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#### **Short Communication**

# S1PR4-dependent CCL2 production promotes macrophage recruitment in a murine psoriasis model

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The sphingolipid sphingosine-1-phosphate (S1P) fulfills distinct functions in immune cell biology via binding to five G protein-coupled receptors. The immune cell-specific sphingosine-1-phosphate receptor 4 (S1pr4) was connected to the generation of IL-17producing T cells through regulation of cytokine production in innate immune cells. Therefore, we explored whether S1pr4 affected imiquimod-induced murine psoriasis via regulation of IL-17 production. We did not observe altered IL-17 production, although psoriasis severity was reduced in S1pr4-deficient mice. Instead, ablation of S1pr4 attenuated the production of CCL2, IL-6, and CXCL1 and subsequently reduced the number of infiltrating monocytes and granulocytes. A connection between S1pr4, CCL2, and Mø infiltration was also observed in Zymosan-A induced peritonitis. Boyden chamber migration assays functionally linked reduced CCL2 production in murine skin and attenuated monocyte migration when S1pr4 was lacking. Mechanistically, S1pr4 signaling synergized with TLR signaling in resident M $\phi$ s to produce CCL2, likely via the NF- $\kappa$ B pathway. We propose that S1pr4 activation enhances TLR response of resident  $M\phi s$  to increase CCL2 production, which attracts further M $\phi$ s. Thus, S1pr4 may be a target to reduce perpetuating inflammatory responses.

Keywords: Møs · psoriasis · S1PR4

Additional supporting information may be found online in the Supporting Information section at the end of the article.

#### Introduction

The lipid mediator sphingosine-1-phosphate (S1P) is the ligand for five G-protein coupled receptors [1]. Of these, S1pr4 is predominantly expressed on cells of the immune system. S1P coupling to its receptors affects survival, proliferation, activation and, most

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prominently, migration of immune cells. S1pr4 appears to cause activation and polarization of immune cells rather than immune cell migration, which, as a consequence affects adaptive immunity. Accordingly, T cells activated by S1pr4-deficient DCs produced less IL-17 [2].

IL-17 is an important mediator of chronic inflammatory diseases such as psoriasis, which is an immune-mediated chronic, relapsing disease of the skin [3]. The majority of symptoms are caused by T lymphocytes activated via IL-23 toward a  $T_{\rm H}17$  phenotype, which is characterized by the production of IL-17A.  $T_{\rm H}17$ 

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cytokines act on the local epithelium to produce chemokines that recruit myeloid cells to the skin leading to chronic disease [3]. The role of S1P in general and S1pr4 in particular during psoriasis is not known and current literature suggests conflicting roles for S1P. Some propose that its application might be beneficial [4]. Others showed that S1P enhanced psoriasis in patients via S1PR1 [5]. The S1PR1 inhibitor ponesimod, however, did not progress to phase 3, since its oral application caused lymphopenia, directly affecting T cell migration [6]. Targeting S1PR4 may attenuate IL-17 production, while effects on T cell migration will be unlikely. This may hold potential for the treatment of psoriasis, a hypothesis we tested using S1pr4<sup>-/-</sup> mice in a murine psoriasis model.

#### **Results and Discussion**

### S1pr4-deficiency does not attenuate IL-17 production in psoriasis

To analyze whether S1pr4 promotes psoriasis through regulating IL-17 production, WT versus S1pr4<sup>-/-</sup> mice were subjected to the IMQ psoriasis model. Quantifying the severity of the inflammatory response, using the previously described simplified PASI score [7], we observed reduced inflammation in  $S1pr4^{-/-}$  animals, significantly at day 3 (Fig. 1A), which was accompanied by reduced epidermal thickening and dedifferentiation (Fig. 1B). We therefore focused on the early, inflammatory phase of the model for further studies and chose experimental endpoints after 6, 24, and 72 h. LC-MS/MS analysis of ground skin samples confirmed increased S1P levels in psoriatic versus healthy skin 24 h after IMQ application (Fig. 1C). Unexpectedly, mRNA expression of IL-23 (Fig. 1D) and IL-17A (Fig. 1E) were similar between psoriatic WT and S1pr4<sup>-/-</sup> animals. IL-23 was upregulated after 6 h followed by IL-17A upregulation at 24 h. Using cytometric bead arrays (CBA), we did not observe a decrease of IL-17A protein in extracellular fluid of S1pr4-/- animal skin (Fig. 1F). IL-23 protein was not detected. Analyzing the immune infiltrate in psoriatic skin via FACS revealed unaltered T cell populations comparing WT and  $S1pr4^{-/-}$  animals 72 h after disease induction (Fig. 1G; Supporting Information Fig. 1). S1pr4 in other models promoted IL-6-dependent IL-17 production from CD4<sup>+</sup> T cells [2]. In IMQinduced psoriasis, γδ T cells are a major source of IL-17 and depend on IL-23 rather than IL-6 [7], which was not affected by S1pr4 KO.

#### S1pr4 KO reduces Mø infiltration and CCL2 production

We noticed that S1pr4 ablation caused an unexpected reduction in infiltrating neutrophils and M $\phi$ s 72 h after IMQ application (Fig. 1G and H). This was confirmed via histology, where differences in monocyte/M $\phi$  numbers were already detected after 24 h (Fig. 1I–K). In order to understand reduced myeloid cell infiltration, we analyzed mRNA expression of chemokines and inflammatory cytokines and found reduced levels of CCL2 (Fig. 2A), CXCL1 (Fig. 2B), and IL-6 (Fig. 2C) in the skin of S1pr4<sup>-/-</sup> mice. CCL2

and CXCL1 are chemotactic agents for monocytes and neutrophils, respectively, while IL-6 is an important mediator in psoriasis [8]. While all three cytokines were upregulated 6 h after induction of inflammation, the expression of CCL2 decreased rapidly and was undetectable after 24 h. CXCL1 expression increased persistently for 72 h. IL-6 expression was highest at 24 h and returned to basal levels at 72 h. CCL2 protein peaked 6 h after IMO application, with less protein in S1pr4-/- animals compared to the WT (Fig. 2D), closely following its mRNA profile. CXCL1 protein also followed its mRNA expression pattern 6 and 24 h after disease onset (Fig. 2E). IL-6 protein was present in large amounts after 24 h in WT animals that persisted until 72 h, with S1pr4<sup>-/-</sup> mice producing drastically reduced levels (Fig. 2F). We then validated findings in a Zymosan-induced acute peritonitis model. Zymosaninduced peritonitis is defined by rapid granulocyte influx within the first 24 h, followed by a delayed influx/return of monocytes and M $\phi$ s into the peritoneal cavity. In S1pr4<sup>-/-</sup> mice, we again saw significantly reduced Mo numbers 72 h after Zymosan injection (Fig. 2G and H). This was preceded by reduced levels of CCL2 in the peritoneal lavage after 24 h. Neither IL-6 nor CXCL1 was changed by the ablation of S1pr4 in this model (Fig. 2I). Thus, S1pr4 deficiency reduced CCL2 production and Mo numbers in two independent inflammatory mouse models. While CCL2 is a potent inducer of monocyte migration [9], we aimed to confirm that reduced CCL2 production in S1pr4-/- animals sufficed to reduce monocyte migration. In a transwell migration assay, supernatants of WT skin explants induced migration of primary BM monocytes, while supernatants of  $S1pr4^{-/-}$  explants did not. However, recombinant CCL2 restored monocyte migration toward S1pr4 $^{-/-}$  skin explant supernatants (Fig. 2J).

#### Reduced CCL2 production by $S1pr4^{-/-} M\phi s$

One common feature of both models is resident  $M\phi$ s that rapidly react to the initial insult. While CCL2 can be produced by many cell types, the major producers are monocytes and Mos [9]. We therefore postulated that S1pr4 is upstream of a TLR response in Mos and amplifies CCL2 production and concomitant monocyte infiltration. Using in situ hybridization (RNAScope) of CCL2 mRNA, we observed CCL2 expression 6 h after IMQ application, which was limited to a subset of dermal cells (Fig. 3A). Sequential staining of CCL2 mRNA and MHC-II protein ruled out dendritic cells or inflammatory Møs as source of CCL2 (Fig. 3B). Combining RNAScope with immunofluorescence staining of CD3 or  $\alpha$ SMA excluded T cells (Fig. 3C) or activated fibroblasts (Fig. 3D), while CCL2 and MerTK mRNA co-staining revealed resident Møs as the cell type of interest (Fig. 3E and F). Activation of peritoneal Mos from WT or S1pr4-/- animals, in the presence or absence of S1pr4 agonist Cym50308, revealed that Zymosan upregulated CCL2, and the additional activation of S1pr4 further increased CCL2. S1pr4<sup>-/-</sup> M $\phi$ s produced less CCL2 than WT M $\phi$ s (Fig. 3G). Normally, CCL2 upregulation requires activation of NF-κB [10]. WB analysis of peritoneal Mos for ikBa, which restricts NF-kB activation, showed that stimulation with Zymosan A and Cym50308



**Figure 1.** S1pr4-/- reduces myeloid cell infiltrates into psoriatic skin. (A–J) WT and S1pr4<sup>-/-</sup> mice were treated daily with 62.5 mg IMQ topically on the backskin for up to 5 days. (A) Cumulative Psoriasis Area Severity Index (PASI) scores are shown. Data are from five individual animals of each genotype pooled from three independent experiments. (B) PhenOptics images of skin sections from control mice (day 0) and IMQ-treated mice (representative of five animals each) at the times indicated, stained with anti-Ki67 (blue), anti-Keratin14 (green), anti-Keratin10 (yellow), anti-Involucrin (orange), anti-Loricrin (red), and DAPI (white). Scale bars represent 50  $\mu$ m. (C) LC-MS/MS analysis of S1P concentrations in murine skin. Data are from five or four (S1pr4<sup>-/-</sup>, 72 h) individual animals per group pooled from two independent experiments. (D) IL-23 and (E) IL-17 expression in total skin assessed by qPCR at individual timepoints. Data are from five (0 h), three (6 h), or seven (24 and 72 h) individual animals of each genotype pooled from two (6 and 24 h), or three (0 and 72 h) independent experiments. (F) IL-17 protein levels in ground skin determined by cytometric bead array (CBA) at the indicated timepoints. Data are from nine (24 h) or four (72 h) individual animals of each genotype pooled from three independent experiments. (G and H) Immune cell infiltrate in total skin 72 h after IMQ application quantified by flow cytometry analysis. Representative FACS Plots (G) and the quantification (H) from four (WT) or six (S1pr4-/-) individual animals pooled from three independent experiments are shown. (I and DAPI (blue). Scale bars represent 100  $\mu$ m. (K) Quantification of anti-CD163 stained cells in histological skin sections from 24 h IMQ-treated mice. Data are from five (WT) or four (S1pr4<sup>-/-</sup>) individual animals pooled from two independent experiments. All data are biological replicates shown as mean  $\pm$  SEM. \*p < 0.05; \*p < 0.01; p-values were calculated using Fishers LSD test (A), Student's t-test (C,



**Figure 2.** S1pr4 KO reduces CCL2 production during the onset of inflammation. (A–F) WT and S1pr4<sup>-/-</sup> mice were treated daily with 62.5 mg IMQ on the backskin for up to 3 days. (A–C) mRNA expression of CCL2 (A), CXCL1 (B), and IL-6 (C) in total skin determined by qPCR at indicated timepoints. Data are from five (0 and 6 h) or seven (24 and 72 h) individual animals of each genotype pooled from two (6 and 24 h) or three (0 and 72 h) independent experiments. n.d., not detected. (D–F) Protein levels of CCL2 (D), CXCL1 (E), and IL-6 (F) in total skin assessed by CBA at the indicated timepoints. Data are from three (0 h), five (6 h), seven (24 h), or four (72 h) individual animals of each genotype pooled from two (0, 6, and 72 h) or three (24 h) independent experiments. (G, H) WT and S1pr4<sup>-/-</sup> mice were injected intraperitoneally with 10 mg Zymosan/kg body weight. (G and H) Immune cell numbers in peritoneal lavage of mice 72 h after Zymosan injection quantified by flow cytometry. The quantification (G) and representative FACS plots showing M $\phi$ s (H) are shown. Data are from six individual animals of each genotype pooled from two independent experiments. (I) Protein levels of IL-6, CCL2, and CXCL1 in peritoneal lavage 24 h after Zymosan injection. Data are from four (WT; only three for CXCL1), or nine (S1pr4<sup>-/-</sup> individual animals pooled from four independent experiments. (J) Migration of BM from WT and S1pr4<sup>-/-</sup> mice toward skin explant conditioned medium in 96-well transwell plates. Data shown are cells from three individual animals each. One representative experiment out of two independent experiments is shown. All data are biological replicates and are shown as mean + SEM. \* p < 0.05; \* p < 0.01; p-values were calculated using Student's t-test (I), and ratio paired Student's t-test (J).



**Figure 3.** Early CCL2 production from M $\phi$ s demands S1PR4. (A–D) WT and S1PR4<sup>-/-</sup> mice were treated daily with 62.5 mg IMQ on the back skin for 6 h. (A and B) Representative PhenOptics images of sequential 3 µm thick skin sections from 6 h IMQ-treated mice stained for (A) CCL2 mRNA (red) and DAPI (white) and (B) anti-MHC II (red) and DAPI (white). Scale bars represent 100 µm. (C and D) Representative PhenOptics images of skin sections from 6 h IMQ-treated mice stained for CCL2 mRNA (red), together with (C) anti-CD3 (yellow), (D) anti- $\alpha$ SMA (yellow), or (E) MerTK mRNA and DAPI (white). Scale bars represent 100 µm. For representative histological images, samples from three individual WT and KO animals were analyzed. (F) Quantification of CCL2<sup>+</sup> MerTK<sup>+</sup> cells in histological skin sections from 6 h IMQ-treated with either Zymosan aloc or Zymosan and Cym50308 for 24 h. CCL2 protein in cell supernatants were assessed by CBA. Data are from six (WT) or four (S1pr4<sup>-/-</sup>) individual animals, pooled from two independent experiments. (H) Peritoneal M $\phi$ s of WT and S1PR4<sup>-/-</sup> mice were treated with Zymosan and Cym50308 for 30 min or were controls. Western analysis of ikBa and actin, and fold change of ikBa normalized to controls and actin. Data are from four individual animals of each genotype pooled from two independent experiments. All data are biological replicates and actin. Data are from four individual animals of each genotype pooled from two independent experiments. All data are biological replicates and actin. Data are from four individual animals of each genotype pooled from two independent experiments. All data are biological replicates and actin. Data are from four individual animals of each genotype pooled from two independent experiments. All data are biological replicates and actin. Data are from four individual animals of each genotype pooled from two independent experiments. All data are biological replicates and actin. Data are from four individual animals of each genotype pooled from two independent

for 30 min decreased i $\kappa$ B $\alpha$  protein in WT, but not S1pr4<sup>-/-</sup> M $\phi$ s (Fig. 3H; Supporting information Fig. S3). Thus, M $\phi$ s produce CCL2 via S1pr4, likely via the NF $\kappa$ B pathway.

#### **Concluding remarks**

In summary, we show that S1pr4 is negligible for IL-17 production during psoriasis but promotes CCL2 production in resident M\u03c6s during early inflammation. Our data suggest that attenuated CCL2 production translates to reduced infiltration of M\u03c6s into the inflamed tissue. M\u03c6s and monocytes are critically involved during auto-immune diseases such as psoriatic arthritis or MS [11,12]. Increased CCL2 production in resident M\u03c6s after activation of S1pr4 suggests that S1pr4 acts as an enhancer of the initial inflammatory response, which fits to the observation that S1P levels are often elevated in inflammatory diseases [13]. S1pr4 might therefore be a promising target in inflammatory diseases that are

driven by CCL2 and M $\phi$  recruitment. The reduction of T<sub>H</sub>17 cells upon S1pr4 ablation observed before [2] may add to an antiinflammatory potential of blocking S1pr4. This may not only be interesting for auto-immune diseases but also for cancer, where CCL2 production correlates with increased numbers of tumorassociated M $\phi$ s, which promote the formation of metastasis [14].

#### Material and methods

#### Animal experiments

For animal experiments, the guidelines of the Hessian animal care and use committee were followed (FU/1059). Psoriasis-like skin inflammation was induced as described by van der Fits et al. and severity evaluated according to their PASI scoring system [15]. Mice were euthanized and skin samples harvested after 6, 24, or 72 h for further analysis. For induction of Zymosan A-dependent peritonitis, WT and S1pr4<sup>-/-</sup> mice were injected i.p. with 10 mg/kg Zymosan A (Sigma–Aldrich, St. Louis, USA). After up to 72 h, peritoneal lavage was obtained for further analysis.

#### Flow cytometry analysis

Flow cytometry experiments followed the "Guidelines for the use of flow cytometry and cell sorting in immunological studies" [16]. To create single cell suspensions from murine skin, the epidermis dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to the manufacturer's protocol. Peritoneal lavage was analyzed directly after erythrocyte removal. Single cell suspensions were blocked with FcR blocking reagent (Miltenyi Biotec) in 0.5% PBS-BSA for 20 min, stained with fluorochrome-conjugated antibodies and analyzed on a LSR II/Fortessa flow cytometer (BD Biosciences, Franklin Lakes, USA). For gating, fluorescence minus one controls were performed. The following antibodies were used: anti-CD3-PE-CF594, anti-CD4-BV711, anti-CD11b-BV605, anti-CD11c-AlexaFluor700, anti-CD326-BV711, anti-NK1.1-BV510 (all BD Biosciences), anti-CD19-APC-H7, anti-Ly6C-Per-CP-Cy5.5, anti-F4/80-PE-Cy7, antiγδTCR-APC, anti-Ly6G-APC-Cy7, anti-SiglecH-FITC (all BioLegend), anti-CD45-VioBlu, anti-CD90.2-PE, and anti-MHC-II-APC (all Miltenyi Biotec). Gating strategy is shown exemplary in Supporting Information Figure 1.

#### Cytokine quantification

Cytokine levels in cell culture supernatants, peritoneal lavage, or murine skin were measured as described before [17]. Murine IFN $\gamma$ , IL-1 $\beta$ , IL-6, IL-10, IL-17A, IL-23, CCL2, CXCL1, G-CSF, and GM-CSF Cytometric Bead Array Flex-Sets (BD Biosciences) or LegendPlex Chemokine Arrays (Biolegend) were used. Samples were acquired with a LSR Fortessa flow cytometer and data analyzed using BD Biosciences FCAP software or FlowJo V10.6 (BD Biosciences).

#### **Quantitative PCR**

Total RNA from skin and tumor samples was extracted using peg-GOLD RNAPure (Peqlab Biotechnologie, Erlangen, Germany) and reverse transcribed with the Maxima First Strand cDNA Synthesis kit (Thermo Fisher, Waltham, USA). Quantitative real-time PCR reactions were conducted with the iQ SYBR Green Supermix (Bio-Rad, Hercules, USA) on a CFX96 Connect system (Bio-Rad). Relative mRNA expression was analyzed based on the  $\Delta\Delta$ Ct method and normalized to Rps27A.

#### Histology

In situ hybridization by RNAScope was performed according to the manufacturer's instructions (Bio-Techne, Minnesota, USA). Antigen retrieval and protease treatment was done according to the manufacturer's instructions. For murine skin sections, probes to Mm-Ccl2 (Bio-Techne, ACD#311791) and Mm-Mertk (Bio-Techne, ACD#474541) were used. The signal was detected with RNAScope multiplex fluorescent detection reagent kit v2 (Bio-Techne, ACD#323110) using Opal dyes (PerkinElmer, Waltham, USA). Following RNAScope, skin sections were stained using the Opal staining system (PerkinElmer) according to the manufacturer's instructions. For analyzing epidermal architecture, slides were stained with primary antibodies targeting Ki67, Keratin10 (both Abcam), Keratin14, Loricrin, and Involucrin (Biolegend) as described before [7]. The Vectra 3 automated quantitative pathology imaging system (Perkin-Elmer) was used for acquisition at  $20 \times$  and images were analyzed using inForm2.4 Software (Perkin-Elmer).

#### In vitro activation of peritoneal Møs

Peritoneal M $\phi$ s from healthy WT or S1pr4<sup>-/-</sup> mice were cultured in high attachment 24-well plates for 4 h followed by washing and stimulation with Zymosan A (1  $\mu$ g/mL) and S1pr4 agonist Cym50308 (200 nM) for 24 h.

#### Immunoblotting

Peritoneal M $\phi$ s were collected in lysis buffer, incubated for 5 min at 95°C with SDS buffer, and resolved on polyacrylamide gels followed by transfer onto nitrocellulose membranes. Nonspecific binding was blocked with 5% milk, followed by incubation with antibodies against i $\kappa$ B $\alpha$  (Cell Signaling, Danvers, USA; #4814), and actin (Sigma, St. Louis, USA, A2066). Proteins were visualized by IRDye secondary antibodies using the Li-Cor Odyssey imaging system (all from LICOR Bioscience, Bad Homburg, Germany). Supporting information Figure 3 shows an uncropped blot.

#### **Migration assay**

Full skin explants were extracted as described before [18] and were individually placed into 24-well untreated tissue culture dishes (Falcon, Becton Dickinson, Franklin Lakes, USA). After 5 min to allow adherence, 200  $\mu$ L medium was added. Plates were incubated at 37°C, 5% CO<sub>2</sub>. After 24 h, 1.5 mL medium was added to each well. Supernatants were used for Boyden chamber migration assays after another 24 h. A total of  $3.5 \times 10^5$  total BM cells were added in 96-transwell inserts (5  $\mu$ m, Corning) and allowed to migrate toward explant supernatant for 2 h. Rm-CCL2 (10 ng/mL) was used as a positive control. Migrated and non-migrated cells were counted using flow cytometry with Flow-Count Fluorospheres (Beckman Coulter, Krefeld, Germany) as internal counting standard.



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**Conflict of Interest:** The authors declare no commercial or financial conflicts of interest.

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Abbreviation: S1P: sphingosine-1-phosphate

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