Signal Inhibitory Receptor on Leukocytes-1 is highly expressed on lung monocytes, but absent on mononuclear phagocytes in skin and colon

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ABSTRACT

Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) is expressed on human blood monocytes and granulocytes and inhibits myeloid effector functions. On monocytes, but not granulocytes, SIRL-1 expression is low or absent in individuals with the single nucleotide polymorphism (SNP) rs612529C. The expression of SIRL-1 in tissue and the influence of rs612529 hereon is currently unknown. Here, we used flow cytometry to determine SIRL-1 expression on immune cells in human blood and three barrier tissues; skin, colon and lung. SIRL-1 was expressed by virtually all neutrophils and eosinophils in these tissues. In contrast, SIRL-1 was not expressed by monocyte-derived cells in skin and colon, whereas it was highly expressed by lung classical monocytes. Lung monocytes from individuals with a rs612529C allele had decreased SIRL-1 expression, consistent with the genotype association in blood. Within the different monocyte subsets in blood and lung, SIRL-1 expression was highest in classical monocytes and lowest in nonclassical monocytes. SIRL-1 was not expressed by dendritic cells in blood and barrier tissues. Together, these results indicate that SIRL-1 is differentially expressed on phagocyte subsets in blood and barrier tissues, and that its expression on monocytes is genotype- and tissue-specific. Immune regulation of monocytes by SIRL-1 may be of particular importance in the lung.

1. Introduction

Phagocytes, a heterogeneous cell population including mononuclear phagocytes and polymorphonuclear granulocytes, play a crucial role in the host defense at barrier tissues. Phagocytes internalize and process invading pathogens, and thereby contribute directly to pathogen clearance as well as to activation of the adaptive immune response via antigen presentation [1,2]. However, the defense mechanisms of phagocytes can also be harmful to the host. For example, excessive production of Reactive Oxygen Species (ROS) by phagocytes causes tissue injury and has been implicated in the pathogenesis of several inflammatory diseases, such as pulmonary fibrosis, atherosclerosis and atopic dermatitis (AD) [3,4]. Therefore, the activity of phagocytes needs to be tightly regulated.

Phagocytes are regulated, among others, by inhibitory receptors, also known as immune checkpoints. These receptors inhibit several phagocyte effector functions, including ROS production, cytokine production and phagocytosis [5]. Most inhibitory receptors relay inhibitory signals via immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in the intracellular tail [6]. The expression pattern of immune inhibitory receptors varies: receptors can be widely expressed or restricted to specific cell types, tissues or activation states. For example, the inhibitory collagen receptor LAIR-1 is constitutively expressed on most lymphocytes [7], but on neutrophils it is only expressed upon activation and in inflamed tissue [8]. The expression pattern of a receptor is pivotal for its biology, as it dictates where and when the receptor can exert its function.

We have previously identified Signal Inhibitory Receptor on

Leukocytes-1 (SIRL-1) as an ITIM-bearing inhibitory receptor that is expressed on circulating human monocytes and granulocytes, but absent on lymphoid cells [9–11]. Crosslinking of SIRL-1 with an agonistic antibody inhibits Fc Receptor (FcR) induced ROS production [12,9,13] and neutrophil extracellular trap (NET) formation [12,14]. The gene encoding SIRL-1, *VSTM1*, contains a single nucleotide polymorphism (SNP) rs612529T/C in its promoter region that dictates expression (Frequency of C-allele: in Caucasians 15.8%, in Chinese 36.7%, in Japanese 25.6% [9]). In individuals with rs612529C/C genotype, SIRL-1 expression on monocytes is almost completely absent, whereas SIRL-1 expression on granulocytes does not associate with rs612529 [9]. rs612529C also associates with an increased risk at AD [9].

Even though SIRL-1 expression has been well characterized on peripheral blood granulocytes and monocytes, SIRL-1 expression in barrier tissues and the influence of rs612529 hereon is currently unknown. In this study, we used flow cytometry, cell sorting and qRT-PCR to examine SIRL-1 expression on human phagocytes in peripheral blood and in the barrier tissues skin, colon, and lung. We show that SIRL-1 is expressed on granulocytes in these tissues, but that SIRL-1 expression on mononuclear phagocytes differs between tissues and cell types.

2. Materials and methods

2.1. Tissue source

Peripheral blood was obtained from healthy volunteers. Healthy skin from Caucasian donors was obtained from discarded material after cosmetic abdominal or breast reduction surgery. AD patient skin was obtained from 4 mm punch biopsies from lesional skin, non-lesional skin, and non-lesional skin 24 h after the initiation of an atopy patch test (APT). APT was performed by application of house dust mite extract to the patients' back, as previously described [15]. Colon biopsies were obtained from Ulcerative Colitis or Crohn's disease patients who were in remission for at least four years. Lung tissue was obtained from lung cancer patients during surgical tumor removal; for this study a piece of non-malignant tissue was used. All samples were collected in accordance with the Institutional Review Board of the University Medical Center (UMC) Utrecht and Amsterdam UMC.

2.2. PBMC isolation

PBMCs were isolated by density gradient centrifugation of sodium-heparinized peripheral blood over Ficoll-Paque (Amersham Biosciences). PBMCs were washed and suspended in PBS containing 2% (v/v) heat-inactivated fetal calf serum (FCS; Sigma-Aldrich) before further use.

2.3. Digestion of skin tissue

For flow cytometric analysis, healthy whole skin was digested with a Whole Skin Dissociation Kit (Miltenyi) according to manufacturer's instructions, using overnight incubation without Enzyme P.

For qRT-PCR analysis of sorted healthy skin cell populations, dermis and epidermis were digested separately. Skin was shaved with a dermatome to get the upper 0.3 mm, washed with PBS, and incubated 5 min with 100 mg/mL gentamycin (Duchefa) to kill bacteria. Skin was incubated overnight at 4 °C with 0.2% (w/v) dispase II (Roche) to separate dermis and epidermis. Dermis was digested for 1.5 h in IMDM (Lonza) supplemented with 0.5% (w/v) collagenase D (Sigma-Aldrich), 30 U/mL DNAse-I (Roche) and 1% (v/v) FCS. Epidermis was digested for 0.5 h in PBS supplemented with 0.25% (w/v) Trypsin (Invitrogen) and 0.2% EDTA (Invitrogen). Both digestions were performed at 37 °C under gentle agitation, and quenched by adding FCS. Single cell suspensions were obtained by thorough vortexing and filtering of the cells over a single cell filter chamber and a 70 μm cell strainer. Next, epidermal cells were treated with 12 U/mL DNAse-I and again filtered over

a 70 μm cell strainer, followed by Ficoll gradient centrifugation and harvesting of cells from the interphase. Finally, epidermal and dermal cells were washed and suspended in FACS buffer (PBS supplemented with 2% (v/v) FSC, 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich), 1% (v/v) human serum (HS; Sigma-Aldrich) and 0.1% (v/v) sodium azide) before further use.

2.4. Digestion of colon tissue

Biopsies from the colon were collected in ice-cold Hank's Balanced Salt Solution (HBSS; Gibco) supplemented with 2% (v/v) FCS, 1% (v/v) penicillin–streptomycin (PS; Gibco) and 1% (v/v) Amphotericin B (Gibco). Next, 1 mM DTT (Sigma-Aldrich) was added to the solution and the biopsy was incubated 10 min at 4 °C under gentle agitation. After thorough vortexing and one wash with supplemented HBSS, the biopsy was digested with 1 mg/mL collagenase IV (Sigma-Aldrich) in supplemented RPMI 1640 (10% FCS, 1% PS, 1% Amphotericin B), for 1 h at 37 °C under gentle agitation. The biopsy was suspended by pressing it through a 18.5-19G needle and a 70 μm cell strainer. Cells were then washed and suspended in supplemented RPMI 1640 before further use.

2.5. Digestion of lung tissue

Lung biopsies were rinsed with PBS, cut thoroughly, and digested in IMDM supplemented with 125 ug/mL liberase (Roche), 100 ug/mL DNAse-I and 5% (v/v) HS, for 45 min at 37 °C or overnight at 4 °C. The digestion was quenched with FCS, and a single cell suspension was obtained by vortexing and filtering of the cells over a 70 μm cell strainer. Erythrocytes were lysed by incubation with erythrocyte lysis buffer (containing 0.155 mM NH_4Cl (Sigma-Aldrich), 1 mM $KHCO_3$ (Merck KGaA), and 80 μM EDTA) for 10 min on ice. After one wash with PBS and one wash with MACS buffer (PBS supplemented with 0.5% BSA, 2% FCS, 1% HS and 2 mM EDTA), cells were frozen in HBSS with 50% (v/v) FCS and 10% (v/v) DMSO (Sigma-Aldrich) and stored in liquid N_2. Before flow cytometry, cells were thawed by drop wise addition of HBSS with 10% (v/v) FCS and washing with MACS buffer.

2.6. Flow cytometry and cell sorting

Cells were incubated with 5% (v/v) mouse serum (Equitech-Bio) in PBS to block aspecific binding by Fc receptors. For cells from digested tissue, this incubation was combined with viability dye APC-eFluor 780 (eBioscience). Subsequently, cells were stained with fluorochrome-conjugated antibodies diluted in Brilliant Stain buffer (BD Biosciences), see Supplementary Table 1 for an overview of the antibodies used. Flow cytometry was performed on an LSRFortessa (BD Biosciences); cell sorting was performed on a FACSAria III (BD Biosciences). Data were analyzed using FlowJo software (Treestar, Ashland, OR). Cell subsets were gated based on their typical surface marker expression [16,1,17], see Supplementary Figs. 1–4 for the gating strategy used).

2.7. qRT-PCR

Sorted cells from the skin were collected in FACS buffer, centrifuged and taken up in 350 μl RTL buffer (Qiagen) supplemented with 0.14M 2-mercaptoethanol (Sigma-Aldrich). RNA was isolated with the RNeasy mini KiT (Qiagen) and cDNA was synthesized with the iScript reverse transcription kit (Bio-Rad). Subsequently, qRT-PCR was performed with SYBR Select Master Mix (Life Technologies) on 10 ng cDNA input per reaction on a QuantStudio 12 K Flex (Applied Biosystems). Primers were used that specifically amplify VSTM1-v1, the primary splice form that encodes membrane-bound SIRL-1.

SIRL-1 mRNA expression was represented relative to GAPDH expression, using the formula 'relative expression = $2^{(Ct(\text{average of reference genes})-Ct(\text{target}))}$.

Target	Forward primer	Reverse primer
VSTM1-v1 (SIRL-1)	gacaacagcctcccatgagt	tgaagatggcgacaaagatg
GAPDH	agaaggctggggctcattt	gaggcattgctgatgatcttg

2.8. Genotyping

DNA was isolated with the DNA Extract All Reagents Kit (Thermo Fisher Scientific). The genotype of rs612529 was determined using a TaqMan SNP Genotyping Assay and TaqMan GTXpress Master mix (Thermo Fisher Scientific). All procedures were performed according to manufacturer's instructions.

2.9. Immunohistochemistry

Whole skin cryosections (7 μm) were fixed by immersion in 10% (v/v) Neutral Buffered Formalin (Sigma-Aldrich). Next, slides were incubated for 10 min in 0.03% (v/v) hydrogen peroxide (Sigma-Aldrich) and blocked for 20 min with 2.5% (v/v) horse serum (ImmPress kit, Vector Laboratories). Slides were stained by a 1 h incubation with anti-SIRL-1 (clone 1A5, produced in house [10], anti-elastase (clone NP57, DAKO) or an isotype control (SouthernBiotech), followed by a 30 min incubation with horse anti-mouse IgG-HRP (ImmPress kit, Vector Laboratories) and a 10 min incubation with AEC + Substrate-Chromogen (DAKO). Slides were counterstained with Mayer's Hematoxylin solution (Sigma-Aldrich) and mounted with Entellan (VWR). Between each step, slides were washed extensively with PBS 0.05% (v/v) Tween-20. All procedures were performed at room temperature. Images were acquired on a Zeiss Axiovision.

2.10. Data analysis

Statistical analysis was performed using GraphPad Prism software (version 8.3.0). A mixed-effects model with Sidak's correction for multiple testing was used to compare SIRL-1 expression between peripheral blood mononuclear phagocytes subsets of donors with rs612529T/T genotype. The same test was used to compare SIRL-1 expression between donors with rs612529T/T and rs612529T/C genotypes in each lung phagocyte subset. P values of < 0.05 were considered statistically significant. Statistics in the text indicate the percentage of SIRL-1$^+$ cells as compared to the isotype control and are reported as mean $\pm$ standard deviation.

3. Results

3.1. Monocyte subsets, but not pDCs and cDCs, express SIRL-1 in peripheral blood

We have previously shown that SIRL-1 is highly and homogeneously expressed on peripheral blood neutrophils and eosinophils [12,8,9,13,10,18]. Here, we used flow cytometry to measure SIRL-1 expression on mononuclear phagocyte subsets in peripheral blood (For gating strategy see Supplementary Fig. 1). SIRL-1 was expressed by all monocyte subsets, with highest expression on classical (c) monocytes (CD14$^+$CD16$^-$), intermediate expression on intermediate (i) monocytes (CD14$^+$CD16$^+$), and lowest expression on nonclassical (nc) monocytes (CD14^{dim}CD16$^+$) and slanMonocytes, a subset of ncMonocytes (Fig. 1A, B). SIRL-1 was absent on plasmacytoid dendritic cells (pDC), CD141$^+$ DCs ('cDC1') and CD1c$^+$ DCs ('cDC2') (Fig. 1A, C). However, a subset of CD141$^-$CD1c$^-$ DCs expressed SIRL-1 in all donors (Fig. 1A, D). As CD141$^-$CD1c$^-$ DCs are not well described, we backgated these SIRL-1$^+$ DCs (data not shown) and confirmed their correct gating as CD3$^-$CD19$^-$CD56$^-$HLA-DR$^+$CD14$^-$CD16$^-$CD11c$^+$CD141$^-$CD1c$^-$ cells (Supplementary Fig. 1).

In line with our previous findings [9], we found that SIRL-1 expression was lower on monocytes from a donor with rs612529T/C

genotype and absent on monocytes from a donor with rs612529C/C genotype (Fig. 1B). The percentage of SIRL-1$^+$ CD141$^-$CD1c$^-$ DCs was not clearly affected by rs612529 (Fig. 1D). Taken together, these data show that SIRL-1 is expressed by all monocyte subsets in peripheral blood, but not by cDCs and pDCs.

3.2. SIRL-1 is expressed by very few mononuclear phagocytes in the skin

We previously reported that rs612529C associates with an increased risk at AD [9], which led to the hypothesis that low SIRL-1 expression on monocytes or monocyte-derived cells in the skin predisposes for AD. Therefore, we used flow cytometry to examine SIRL-1 expression in healthy human skin (For gating strategy see Supplementary Fig. 2). SIRL-1 was not detected on autofluorescent dermal macrophages (MΦ), CD1c$^+$ DCs or CD141$^+$ DCs in the skin (Fig. 2A). Low SIRL-1 expression was found on a very small percentage of CD14$^+$ monocyte-derived macrophages (Mo-MΦ) in all four donors with rs612529T/T genotype (2.13 $\pm$ 0.54% SIRL-1$^+$ cells). Of note, in CD14$^+$ Mo-MΦ from an individual with rs612529T/C genotype, an even lower percentage of cells expressed SIRL-1 (0.42% SIRL-1$^+$ cells).

Tissue digestion may have altered surface receptor expression, so to validate these results we used qRT-PCR to determine SIRL-1 mRNA levels in sorted mononuclear phagocytes from the skin. Compared to PBMC, SIRL-1 mRNA levels were approximately 100-fold lower in CD14$^+$ Mo-MΦ from donors with rs612529T/T genotype, and levels were 500–1000-fold lower or undetectable in CD14$^+$ Mo-MΦ from donors with rs612529T/C genotype and in MΦ, dermal DCs and Langerhans cells (LCs) (Fig. 2B). Together, this confirmed that SIRL-1 expression on mononuclear phagocytes in healthy skin is very low or absent, and is lower in CD14$^+$ Mo-MΦ from donors with rs612529T/C genotype.

Lastly, we used immunohistochemistry (IHC) to examine SIRL-1 expression in AD skin. No SIRL-1 staining was detectable in healthy skin, AD non-lesional skin, and AD lesional skin, compared to isotype control (Fig. 2C). In contrast, several SIRL-1$^+$ cells and elastase$^+$ granulocytes were found in skin from an AD patient who received an atopy patch test [15] (Fig. 2C), suggesting that SIRL-1 may be expressed by skin infiltrating granulocytes.

In summary, we show that SIRL-1 is expressed on a very low number of cells in healthy skin and AD skin.

3.3. Eosinophils in the colon express SIRL-1

To investigate SIRL-1 expression in another human barrier tissue, we analyzed cells from a colon biopsy by flow cytometry (For gating strategy see Supplementary Fig. 3). Because of varying degrees of background signal in tissue cells, the MFI of anti-SIRL-1 stained cells was normalized by subtracting the MFI of isotype stained cells. As in skin, SIRL-1 was completely absent on CD11c$^+$ DCs and lowly expressed on a small percentage of mature CD206$^+$ MΦ and immature CD206$^-$ Mo-MΦ (Fig. 3A). More SIRL-1$^+$ cells were found in immature CD206$^-$ Mo-MΦ from two donors with rs612529T/T genotype (8.29% and 11.90% SIRL-1$^+$ cells) compared to two donors with rs612529T/C genotype (1.28% and 0% SIRL-1$^+$ cells)

Eosinophils reside in the colon under homeostatic conditions [19]. In contrast to mononuclear phagocytes in the colon, SIRL-1 was highly expressed on these colonic eosinophils. This expression was unaffected by rs612529C (Fig. 3B), which corresponds with our earlier findings that SIRL-1 expression on blood granulocytes does not associate with rs612529C [9]. Together, these results show that in the colon, SIRL-1 is mostly absent on mononuclear phagocytes, but highly expressed on eosinophils.

3.4. Lung monocytes express SIRL-1

Lastly, we determined SIRL-1 expression on immune cells in human
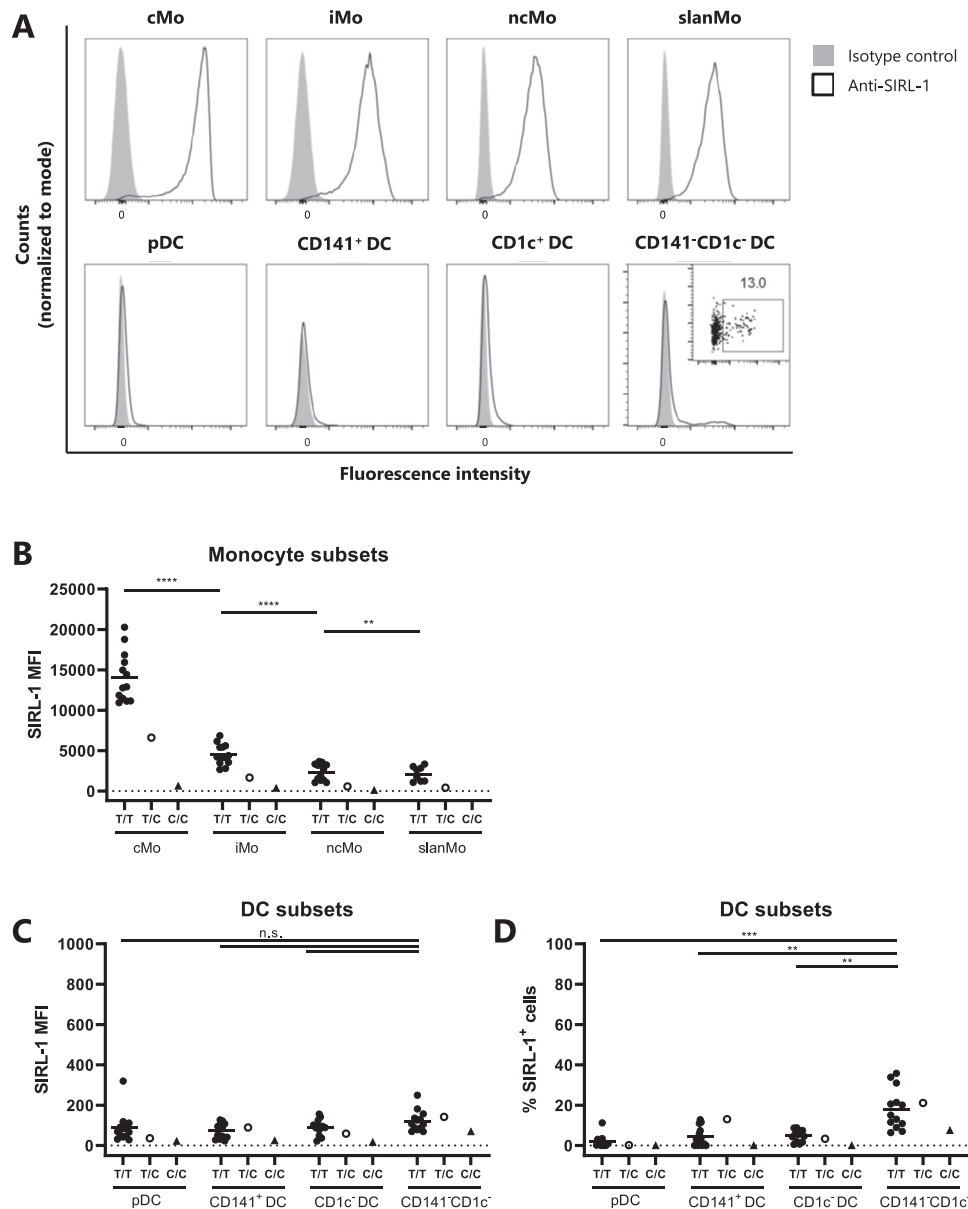
Fig. 1. SIRL-1 is expressed on monocyte subsets, but not on pDCs and cDCs in peripheral blood. PBMCs were isolated from peripheral blood of healthy human donors and analyzed by flow cytometry (See Supplementary Fig. 1 for the gating strategy). (A) Panels show representative histograms of the fluorescence intensity of mononuclear phagocyte subsets stained with a SIRL-1 antibody (open histogram) or an isotype control (closed histogram). The dot plot insert of CD141⁻CD1c⁻ DCs shows the fluorescence intensity of anti-SIRL-1 stained cells on the x-axis, and SSC-A on the y-axis. The gate shows the percentage of SIRL-1$^+$ cells compared to the isotype control. The donor's genotype is rs612529T/T (n = 8–13). (B-C) Median fluorescence intensity (MFI) of monocyte subsets (B) and DC subsets (C) stained with a SIRL-1 antibody. (D) Percentage of SIRL-1$^+$ cells in DC subsets. (B-D) Each symbol represents one donor, the bar indicates the mean. Genotypes of rs612529 are indicated with filled circles (T/T, n = 13), open circles (T/C, n = 1) or filled triangles (C/C, n = 1). SlanMo's were analyzed in less donors: T/T, n = 8; T/C, n = 1; C/C, n = 0. A mixed-effects model with Sidak's correction for multiple testing was used to compare SIRL-1 expression between cell subsets of donors with T/T genotype, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$. cMo, classical monocytes; iMo, intermediate monocytes; ncMo, nonclassical monocytes; slanMo, slan monocytes; pDC, plasmacytoid DC.

lung by flow cytometry (for gating strategy see Supplementary Fig. 4). In contrast to mononuclear phagocytes in skin and colon, SIRL-1 was highly expressed on lung cMonocytes (CD206⁻CD14$^+$CD16⁻) (Fig. 4A, B). Similar to blood, SIRL-1 expression was intermediate on iMonocytes and lowest on ncMonocytes (Fig. 4B). SIRL-1 expression was significantly lower on lung cMonocytes and ncMonocytes from donors with rs612529T/C genotype compared to donors with rs612529T/T genotype (Fig. 4B). SIRL-1 was also expressed by a subset of interstitial macrophages (12.54 ± 10.77% SIRL-1$^+$ cells), but absent on alveolar macrophages, pDCs and CD11c$^+$ DCs (Fig. 4A, B).

SIRL-1 was undetectable on mast cells in the lung, but highly expressed by neutrophils and, although at more variable levels, by eosinophils (Fig. 4A, C). In line with findings from blood [9] and colon, there was no effect of rs612529C on SIRL-1 expression by neutrophils and eosinophils (Fig. 4C). In summary, these results show that SIRL-1 is expressed by subsets of mononuclear phagocytes and granulocytes in the lung.

3.5. SIRL-1 is differentially expressed between non-diseased tissues from the GTEx consortium

The lung tissue in which we examined SIRL-1 expression was derived from lung cancer patients, and malignant tissue might affect SIRL-1 expression in healthy adjacent tissue. Therefore we used the Genotype-Tissue Expression (GTEx) RNA sequencing database of gene expression in non-diseased tissues to analyze *VSTM1* expression [20,21]. *VSTM1* mRNA expression was highest in blood, pituitary gland, spleen, lung and testis, whereas it was mostly absent in colon and skin (Supplementary Fig. 5). This corresponds to the SIRL-1 protein expression we found on mononuclear phagocytes in blood, skin, colon and lung, suggesting that the SIRL-1 expression we report is representative for SIRL-1 expression in healthy tissues.

4. Discussion

Phagocytes are pivotal players in host defense, but excessive use of their effector mechanisms can cause immunopathology. Therefore, phagocyte activity is controlled by several mechanisms, including the
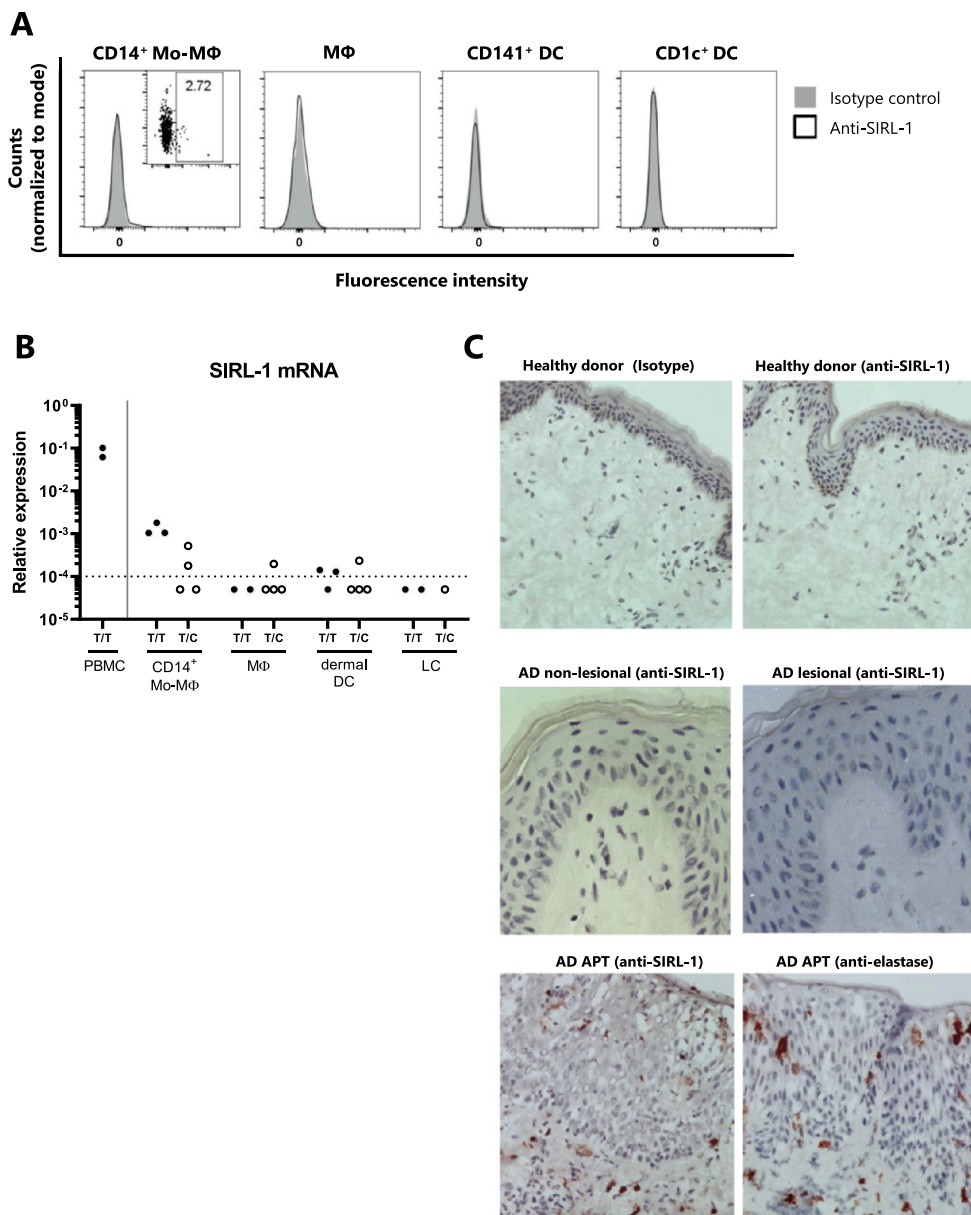
Fig. 2. SIRL-1 expression on mononuclear phagocytes in skin is very low or absent. (A) Healthy human skin biopsies were digested and analyzed by flow cytometry (See Supplementary Fig. 2 for the gating strategy). Panels show representative histograms of the fluorescence intensity of mononuclear phagocyte subsets stained with a SIRL-1 antibody (open histogram) or an isotype control (closed histogram). The dot plot insert of CD14$^+$ Mo-MΦ shows the fluorescence intensity of anti-SIRL-1 stained cells on the x-axis, and SSC-A on the y-axis. The gate shows the percentage of SIRL-1$^+$ cells compared to the isotype control. The donor's genotype is rs612529T/T (n = 4). (B) Human skin biopsies were separated into dermis and epidermis, followed by digestion and FACS sorting. mRNA was isolated from the sorted cell subsets and SIRL-1 mRNA expression was analyzed by qRT-PCR. Expression was normalized to GAPDH expression, and expression below the detection limit was set at a value below 10^{-4}. Each symbol represents one donor. Genotypes of rs612529 are indicated with filled circles (T/T) or open circles (T/C). PBMC, n = 2; CD14$^+$ Mo-MΦ, n = 7; MΦ, n = 6, dermal DCs, n = 7; LCs, n = 3. The vertical line indicates that PBMCs were collected from different donors and used for reference. (C) Human skin cryosections were stained for presence of SIRL-1 or elastase. The sections derived from donors with rs612529T/T genotype and include healthy donor skin (upper panels), atopic dermatitis (AD) non-lesional skin (middle left panel), AD lesional skin (middle right panel) or skin from an AD patient that underwent an atopy patch test (APT, lower panels). Shown are representative images of ≥ 3 donors. Magnification: upper and lower panel 200x, middle panel 400x. PBMC, peripheral blood mononuclear cell; MΦ, macrophage, Mo-MΦ, monocyte-derived macrophage; LC, Langerhans cell.

expression of inhibitory receptors [5]. SIRL-1 is a functional inhibitory receptor that is expressed on circulating monocytes and granulocytes [9,10]. Here, SIRL-1 expression was examined in peripheral blood and barrier tissues, including skin, colon and lung. SIRL-1 was expressed by virtually all neutrophils and eosinophils in these barrier tissues. In contrast, SIRL-1 was expressed by few mononuclear phagocytes in skin and colon, yet highly expressed by lung classical monocytes.

SIRL-1 was not expressed on pDCs and cDC subsets in peripheral blood and barrier tissues (Fig. 1A, Fig. 2A, Fig. 3A, Fig. 4A). This is in apparent conflict with a previous report, in which we showed SIRL-1 expression by 30% of peripheral blood DCs [10]. These outcomes can be explained by different gating strategies that were used to identify DCs. At the time, DCs were defined as HLA-DR$^+$CD14$^-$, without excluding CD16$^+$ cells. However, in recent protein and RNA single-cell analyses, CD16$^+$ DCs were found to be more similar to ncMonocytes [22,23]. Therefore, cells that were previously gated as SIRL-1 positive CD16$^+$ DCs were in this study defined as ncMonocytes. Remarkably, a small subset of CD141$^-$CD1c$^-$ DCs did express SIRL-1 in all donors examined (Fig. 1D). These 'double negative' DCs are poorly described in literature, but a single cell RNA sequencing study described a CD141$^-$ CD1c$^-$ DC subset in peripheral blood, which were named DC4s [24]. However, DC4s expressed CD16 and were therefore in later studies also described as ncMonocytes [22,23]. In contrast, the CD141$^-$CD1c$^-$ DCs we describe here are CD16 negative. Further research is warranted to clarify whether SIRL-1 expressing CD141$^-$CD1c$^-$ DCs are a distinct subset.

We previously reported that rs612529C associates with low SIRL-1 expression on monocytes [9]. Here, we extended this into tissue. SIRL-1 expression was significantly lower in lung cMonocytes and ncMonocytes from individuals carrying a rs612529C allele (Fig. 4B). A similar trend was observed in CD14$^+$ Mo-MΦ in the skin and immature CD206$^-$ Mo-MΦ in the colon, even though SIRL-1 levels were overall very low in these cells. On eosinophils and neutrophils in colon and lung, SIRL-1 expression was not affected by the genotype of rs612529 (Fig. 3B, Fig. 4C), which is in correspondence with findings from peripheral blood granulocytes [9].

Notably, rs612529C also associates with an increased risk at AD [9]. This association suggests a relationship between rs612529C, abrogated SIRL-1 expression on monocytic cells, and AD pathogenesis. We hypothesized that SIRL-1 is expressed by skin-resident mononuclear
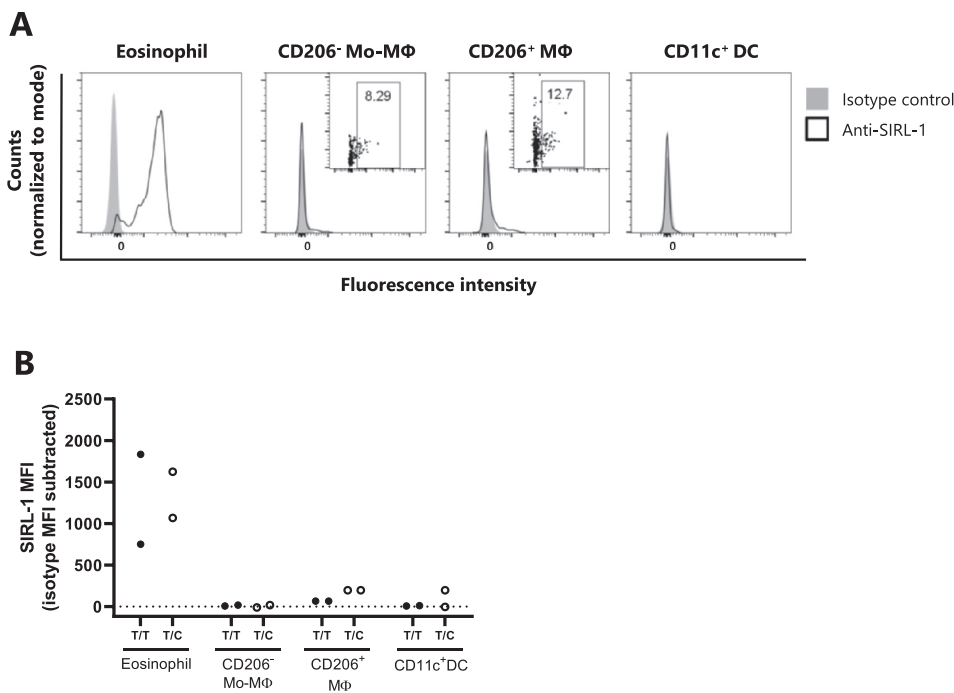
Fig. 3. SIRL-1 is expressed by eosinophils in the colon. Human colon biopsies were digested and analyzed by flow cytometry (See Supplementary Fig. 3 for the gating strategy). (A) Panels show representative histograms of the fluorescence intensity of immune cells stained with a SIRL-1 antibody (open histogram) or an isotype control (closed histogram). The dot plot inserts of CD206$^-$ Mo-MΦ and CD206$^+$ MΦ show the fluorescence intensity of anti-SIRL-1 stained cells on the x-axis, and SSC-A on the y-axis. The gate shows the percentage of SIRL-1$^+$ cells compared to the isotype control. The donor's genotype is rs612529T/T (n = 2). (B) Median fluorescence intensity (MFI) of immune cells stained with a SIRL-1 antibody, minus the MFI of cells stained with an isotype control. Each symbol represents one donor. Genotypes of rs612529 are indicated with filled circles (T/T, n = 2) or open circles (T/C, n = 2). Mo-MΦ, monocyte-derived macrophage.

phagocytes, and that abrogation of this expression in individuals with rs612529C genotype leads to lack of inhibitory signaling via SIRL-1. This, in turn, could lead to hyperactivation of skin-resident mononuclear phagocytes and thereby contribute to skin inflammation in AD. However, mononuclear phagocytes in the skin expressed no or very low levels of SIRL-1, in donors from all rs612529 genotypes, which argues against a major role of SIRL-1 in the regulation of these cells in the skin (Fig. 2A). Therefore it remains to be elucidated if and how the absence of SIRL-1 expression on monocytes in individuals with rs612529C genotype contributes to the development of AD. Possibly, monocytes that are recruited to inflamed skin express SIRL-1, and abrogation of SIRL-1 expression in this time frame could predispose for AD. Alternatively, mononuclear phagocytes with abrogated SIRL-1 expression could play a role in AD pathogenesis in a different location than in the skin, for example in the lymph nodes. Of note, CD14$^+$ Mo-MΦ in the skin expressed SIRL-1 mRNA, albeit at very low levels compared to PBMCs, indicating that these cells have the potential to express SIRL-1 protein (Fig. 2B). In the flow cytometry analysis, this protein expression may have been partially lost by the tissue digestion.

This study is limited by the availability of healthy human tissue. Firstly, the sample size of tissue biopsies is relatively small. Secondly, colon and lung tissue were derived from patients with (former) disease: colon biopsies were taken from Ulcerative Colitis or Crohn's disease patients that were in remission for at least four years, and lung biopsies were obtained from non-malignant tissue of lung cancer patients. Because these disease states might alter SIRL-1 expression, we compared our data to SIRL-1 mRNA expression found in the GTEx dataset, which contains RNA sequencing data of human non-diseased tissues [20]. In the GTEx dataset, SIRL-1 expression was relatively high in blood and lung, and absent in colon and skin (Supplementary Fig. 5), resembling the SIRL-1 protein expression we found on mononuclear phagocytes in these tissues. This supports that the SIRL-1 expression we measured in this study is representative for SIRL-1 expression in healthy tissues. SIRL-1 expression was absent in the GTEx analysis of the colon, whereas we detected SIRL-1 expression on colon eosinophils. Granulocytes contain low total transcript levels [25], and therefore the SIRL-1 transcripts of eosinophils may have been underrepresented in the RNA sequencing analysis.

Mononuclear phagocyte subsets have distinct ontogenies. For

example, macrophages can originate either from circulating monocytes that extravasate into the tissue, or from yolk-sac or fetal liver-derived cells that already populate the tissue during embryonic development [1]. We show that most macrophages in tissues do not express SIRL-1. However, a small percentage of macrophages expressed low levels of SIRL-1, including CD14$^+$ Mo-MΦ in the skin, immature CD206$^-$ Mo-MΦ and mature CD206$^+$ MΦ in the colon, and interstitial MΦ in the lung. These macrophage subsets are all considered to be of monocytic origin [16,26,1,27,28]. In contrast, autofluorescent dermal MΦ and alveolar MΦ, both considered to be of fetal origin [26,1], did not express SIRL-1. Even though we cannot confirm the development of SIRL-1 expression over time, these results suggest that monocytes downregulate SIRL-1 expression when they enter the tissue and differentiate into macrophages.

We can only speculate as to why monocytes in lung express SIRL-1, whereas mononuclear phagocytes in colon and skin do not. Mechanistically, SIRL-1 expression may be directly maintained by a factor specifically present in the lung. Alternatively, SIRL-1 may be predominantly expressed by undifferentiated monocytes, and a factor in the lung keeps monocytes in this state. Indeed, even though it has long been thought that all monocytes which enter the tissue differentiate into MΦ or DCs, healthy human lungs contain undifferentiated monocytes in steady state [29]. Of note, lung interstitial macrophages, which are at least partially monocyte-derived (reviewed by [28], express very low levels of SIRL-1 (Fig. 4A, B). This argues that it is not the lung environment per se which maintains SIRL-1 expression on mononuclear phagocytes, but rather the differentiation status of the monocytes.

Functionally, SIRL-1 may inhibit ROS production in lung monocytes and granulocytes, as it has been described for these cells from healthy donor peripheral blood [9,13] and sputum from infants with severe Respiratory Syncytial Virus bronchiolitis [12]. In addition, SIRL-1 may have yet unexplored effects on the regulatory function of monocytes, for example cytokine production. Based on intravital imaging in mice, lung monocytes were suggested to be mainly involved in scavenging of particles to clean up airways and blood [30]. Lungs are continuously exposed to inhaled particles, many of which are non-pathogenic. Inhibiting the immune response to such particles, for example by inhibitory receptor signaling, is therefore likely to be beneficial to the host.
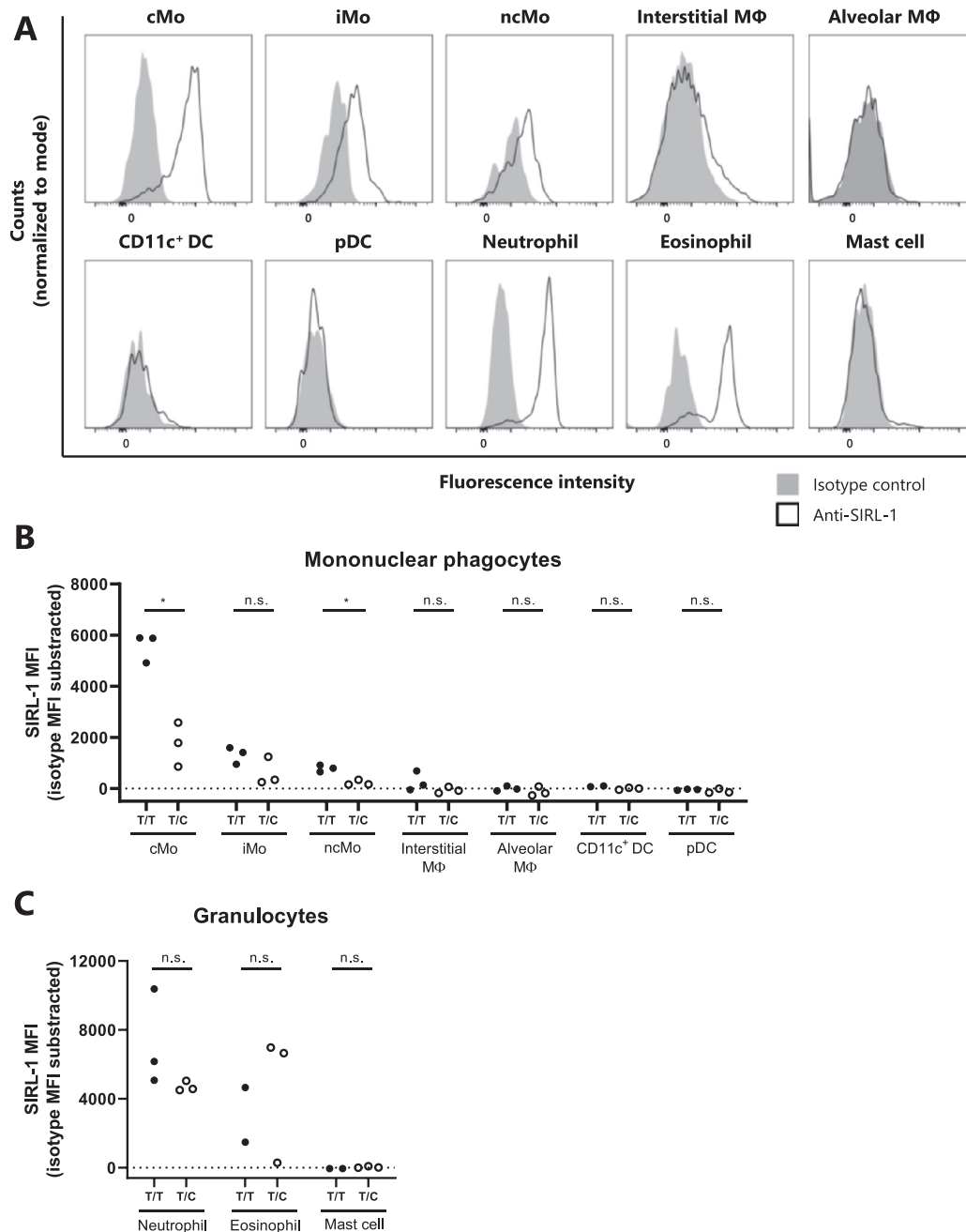
Fig. 4. SIRL-1 is expressed by lung monocytes. Human lung biopsies were digested and analyzed by flow cytometry (See Supplementary Fig. 4 for the gating strategy). (A) Panels show representative histograms of the fluorescence intensity of mononuclear phagocytes and granulocytes stained with a SIRL-1 antibody (open histogram) or an isotype control (closed histogram). The donor's genotype is rs612529T/T (n = 3). (B-C) Median fluorescence intensity (MFI) of mononuclear phagocytes (B) and granulocytes (C) stained with a SIRL-1 antibody, minus the MFI of the same cell type stained with an isotype control. Each symbol represents one donor. Genotypes of rs612529 are indicated with filled circles (T/T, n = 3) or open circles (T/C, n = 3). A mixed-effects model with Sidak's correction for multiple testing was used to compare SIRL-1 expression between donors with rs612529T/T and rs612529T/C genotype in each cell subset, *p < 0.05. cMo, classical monocytes; iMo, intermediate monocytes; ncMo, nonclassical monocytes; MΦ, macrophage.

In conclusion, we show that SIRL-1 is differentially expressed on human phagocyte subsets in barrier tissues. SIRL-1 is ubiquitously expressed on granulocytes, whereas expression on monocytes is genotype- and tissue-specific. Identification of the ligand of SIRL-1 will add to the understanding of the context in which SIRL-1 exerts its biological functions.

5. Authorship

LM, EJ and MV designed the study. DG, TC, BG and GW carried out the experiments. HR analyzed the data. LL, BO and DH recruited patients and gave advice on the study design. HR, DG and LM interpreted the results. All authors contributed to writing of the manuscript and gave final approval of the version to be submitted.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2020.104199.

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