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 (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.

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 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’-TTGGTTCGGCAGGAAGGGACG-3’) and reverse (5’-AGAATGGATGATGTTCTTC3’) primers and the AmpliTaq Gold DNA Polymerase system (Applied Biosystems). Each amplification reaction underwent 40 cycles of denaturation at 96°C for 30 s, annealing for 30 s at 58°C, and elongation at 72°C for 1 min. SIRL-1 cDNA obtained from these samples was cloned into pGEM-T Easy vectors using the Gold DNA Polymerase system (Applied Biosystems). The cDNA mixtures were amplified by PCR using SIRL-1–specificforward (5’-TTTCAATTGCTACACTTTCAGTGCCGC-3’) and reverse (5’-CATTGATTCAGGATGTTCTTC3’) primers. To create a N-terminal flag-tagged SIRL-1, SIRL-1 cDNA lacking a leader sequence was ligated in a PMX vector containing a CDB-leader-flag construct using the SIRL-1–specific forward primer (5’-CATCGATGATGTTCTTC3’) and reverse (5’-CGATGATGTTCTTC3’), and reverse (5’-CATCGATGATGTTCTTC3’). Tyrosines at positions Y206 and Y231, both part of a (semi) ITIM, were mutated to phenylalanines by 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 (Promega, Madison, WI) and subsequently sequenced. cDNA sequences were compared with those published in the GerDB sequence database (identification number 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 European Molecular Biology Laboratory-European Bioinformatics Institute database (www.ebi.ac.uk/embl) using the accession number FN998145.

cDNA constructs

SIRL-1 cDNA was cloned into a retroviral PMX vector using SIRL-specific forward (5’-CGGCCATCACCATCCGCACTTGAGGAGTGGACG-3’) and reverse (5’-TTGGTTCGGCAGGAAGGGACG-3’) primers. To generate a N-terminal flag-tagged SIRL-1, SIRL-1 cDNA lacking a leader sequence was ligated in a PMX vector containing a CDB-leader-flag construct using the SIRL-1–specific forward primer (5’-CAATGGATGATGTTCTTC3’) and reverse (5’-CATCGATGATGTTCTTC3’). Tyrosines at positions Y206 and Y231, both part of a (semi) ITIM, were mutated to phenylalanines by 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–flag protein was created by fusing the extracellular domain of SIRL-1, consisting of aa 1–132, to the Fe region of human IgG1. SIRL-1–flag 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–tagged SP2/0 cells were cultured in DMEM; other cells were cultured in RPMI 1640 medium (Life Technologies, Paisley, U.K.) supplemented with 10% FCS (Integro, Dieren, The Netherlands) and antibiotics.

Generation of mAbs

mAbs specific for SIRL-1 were produced as described previously (17). Briefly, 50 μg purified SIRL-1–flag fusion protein in PBS was mixed with CFA (Difco, Lawrence, KS) and injected s.c. into BALB/c mice. A second injection was repeated after 2 wk using SIRL-1–flag fusion protein mixed withIFA. One week after the second injection, mice were boosted with an injection of 50 μg fusion protein in PBS. Mice were sacrificed 3 d 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 nontransfected 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 Europe, Diegem, Belgium). Part of the Ab was FITC conjugated (Molecular Probes, Eugene, OR) 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 coexpression 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 eBioscience (San Diego, CA). Anti-CD123 was from Miltenyi Biotec (Auburn, CA), and anti-CD16 was from Sanquin (Amsterdam, The Netherlands). FITC-conjugated mouse IgG1 isotype control mAb (BD Biosciences, San Jose, CA) 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 Abs were from BD Biosciences.

Immunoprecipitation and Western blotting

Isolated granulocytes were used to study SIRL-1 expression. RBL-2H3 cells transfected with SIRL-1 and SIRL-1–tyrosine-to-phenylalanine (tyr-phe) mutants and monoclonal antibodies 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 min at 37°C. After the incubation period samples were kept on ice. Cells were lysed in Triton xylis buffer (1% Triton X-100, 10 mM Tris, and 150 mM NaCl) supplemented with protease inhibitors (complete Mini EDTA–free protease inhibitor mixture tablets; Roche, Mannheim, Germany), 1 mM PMSF and 50 μM pervanadate. For immunoprecipitation anti-flag M2 Affinity Gel (Sigma-Aldrich, Munich, Germany) was used, or protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA), coated with 2 μg IgA anti–SIRL-1 or an isotype control MAb for 2 h. Gel or beads were washed with PBS and blocked with 50 μl 10% BSA. Samples were immunoprecipitated for 90 min. Immune complexes were washed five times with 0.1% Triton X-100, supplemented with 1 mM PMSF and 250 μM sodium orthovanadate and boiled in non-reducing sample buffer. Proteins were resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blots were incubated with Abs specific for phosphotyrosine, SIRL-1, flag M2, SHP-1, and SHP-2, followed by HRPLinked secondary Abs. Antiphosphotyrosine 4G10 was from Upstate Biotechnology (Lake Placid, NY), anti-flag M2 HRP was from Sigma-Aldrich, and polyclonal rabbit anti–SHP-2 and anti–SHP-2 Abs were purchased from Santa Cruz Biotechnology. HRP-linked secondary Abs were from DakoCytomation (Fort Collins, CO). ECL (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 transfected 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 Iα5 anti–SIRL-1 Ab 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 100 × (OD450 TNP with isotype cross-linking – OD450 spontaneous release). A value of p < 0.05 was considered statistically significant.

Intracellular cytokine staining

Whole blood was diluted 1:5 in RPMI 1640 medium supplemented with FCS and transfected to plates coated with Iα5 anti–SIRL-1 Ab or an isotype control. TNF-α production was induced by addition of 10 μg/ml Curdlan (Wako Chemicals) or 5–10 ng/ml LPS (Sigma-Aldrich). TNF-α secretion was prevented by addition of Golgiplug (BD Biosciences) to the culture. After 4 h of stimulation at 37°C, erythrocytes were lysed, and cells were stained for surface expression of SIRL-1 and CD14, after which cells were permabilized with cytfix/perm (BD Biosciences), stained with anti–TNF-α (BD Biosciences), and analyzed by flow cytometry.

Statistical analysis

Data were analyzed using SPSS 15.0 software (SPSS, Chicago, IL). Differences between SIRL-1high and SIRL-1low monocytes after stimulation were analyzed using Wilcoxon signed-rank test. A value of p < 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 LRC 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 157 (Fig. 1A). SIRL-1 is a type 1a membrane protein that contains one extracellular 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 (VYaaL) and a putative ITIM (HeYaaL), respectively. To investigate whether SIRL-1 had close family members or homologs, we searched the NCBI human blast program with a blastp algorithm for sequences homologous with SIRL-1 protein sequence. The protein with the highest amino acid homology to SIRL-1 was LAIR-1b, which is ∼25% identical and 55% similar to full-length SIRL-1 (Fig. 1A). The SIRL-1 gene spans ∼23.1 kb of genomic sequence and consists of nine exons (Fig. 1B). The gene is located centromeric to the human LRC region on chromosome 19q13.4 (Fig. 1C). The LRC region contains several gene families that are part of the IgSF including LAIRs, leukocyte Ig-like receptors and killer Ig-like receptors (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 Ab of the IgG1 type was purified and used for our studies (see Materials and Methods). SIRL-1–transfected but not nontransfected RBL-2H3 cells stained with 1A5 anti–SIRL-1-FITC mAb as analyzed by flow cytometry, demonstrating the specificity of the Ab (Fig. 2A). In addition, the SIRL-1–specific Ab detected a ∼45-kDa and a fainter ∼37-kDa protein by Western blot analysis in SIRL-1 transfected but not in the nontransfected RBL-2H3 cells (Fig. 2B). 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 (Fig. 3A). We found that SIRL-1 was highly expressed by both CD11b+CD16hi neutrophils and CD11b+CD16lo eosinophils (Fig. 3A, 3B). Likewise, most CD14+ monocytes highly expressed SIRL-1, although a subset of monocytes was low/intermediate for SIRL-1 expression (Fig. 3A, 3B). Expression of SIRL-1 on CD3+CD14+CD19−CD56+CD11c+HLA-DR+ myeloid dendritic cells differed per donor: some donors showed intermediate expression of SIRL-1, whereas other donors did not express SIRL-1 on the myeloid dendritic cells (Fig. 3A, 3B). In contrast, CD3+CD14+CD19−CD56−CD11c−HLA-DR−CD123+ plasmacytoid dendritic cells did not express SIRL-1 (data not shown). Lymphocytes, including CD3+CD56− NK cells, CD3−CD19+ B cells, and CD3+ T cells, also did not express SIRL-1 (Fig. 3A, 3B).
SIRL-1, A NOVEL INHIBITORY RECEPTOR ON PHAGOCYTES

The capacity of inhibitory immune receptors to inhibit cellular activation depends on effector molecules, generally recruited to the ITIMs. Because most phosphorylated ITIMs can recruit the phosphatases SHP-1, SHP-2, or SHIP, we tested whether these molecules were indeed recruited to SIRL-1. Nontransfected 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 (Fig. 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 FF), 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-1 or SIRL-1 tyr-phe mutants, which were expressed at comparable levels on the cell surface (Fig. 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 (Fig. 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 (Fig. 4C). 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.

Because 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 (Fig. 4D). Immunoprecipitations using tyr-phe mutants of SIRL-1 once more suggest that two intact ITIMs are required for the interaction, because 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 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, whereas an isotype control had no effect (Fig. 5A). We next used SIRL-1 tyr-phe mutants to assess the capacity of

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**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 min 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 Abs. 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). Nontransfected 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 PV, lysed, and immunoprecipitated as described in A. WB was performed using anti–SHP-1 (top panel and bottom panel loading control), 4G10 antiphosphotyrosine (second panel), and 1A5 anti–SIRL-1 Abs (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 FcεRIIB. After PV treatment, cells were lysed and immunoprecipitated with anti–flag M2 Affinity Gel. WB 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.

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**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 and an isotype control (1–0.12 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. Error bars represent SEM. 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). Nontransfected 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 (1–0.12 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 – OD405 spontaneous release) × 100. Error bars represent SEM.
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 with the isotype control, demonstrating that both ITIMs have inhibitory potential (Fig. 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.

**SIRL-1**

monocytes produce less TNF-α than SIRL-1

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 (Fig 6A). Notably, SIRL-1high monocytes consistently express less TNF-α than SIRL-1low monocytes (p = 0.018 for Curdlan-stimulated samples and p = 0.028 for LPS-stimulated samples) (Fig 6B). Cross-linking of SIRL-1 by plate-bound 1A5 anti-SIRL mAb did not decrease TNF-α expression in total CD14+ monocytes (Fig 6C) or in SIRL-1high or SIRL-1low 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, because similar results were obtained when we stimulated isolated PBMCs and analyzed intracellular TNF-α production in monocytes, nor did plate-bound 1A5 anti-SIRL mAb decrease TNF-α expression in either SIRL-1high or SIRL-1low 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 (Fig. 6D, top panel). Notably, in three 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 (Fig. 6D, bottom panel). In conclusion, SIRL-1 recruits SHP-1 but not SHP-2 in monocytes, and SIRL-1high-monocytes produce less TNF-α than SIRL-1low monocytes.

**Discussion**

In this paper, 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.

On the basis of the 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 by Meynard (8)]. The broad expression pattern of LAIR-1 is quite dissimilar to the expression profile of SIRL-1, which is restricted to myeloid cells (Fig. 3). Furthermore,
with no more than 25% 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/LxSxYxxV/L, 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 SH2 domain-containing phosphatases, Sweeney et al. (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 SH2 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 SIRPα and SHP-2, suggesting that the second tyrosine participates in SHP-1 and SHP-2 binding (Fig. 4). An alternative explanation for the abrogated phosphatase recruitment in the SIRL-1 FY 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 FY 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, because the abrogation of SHP-1 recruitment by disrupting one 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 (Fig. 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 Ab. 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-2 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 (Fig. 4). Because 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 (Fig. 6D). Additionally, our data suggest that besides SHP-1 and SHP-2, SIRL-1 may recruit other molecules to establish its inhibitory effect, because 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 α (SIRPα) (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 SIRPs and CD200R have crucial functions in immune regulation and 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). SIRPα, in contrast, has a well-established role in the inhibition of host cell phagocytosis by macrophages (33). Furthermore, SIRPs cross-linking has shown to inhibit LPS-induced TNF-α production in macrophages (34), whereas knockdown of SIRPα in mouse macrophages results in increased production of TNF-α, IL-6, NO, and IFN-β in response to LPS. Moreover, transfer of SIRPα knockdown macrophages into wild-type mice results in an increased susceptibility to lethal LPS shock (35).

In this study, we demonstrate that SIRL-1 high monocytes express less TNF-α than SIRL-1 low monocytes after LPS or Curdlan stimulation (Fig. 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, leading to suppression of cell activation. This notion is supported by the finding that additional Ab-mediated cross-linking of SIRL-1 did not further affect TNF-α expression and by the finding that SIRP-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 toward unraveling this function would be the identification of SIRL-1 biological ligand.

Disclosures
T.A.M.S., R.J.L., and L.M. are named as inventors on a patent application on SIRL-1 function. All other authors have no conflicting financial interests.

References


