Leukocyte-associated Ig-like receptor-1 is a novel inhibitory receptor for surfactant protein D

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ABSTRACT

The collagenous C-type lectin, SP-D, is a multitrimeric glycoprotein present at mucosal surfaces and is involved in host defense against infections in mammals. SP-D has immunomodulatory properties, but the underlying mechanisms are incompletely understood. SP-D contains collagen domains. LAIR-1 is an inhibitory immune receptor at the cell surface of various immune-competent cells that binds collagen. We hypothesized that the immunomodulatory functions of SP-D can be mediated via interactions between its collagen domain and LAIR-1. Binding assays show that SP-D interacts via its collagenous domain with LAIR-1 and the related LAIR-2. This does not affect the mannan-binding capacities of SP-D, which induces cross-linking of LAIR-1 in a cellular reporter assay. Functional assays show that SP-D inhibits the production of FcεR-mediated reactive oxygen via LAIR-1. Our studies indicate that SP-D is a functional ligand of the immune inhibitory receptor LAIR-1. Thus, we have identified a novel pathway for the immunomodulatory functions of SP-D mediated via binding of its collagenous domains to LAIR-1. This may provide a mechanism for the unexplained immunomodulatory function of the collagenous domains of SP-D. J. Leukoc. Biol. 96: 105–111; 2014.

Introduction

The lung contains a large alveolar surface area that is constantly exposed to pathogens, such as viruses and bacteria. SP-D belongs to the family of the “collectins” and functions as a first-line (innate) defense component that contributes to protection against pathogens in various mucosal tissues, such as the lung and gastrointestinal tract [1]. SP-D is composed of four domains: a short cysteine-rich N-terminal region, a 177-aa-long collagenous domain, and an α-helical neck domain that links the collagen to a C-type lectin domain or CRD (see Fig. 1A) [2]. One SP-D structural subunit is composed of three polypeptide chains, and these trimers assemble further into cruciform-like dodecamers [2]. SP-D functions as an opsonin by binding to various pathogens, such as influenza A virus, but it also binds to the LPS on the cell wall of Gram-negative bacteria (reviewed in ref. [3]). Besides functioning as an opsonin, SP-D has immunomodulatory properties. In human systems, SP-D inhibits T cell proliferation, as well as IL-2 production in vitro [4, 5]. SP-D also functions as a chemoattractant and can modulate the surface expression of TLR4 (reviewed in refs. [3, 6]). In a human epithelial airway model, SP-D-diminished suppollen particles induced IL-8 secretion [7]. SP-D−/− mice show a clear defect in immune regulation, as the lungs show a state of constitutive immune activation. Immune cell lesions in the lungs of SP-D−/− show abnormalities, such as the constitutive release of cytokines and an altered morphology. Despite the exaggerated inflammatory response, SP-D−/− mice have impaired host defense (reviewed in ref. [8]). Attempts to correct the phenotype by administrating or overexpressing truncated forms of SP-D showed that the collagenous domain is essentially involved in immune homeostasis [9, 10]. There are several candidate receptors that may exert the immunomodulatory functions of SP-D. Most candidate receptors have been shown to bind SP-D via its CRD and require the presence of calcium ions. Gardai et al. [11] showed binding of SP-D to the inhibitory SIRPα, resulting in a reduced capacity of alveolar macrophages to ingest apoptotic...
cells. Furthermore, SP-D binds to the SIRPα homologue SIRPβ, although the functionality of this binding remains unclear [12]. SP-D binds CD14 and rough LPS via the CRD, whereby the binding of SP-D to CD14 inhibits the binding of rough and smooth LPS [13]. SP-D has been shown to bind to calreticulin/CD91 on macrophages via its collagenous domain, resulting in increased chemotaxis [14]. SP-D function can be modulated through post-translational modification by NO. The altered structure then initiates a proinflammatory response via calreticulin/CD91 and p38 activation [14].

Despite the increasing number of SP-D-binding receptors, the mechanism via which SP-D exerts its immunoinhibitory role is unclear. LAIR-1 is an inhibitory immune receptor that is expressed on most immune cells for which collagens are high-affinity ligands [15–17]. LAIR is capable of binding multiple collagens, and different binding sites on collagens II and III have been mapped [18]. Upon collagen-mediated ligation, LAIR-1 inhibits various cellular functions, including maturation, proliferation, and degranulation of cells [19]. In this study, we set out to investigate whether the collagen-containing innate immune protein SP-D can exert its immunomodulatory functions via the inhibitory receptor LAIR-1.

MATERIALS AND METHODS

Cells
PLB-985 cells, a human myeloid leukemia cell line derived from HL-60, which were a kind gift from Dr. Timo van den Berg (Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam), and 2B4 NFAT-GFP T cell reporters were cultured as described [16, 20, 21]. For granulocytic differentiation, cells were exposed to 0.65% rough and smooth LPS [13]. SP-D has been shown to bind to the SIRPα homologue although the functionality of this binding remains un

Recombinant proteins and antibodies
Anti-LAIR-1 (Clone 8A8; IgG1) [23] and anti-SIRL [24] antibodies, rLAIR-1 proteins [25], rhSP-D, and a truncated NGRD trimERIC fragment of hSP-D [26, 27] were produced and characterized as described previously. F(ab')2 fragments were made by using the Mouse IgG1 F(ab')2, Preparation Kit (Thermo Fisher Scientific). Collagen I was purchased from Sigma-Aldrich (St. Louis, MO, USA), BSA (fraction V) from Roche Diagnostics (Basel, Switzerland), and peroxidase-conjugated goat anti-IgG (Fc fragment-specific) from Jackson ImmunoResearch (Suffolk, UK). Serum-derived IgA was purchased from MP Biomedicals (Santa Ana, CA, USA). Collagen peptides were a part of collagen toolkits obtained from Richard Aldrich and were a kind gift from Dr. Timo van den Berg (Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam), and 2B4 NFAT-GFP T cell reporters were cultured as described [16, 20, 21]. For granulocytic differentiation, cells were exposed to 0.65% rough and smooth LPS [13]. SP-D has been shown to bind to the SIRPα homologue although the functionality of this binding remains unclear. LAIR-1 is an inhibitory immune receptor that is expressed on most immune cells for which collagens are high-affinity ligands [15–17]. LAIR is capable of binding multiple collagens, and different binding sites on collagens II and III have been mapped [18]. Upon collagen-mediated ligation, LAIR-1 inhibits various cellular functions, including maturation, proliferation, and degranulation of cells [19]. In this study, we set out to investigate whether the collagen-containing innate immune protein SP-D can exert its immunomodulatory functions via the inhibitory receptor LAIR-1.

Biacore
Surface plasmon resonance (Biacore Life Sciences, GE Healthcare, Buckinghamshire, UK)-binding studies were performed with the Biacore T100 system (Biacore, GE Healthcare). Approximately 1100 RU of SP-D in 10 mM MES buffer, pH 6.1, were immobilized on a series S CMS sensor chip by the amine coupling kit, according to the manufacturer’s instructions. After coupling, the chip was pulsed for 30 s with ethyl(dimethylaminopropyl)carbodiimide/N-hydroxysuccinimide subsequently, followed by a 30-s pulse with ethanolamine. Analysis was performed in buffer [125 mM NaCl, 2.5 mM CaCl2, 0.005% Tween 20, and 25 mM HEPES, pH 7.4] at 25°C and at a flow rate of 20 µl/min. Injections with increasing concentrations of recombinant proteins (0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 20, and 24 µM for LAIR-2-HIS and 0, 0.051, 0.063, 0.125, 0.25, 0.5, 1, 2, 3, 4, 6, and 8 µM for LAIR-2-Fc) were allowed to bind for 5 min for LAIR-2-HIS and 10 min for LAIR-2-Fc, after which, regeneration by flowing buffer for 10 min for the LAIR-2-HIS or 10 mM formic acid for LAIR-2-Fc occurred. Baseline stability was checked after every experiment. Proteins were injected until binding equilibrium was reached. Binding data were analyzed with Biacore T100 evaluation software (version 2.01). Affinity constants were determined by steady-state analysis. The affinity is calculated using GraphPad Prism (version 5.03). The formula used for a two-site-specific binding is: $Y = \frac{[\text{Bmax}_1 \times X]}{K_{X_1}} + \frac{[\text{Bmax}_2 \times X]}{K_{X_2}}$, where X represents the ligand concentration in µM.

Mannan-immobilized SP-D-binding assay
Costar 9018 high-binding, 96-well plates (Corning, Corning, NY, USA) were coated overnight at 4°C with 10 µg/ml mannan (M-7504; Sigma-Aldrich), dissolved in 100 mM sodium bicarbonate buffer (pH 9.6). The experiment was carried out at RT with 100 µl/well unless indicated otherwise. After removal of the coating buffer, the wells were washed three times with washing buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 5 mM CaCl2) and blocked with 1% BSA in washing buffer for 1 h, followed by the washing procedure.

rhSP-D (1 µg/ml), in washing buffer, was mixed with LAIR-2-Fc or SIRL-Fc (both 0.5 µg/ml) or buffer only, and after incubation in 1 h in solution, the mixtures were applied to the mannan-coated plates. After 1 h incubation, plates were washed. SP-D detection involves incubation for 1 h with rabbit anti-hSP-D polyclonal serum (1:1000) [26], washing, and 1-h incubation with goat anti-rabbit HRP (Nordic Immunological Laboratories, Eindhoven, the Netherlands). HRP detection involves incubation with rabbit-anti-hsp-D HRP signal set to 100% [26]. After a final wash, 100 µl stabilized TMB chromogen (Life Technologies Europe BV, Bleiswijk, the Netherlands). After color development, the reaction was stopped by adding 50 µl 2 M H2SO4. Absorbance was measured at 450 nm. Background values were obtained for all conditions in the absence of mannan coating or in the presence of 5 mM EDTA during SP-D incubation. Data are expressed as relative intensities compared with incubation with SP-D only (goat anti-rabbit HRP signal set to 100%) or with incubation with SP-D, followed by LAIR-2 incubation (rabbit anti-mouse Fab, HRP signal set to 100%).

LAIR-1 reporter assay
2B4 NFAT-GFP TCRs stably expressing LAIR-1-CD3ζ chimeric receptors were used to detect functional LAIR-1 ligand binding as described before [16].

Flow cytometry
Cells were stained with PE-labeled α-hLAIR-1 or a PE-labeled isotype control (BD PharMingen, San Diego, CA, USA) for 30 min in FACS buffer (PBS supplemented with 10 mg/ml BSA and 0.1% w/v sodium azide) at 4°C. Cells were washed three times with ice-cold FACS buffer and analyzed on an LSRII FACs using FCSData software (BD Biosciences, San Jose, CA, USA).

Oxidative burst assay
ROS production was measured using Amplex Red, as described before [28], with minor adjustments. Statistical analysis was performed using SPSS software.
Western blot analysis
Matured PLB-985 cells were lysed in lysis buffer [50 mM Tris, 0.5 mM EDTA, 0.015 mM NaCl, 0.2 mM NaVO₃, 1% Triton (v/v), and 0.25% Na-deoxycholate], and samples were subjected to SDS-PAGE. Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA) and detected using anti-hLAIR-1 or anti-tubulin and HRP-conjugated secondary antibodies. Amersham ECL Prime Western blotting detection reagent (GE Healthcare) was used to visualize bound antibodies.

RESULTS AND DISCUSSION
SP-D binds LAIR proteins with high-affinity
We applied several techniques to establish if SP-D binds to LAIR proteins and to deduce which domain of SP-D would be involved in this interaction (Fig. 1). rLAIR-1, as well as LAIR-2, bound directly to immobilized, full-length SP-D (Fig. 1C). The presence of 10 mM EDTA ruled out Ca²⁺-dependent interactions of the CRDs of SP-D (Fig. 1C). The amount of LAIR-1 needed to reach the same ODs was 16× higher than that for LAIR-2, indicating a lower affinity of SP-D for LAIR-1 than for LAIR-2, in line with our previous study showing a higher affinity of LAIR-2 for collagens [25]. Binding of LAIR proteins to SP-D was slightly lower than binding to collagen I. To characterize further the binding mechanism of LAIR to SP-D, we also tested a SP-D-mutant that lacks the collagenous domain (rhNCRD; Fig. 1B) for LAIR binding. rhNCRD is a NCRD trimer and binds sugars (data not shown). We observed no LAIR binding to rhNCRD (Fig. 1C). From these data, we can conclude that the binding sites for the LAIR-1 and LAIR-2 proteins are located in the collagenous domain of SP-D.

Figure 1. LAIR-1 and LAIR-2 proteins bind SP-D.
(A) Schematic representation of a SP-D dodecamer molecule. CRD domains are in blue, neck region in red, collagenous domains in black, and N-terminal region in green. (B) Schematic representation of the trimeric rhSP-D-NCRD mutant, which lacks the collagenous domain and N-terminal region. (C) Binding of LAIR-1-Fc (10 μg/ml) and LAIR-2-Fc (0.63 μg/ml) to plate-coated SP-D, rhNCRD, Collagen I, or BSA. Detection with peroxidase-conjugated anti-hFc antibody. Results are of a representative experiment (n=3). (D) Binding of LAIR-2-HIS and (E) LAIR-2-Fc to immobilized SP-D was measured by surface plasmon resonance analysis. In the left panels, individual symbols representing resonance at equilibrium along with the corresponding concentration of proteins are shown. Values are the mean ± SEM. Triplicate-binding curves of LAIR-2-HIS and LAIR-2-Fc are shown in the right panels. (F) Binding of LAIR-bound SP-D to mannan-coated plates. SP-D was preincubated with LAIR-2-Fc or a control inhibitory receptor coupled to a Fc (SIRL) or not. The SP-D/LAIR-2 mixture or the SP-D-control mixture was added to mannan-coated wells. SP-D binding to mannan was detected with rabbit anti-hSP-D serum, followed by goat anti-rabbit HRP incubation and a standard TMB staining solution. Detection of LAIR-2 or control-Fc protein to SP-D was achieved with an HRP-conjugated anti-hFc antibody. Values are the mean ± SD; n = 3.
SP-D functionally engages LAIR-1

We used cells expressing chimeric transmembrane proteins, consisting of the extracellular part of LAIR-1, fused to the intracellular part of the CD3ξ and a NFAT-GFP reporter to investigate if binding of cell-expressed LAIR-1 to the collagen domain of SP-D could also functionally ligate the receptor (Fig. 2A). We used this assay previously to demonstrate functional binding of collagens and collagen-derived peptides to LAIR-1 [18]. As a control, we used cells only containing the NFAT-GFP reporter construct (WT cells) or cells expressing a chimera of SIRL-1 and the reporter construct [24]. Cells were incubated overnight with solid-phase collagen I, SP-D, or BSA (as a control), and GFP expression was assessed by flow cytometry (Fig. 2A). Collagen I induced GFP expression in up to 70% of the LAIR-reporter cells, and SP-D was

![Figure 2](https://www.jleukbio.org/images/figure2.png)

**Figure 2.** SP-D functionally ligates LAIR-1. (A) NFAT-GFP reporter cells, stably transfected with a LAIR-1-CD3ξ, a control protein-CD3ξ chimera, or WT cells, were incubated in 96-well MaxiSorp plates, coated with SP-D, collagen I, or BSA in various concentrations. Overlays of flow cytometry pictures of one representative experiment out of three are shown. GFP expression (MFI) is shown on the y-axis; the amount of coated protein/ml used is indicated adjacent to each row of panels. (B) Calculated percentages of GFP-positive cells. Values shown are the means ± so of three experiments.

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<th>Table 1: Calculated $K_d$ Values of SP-D-LAIR Binding</th>
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Calculated affinities ($K_d$ in µM) from the binding at equilibrium of the surface plasmon resonance experiments. Values are the mean ± sem.
almost as effective, inducing up to 50% GFP-expressing cells (Fig. 2B). Based on these findings, we conclude that SP-D can act as a functional ligand for LAIR-1.

**SP-D inhibits ROS production through LAIR-1**

ROS production by neutrophils and monocytes is an important part of the defense mechanism against pathogens by the innate immune system, although it can also cause severe tissue damage when released into the extracellular milieu [29]. The effect of LAIR-1 ligation on ROS production was not studied previously. To measure the extracellular ROS production, we have performed Amplex Red assays. We activated PLB-985 cells, which were matured with DMF toward a granulocytic lineage via the FcR, to induce an oxidative burst. First, we tested whether ligation of LAIR-1 via collagen regulates this oxidative burst. Collagens II and III have multiple binding sites for LAIR-1. We previously identified trimeric collagen peptides that do (III-38) or do not (II-20) induce inhibitory signaling via LAIR-1 [18]. Simultaneous ligation of FcR and LAIR-1 via collagen peptide III-38 reduced the oxidative burst by 50%, whereas collagen peptide II-20 did not significantly affect the oxidative burst (Fig. 3A and B). Blockade of the LAIR-1-collagen interaction with specific F(ab')2 fragments significantly reversed the inhibition of the oxidative burst, demonstrating that the inhibition is LAIR-1-mediated (Fig. 3A and B). F(ab')2 fragments alone, in combination with IgA (data not shown) or in combination with IgA and collagen peptide II-20, did not significantly affect ROS production (Fig. 3A and B). Thus, LAIR-1 ligation through collagen inhibits the FcR-induced oxidative burst. Next, we tested SP-D for its ability to inhibit ROS production in this setup. As a control, we used the rhNCRD peptide that does not bind LAIR-1 (Fig. 1B). SP-D significantly inhibited the FcR-induced oxidative burst in a concentration-dependent manner, up to 60%, whereas rhNCRD did not (Fig. 3C and D). LAIR-1-specific F(ab')2 fragments prevented SP-D-mediated inhibition of ROS production, whereas addition of F(ab')2 fragments to rhNCRD did not (Fig. 3D), indicating that SP-D inhibits the ROS production via LAIR-1.

To confirm further that the SP-D-mediated inhibition of the oxidative burst is LAIR-1-mediated, we constructed a LAIR-1 knockdown cell line by using shRNA specific for LAIR-1 [22], which resulted in a decreased LAIR-1 surface expression (Fig. 4A). The decrease in LAIR-1 expression in the knockdown cell line was verified on protein level by Western blot analysis (Fig. 4B). We compared the knockdown cell line with the WT cell line in the same experimental setup, as described above. Although SP-D, like collagen peptide III-38, is still able to inhibit the FcR-induced oxidative burst, the inhibition in the LAIR-1 knockdown cell line was significantly lower than in the WT cell line (Fig. 4C and D). This confirmed that the inhibitory action of SP-D is mediated, at least in part, by LAIR-1 (Fig. 4D). Oxidative burst levels were corrected for spontaneous ROS production. Cells were preincubated with 10 μg/ml (final concentration) anti-LAIR F(ab')2 fragments or not. (A) Cells were stimulated with 20 μg/ml IgA in the presence of 5 μg/ml collagen peptides or not. A representative curve of the cumulative ROS production is shown. (B) AUC, Area under the curve; n = 3. (C) Cells were stimulated with 20 μg/ml IgA in the presence of 5 μg/ml or 2.5 μg/ml SP-D or rhNCRD or not. A representative curve of the cumulative ROS production is shown. (D) Area under the curve; n = 3; mean ± sd. Significance was tested by an unpaired two-tailed Student’s t-test; **P ≤ 0.01; ***P ≤ 0.001.
were not completely restored to the condition without SP-D added, which is in agreement with low-level LAIR expression, still observed in the knockdown cell line in Fig. 4A and B.

SP-D has several immunomodulatory functions. However, the mechanisms via which SP-D exerts these functions are incompletely understood. In this study, we assessed the possibility that these functions are partially mediated by the inhibitory immune receptor LAIR-1. We show collagen domain-specific, Ca\(^{2+}\)-inde-

Figure 4. LAIR-1 knockdown significantly reduces the inhibitory effect of SP-D. (A) LAIR-1 expression by PLB-985 WT (dark gray), empty vector (light gray), and LAIR-1 knockdown (black) cells. Dotted lines are unstained cells, dashed lines are isotype-stained cells, and solid lines are LAIR-1-stained cells, with WT LAIR-1-stained cells depicted with filled, dark gray. (B) Western blot analysis of two individually matured WT cell lines, a matured empty vector control, and two individually matured LAIR-1 knockdown cell lines. Tubulin was used as a loading control. (C) Cells were stimulated with 20 μg/ml IgA in the presence of 5 μg/ml collagen peptides or not. Area under the curve is shown; n = 4. (D) Cells were stimulated with 20 μg/ml IgA in the presence of 5 μg/ml or 2.5 μg/ml SP-D or rhNCRD or not. Area under the curve is shown; n = 4; mean ± sd. Significance was tested by an unpaired two-tailed Student’s t-test; **P ≤ 0.01; ***P ≤ 0.001.
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