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Overlapping Signaling Pathways of Sphingosine 1-Phosphate and TGF-β in the Murine Langerhans Cell Line XS52[1]

Heinfried H. Radeke,* Henrik von Wenckstern,† Kirsten Stoidtner,* Bettina Sauer,† Stefanie Hammer,† and Burkhard Kleuser[2]†

TGF-β has been defined as a key mediator for the induction and maintenance of immunological tolerance. Concomitantly, it is essential for homeostasis of specialized epithelial dendritic cells, namely, Langerhans cells (LC). Our data reveal that TGF-β induces migration of the immature LC, XS52, a cell line expressing the signaling components, TGF-β type I and II receptors and Smad2, 3, and 4 mRNA. TGF-β stimulation induced transient Smad3/4 oligomerization and Smad3/DNA binding. Antisense oligonucleotides (ASO) targeting Smad3 abrogated TGF-β-induced XS52 chemotaxis, proving the involvement of this Smad protein in the TGF-β-dependent migration. In contrast, the typical CCR6-dependent chemotaxis of immature LC induced by CCL20/MIP-3α was not affected by Smad3 ASO. Most notably, we also identified the lysophospholipid sphingosine 1-phosphate (S1P) as a potent chemoattractant for immature LC, which expressed mRNA transcripts of lysophospholipid receptors $S1P_{1-4}$. Additional experiments with specific ASO showed that the $G\alpha_i$-coupled receptors $S1P_1$ and $S1P_3$ were dominantly involved in the S1P-induced migration. In contrast, lysophosphatidic acid (LPA), also binding to members of the lysophospholipid receptor family, failed to induce XS52 migration. Intriguingly, we raised evidence that TGF-β and S1P signal transduction pathways are indeed overlapping, as S1P augmented Smad activation and targeted DNA binding with kinetics comparable to TGF-β. Finally, S1P failed to stimulate XS52 chemotaxis when Smad3 protein expression was abrogated. Thus, our data indicate a cross-communication between S1P and TGF-β signaling that might be relevant for more than only migratory activities of immature LC. ***The Journal of Immunology,* 2005, 174: 2778–2786.**

A rapidly increasing number of specialized properties has been assigned to dendritic cells (DC),[3] including sentinel functions with respect to invading microorganisms, Ag-dependent differentiation and maturation, as well as presentation and initiation of primary immune responses after migration to secondary lymphoid organs (1). As intensely debated, this variety of activities may not be conducted by a single cell type, but rather sets of DC from different origins and in alternate differentiation states (2). Despite these controversies, it seems accepted that signals that determine lineage-specific migratory patterns with subsequent influence on DC differentiation, respectively, arise from both the local environment and the antigenic challenge.

Langerhans cells (LC) represent a subpopulation of epithelial tissue-specific DC. Their recruitment to the epidermis has been linked to CCR6 receptor expression on LC precursors and subsequent migration toward its ligand CCL20/MIP-3α, a chemokine predominantly secreted in the skin (3, 4). In addition, TGF-β, a product of local skin or regulatory T cells, is also essential for LC biology since TGF-β null$^{-/-}$ mice lack LC in the epidermis (5–8). Recently, Hacker et al. (9) determined that LC differentiation is regulated by the TGF-β-induced transcription factor Id2, a finding convincingly supported by their data that Id2 knockout mice also lack LC in the skin.

In 1999, Yamada and Katz (10) applied a series of so-called maturation protocols to the LC line (XS52), which was derived from the epidermis of newborn BALB/c mice (11). In addition to former data, they confirmed that at the starting point GM-CSF-grown XS52 share common characteristics with immature LC. Thus, using this model, large numbers of skin-associated DC devoid of other cell types can be generated and used to study molecular events associated with LC maturation. So far, the maturation process is seen as prerequisite for LC migration from epidermis to lymph nodes during a response to inflammatory or antigenic stimuli (12).

Recently, the assumption that a full function of DC is necessarily coupled to such a maturation process has been questioned. Several investigations stressed the migratory potential and tolerogenic role of immature DC (13–15). Results of Geissmann et al. (16, 17) suggest that LC migration and maturation may represent independently regulated events, because immature LC cultivated in the presence of TGF-β accumulated in the lymph nodes under chronic inflammatory conditions. Furthermore, it has been convincingly demonstrated that immature DC promote naive T cells to acquire a tolerogenic phenotype (18, 19). Thus, TGF-β may be a key regulator in the DC-mediated T cell differentiation favoring regulatory T cells while counteracting inflammatory (Th1-driven) and allergic (Th2-driven) responses.

In general, TGF-β responsiveness is based on a system including a family of TGF-β receptor substrates, the Smad proteins, which translocate into the nucleus where they act as transcription

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[3] Abbreviations used in this paper: DC, dendritic cell; ASO, antisense oligonucleotides; EC, endothelial cell; FAK, focal adhesion kinase; GPCR, G protein-coupled receptor; LC, Langerhans cells; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; PTX, pertussis toxin, S1P, sphingosine 1-phosphate.

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factors. Binding of TGF-β links its receptors, TβRII and TβRI, activating the serine/threonine kinase function of TβRII which then phosphorylates TβRI. This process is followed by the anchoring of the receptor-activated Smad2 and Smad3 to the TβRI kinase domain (20, 21). Phosphorylation on the C-terminal SSVS motif of Smad2 and Smad3 causes a conformational change that results in the heteromerization with Smad4 (Co-Smad) and correlates with the movement from the cytoplasm to the nucleus. In this study, the active complex directly or indirectly regulates the transcription of target genes (22, 23).

Among the family of lipid-derived mediators, the group of lysophospholipids, including lysophosphatidic acid (LPA), lysophosphatidylcholine, and sphingosine 1-phosphate (S1P), modulates important immunological processes, such as immune cell proliferation, migration, and inflammatory gene expression (24–28). S1P and LPA evoke their multiple effects through a family of G protein-coupled receptors (GPCRs). To date, five S1P receptors ($S1P_{1-5}$) and three LPA receptors (LPA_{1-3}) have been identified (29, 30). However, control of complex processes is difficult to reconcile with only these receptors, suggesting further unidentified receptors or intracellular targets of S1P and LPA (31, 32). Recently, several reports show that the phosphorylated form of the immunosuppressive drug FTY720 acts as a full agonist on four of five known S1P receptors (33–35). FTY720 is a synthetic analogue of a natural compound derived from the fungus *Isaria sinclairii* and shares some structural characteristics with sphingosine. Interestingly, FTY720-phosphate does not impair lymphocyte proliferation or activation, as do classical immunosuppressive drugs like cyclosporine, but significantly induces lymphocyte homing into lymph nodes and Peyer's patches in vivo (35, 36).

In this study, for the first time we describe overlapping signaling pathways of TGF-β and S1P in the LC line XS52. We have identified TGF-β and S1P but not LPA as potent chemoattractants for immature XS52 cells and resolved receptor and signaling components essential for both TGF-β and S1P. In addition to chemotactic properties, our data might contribute to a better understanding of other biological effects of S1P, which may be supportive for the development of tolerogenic DC.

Materials and Methods

Materials

TGF-β was purchased from R&D Systems. S1P and protein G plus agarose were obtained from Calbiochem. Control and antisense phosphorothioate oligonucleotides (ASO), the biotinylated Smad3 promoter fragment as well as all primers were prepared by TIB Molbiol. Anti-Smad4, anti-Smad1/2/3, and anti-actin Abs as well as HRP-conjugated anti-mouse IgG were purchased from Santa Cruz Biotechnology. GM-CSF and MIP-3α were provided from Strathmann Biotech. FuGENE 6 Transfection Reagent, 4′,6-diamidino-2′-phenylindole dihydrochloride, and poly(dI-dC) were purchased from Roche. Iscove's medium was obtained from Biochrom. FCS was purchased from Cambrex. PBS, L-glutamine, and penicillin/streptomycin were obtained from Invitrogen Life Technologies. Bis-acrylamide, *N*,*N*, *N*′,*N*′-tetramethylethylenediamine, SDS, ethidium bromide, ammonium persulfate, and diethylprocarbonate were purchased from Roth. Polyvinylidene difluoride membranes were obtained from Millipore. Fibronectin, BSA (fatty acid free), pertussis toxin (PTX), streptavidin-agarose, EDTA, Tween 20, Nonidet P-40, deoxycholic acid, sodium pyruvate, β-ME, PMSF, sodium orthovanadate, sodium fluoride, DTT, leupeptin, pepstatin, aprotinin, nonessential amino acids, SB431542, and all other chemicals were purchased from Sigma-Aldrich.

Cell culture

The murine embryonic LC line XS52 (37, 38) (kindly provided by G. Müller, Department of Dermatology, University of Mainz, Mainz, Germany) was cultured in Iscove's medium supplemented with 5% FCS, L-glutamine (2 mM), penicillin/streptomycin (100 IU/ml/100 μg/ml), sodium pyruvate (1 mM), nonessential amino acids (100 μM), β-ME (50 μM), and GM-CSF (5 ng/ml). *Mycoplasma* contamination was monthly proved as described elsewhere (38).

RNA extraction and semiquantitative PCR

XS52 total RNA was isolated using a method described by Chomczynski and Sacchi (40). cDNA was synthesized with Superscript reverse transcriptase (Invitrogen Life Technologies) using oligo$(dT)_n$ primers (Qiagen) and 2 μg of total RNA as per the manufacturer's instructions. Expression analysis was performed in 50-μl reactions using 2 μl of cDNA (equivalent to 0.2 μg of total RNA) in 25–40 cycles of 94°C (1 min), 55°C (1 min), 72°C (2 min) the Platinum *Taq* polymerase kit (Invitrogen Life Technologies). PCR fragments were separated on 12% polyacrylamide gels in Tris-acetate-EDTA buffer and visualized by ethidium bromide staining. Primer sequences are shown in Tables I and II.

Real-time PCR

Real-time PCR assays were conducted using SYBR Green PCR Master Mix on an ABI PRISM 7900HT Sequence Detection System according to the manufacturer's protocol (Applied Biosystems). Amplification was performed in 10-μl reactions (250 nM primer concentration, 1× SYBR Green Master Mix) containing 2 μl of cDNA (equivalent to 10 ng of total RNA) in 40 cycles of 95°C (15 s) and 60°C (1 min). Total RNA of three different sets of XS52 was used to analyze receptor expression. Data normalization was performed using β-actin as a reference gene. Relative mRNA expression was quantified using the comparative threshold cycle method according to the ABI manual. Primer sequences are shown in Table II.

Chemotaxis and chemokinesis assays

XS52 cells were seeded in the upper well of a modified Boyden chamber. For larger series of experiments, the low volume 96-well chemotaxis chambers with 10 μM pore width were used (NeuroProbe). The lower chamber, separated by a fibronectin-coated membrane, contained TGF-β, S1P, LPA,

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Table I. *Primer sequences for semiquantitative PCR analysis*[a]

Gene	Forward Primer	Reverse Primer	Length (bp)
$S1P_1$	5′-TCTCTCTGCATCAGTCTTCAGC-3′	5′-TGATCACCGTCTTCAGCAAG-3′	398
$S1P_2$	5′-ATATCGCTGATTCTGGGTGG-3′	5′-AGAGAAGGATGCTAAAAGCCG-3′	292
$S1P_3$	5′-TTCCCGACTGCTCTACCATC-3′	5′-TCACTACGGTCCGCAGAAG-3′	192
$S1P_4$	5′-GCTGGAACTGTGTGTGCG-3′	5′-CAGGATCCAGTCCATGCC-3′	332
$S1P_5$	5′-CTTGGCCATTGCTTTAGAGC-3′	5′-AGTAAATCCTTGCATAGAGCGC-3′	272
LPA_1	5′-GGCACATCACGGTTTTCC-3′	5′-CCATGTTGGAACAGTGATCG-3′	159
LPA_2	5′-TGCCTCTGTGACTTGGACAG-3′	5′-ATGAGTAGGAAGACAAGCAGGC-3′	101
LPA_3	5′-GATGAGAGTCCACAGCAACTTG-3′	5′-AGATGCGTACGTATACCGCC-3′	230
TβRI	5′-AGTCATGGTTCAGGGAGACG-3′	5′-AGCACAATCCGAAGGCAG-3′	202
TβRII	5′-TCGCCGAGGTCTACAAGG-3′	5′-GAGGTACTCCTGCAGGTTGC-3′	248
Smad2	5′-GCCGTCTTCAGGTTTCACA-3′	5′-TAGTATGCGATTGAACACC-3′	502
Smad3	5′-CGCCAGTTCTACCTCCAGTG-3′	5′-AAAGACCTTCCCCTCCGATGT-3′	518
Smad4	5′-CTCCAGAAATTGGAGAGTTGG-3′	5′-ATCGAATGTCCTTCAGTGGGT-3′	578

[a] cDNA sequences were obtained from the National Institutes of Health Gene Bank. Primer sequences were selected and optimized for RT-PCR by HUSAR software. This table shows resulting primer sequences and the expected PCR product size (length) of $S1P_{1-5}$, LPA_{1-3}, TβRI, and TβRII.

Table II. *Primer sequences for real-time PCR analysis*[a]

Gene	Forward Primer	Reverse Primer	Length (bp)
$S1P_1$	5′-TGGGAGCCTGAGAGAGGGA-3′	5′-TCATGGTATCACCAGGCCG-3′	54
$S1P_2$	5′-ACCTGGAGCTCACAGCAGTCTATC-3′	5′ACCCAACCCTCAGAACACAGA-3′	51
$S1P_3$	5′-GTGGGCACGCTCTTTCATG-3′	5′-GACCTAGACCCACGGCCTC-3′	51
$S1P_4$	5′-CCTGGCTGACATCTTTGGTTC-3′	5′-5′-CATGCCACGCAGGTACTCCT-3′	55
$S1P_5$	5′-TGACGCTTCCCAACCCTTT-3′	5′-TGCGCTTATTTGGCGAGTC-3′	51
LPA_1	5′-AACAGTCTGAGGCAGCCCAA-3′	5′-GGAACCAGTATCTGAGCTAAAGGAAC-3′	51
LPA_2	5′-CCATGTTGAATCAAGAACTGCG-3′	5′-CCAAACTTGTGAGGTCATGCAT-3′	51
LPA_3	5′-TTAGTAAAAGCGCACAGGAAAGG-3′	5′-ACAAGCCGGAACCCACTG-3′	51
TβRI	5′-GGAAATTGCTCGACGCTGTT-3′	5′-TCAACCGATGGATCAGAAGGT-3′	90
TβR-II	5′-GCCGGAAATTCCCAGCTT-3′	5′-CACACGATCTGGATGCCCT-3′	51
β-actin	5′-CATTGCTGACAGGATGCAGAA-3′	5′-ATGGTGCTAGGAGCCAGAGC-3′	51

[a] Primers were designed using the Primer Express software (Applied Biosystems). This table shows resulting primer sequences and the expected PCR product size (length) of $S1P_{1-5}$, LPA_{1-3}, TβRI, TβRII, and β-actin.

or MIP-3α. After 2.5 h the filter was removed, the plate was briefly centrifuged, dried, and stained with 4′,6′-diamidino-2-phenylindole/MeOH solution (1 μg/ml; Roche). The migrated cells were counted by an image analysis algorithm using a fluorescent microscope with computer-driven automatic and heated xy stage (Zeiss Axiovert 200 with Hamamatsu camera and OpenLab 3 image analysis software; Improvision) calculating the average of four square images per well (500 μm in diameter). All samples were tested as triplicates and the data are expressed as the mean ± SD. The specific migration index was calculated as ratio after setting the control vehicle random migration at 1.0. Determination of chemokinesis was performed by treatment with equal concentrations of the cytokine on both sites of the filter followed by checkerboard analysis to distinguish between chemokinetic and chemotactic responses.

For ASO experiments, cells were treated with the indicated oligonucleotides for 3 days at a final concentration of 0.1 μM using FuGENE 6 Transfection Reagent in supplemented Iscove′s medium before chemotaxis assays ($S1P_1$ antisense, 5′-AGTGGACACCATAGCTGCTAA-3′; $S1P_1$ control oligonucleotide, 5′-TTAGCAGCTATGGTGTCCACT-3′; $S1P_2$ antisense, 5′-GGTTCAGACAATTCCAGCCCAGG-3′; $S1P_2$ control nucleotide, 5′-TGGTCAAGCTTAACCAGCAGGCC-3′; $S1P_3$ antisense, 5′-ATG CGTGGTTGCCATGGCTTC-3′; $S1P_3$ control oligonucleotide, 5′-GAAGCC ATGGCAACCACGCAT-3′; $S1P_4$ antisense, 5′-ACCCCAGCACACCACA AAGGCC-3′; $S1P_4$ control oligonucleotide, 5′-CCACAGACCCACAC CAGGCCAA-3′; Smad3 antisense, 5′-GCAGGATGGACGACAT-3′; and Smad3 control oligonucleotide, 5′-GTGGACAGCTAGAGAC-3′).

Immune precipitation and Western blot analysis

Smad immunoprecipitation and blotting were performed as described recently (41). Briefly, cells were seeded in 10-cm dishes and cultured for 72 h in supplemented Iscove′s medium. Then cells were treated, rinsed twice with PBS, and harvested in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS) containing protease inhibitors (1 mM PMSF, 1 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin) and phosphatase inhibitors (1 mM sodium orthovanadate and 50 mM sodium fluoride). Lysates were centrifuged at 14,000 × *g* for 30 min and 1 mg of lysate protein was immunoprecipitated overnight at 4°C with 2 μg of anti-Smad3 Abs, followed by a precipitation with 10 μl of protein G plus agarose at 4°C for 90 min. After four washes with complete radioimmunoprecipitation assay buffer, the immunoprecipitates were eluted by boiling for 5 min in 60 μl of SDS sample buffer.

For Western blot analysis, immunoprecipitates (20 μl) or cell lysates (20 μg) were separated by 10% SDS-PAGE. Gels were blotted overnight onto polyvinylidene difluoride membranes. After blocking with 5% nonfat dry milk in TBS-Tween 20 (0.1%) overnight at 4°C, membranes were incubated with anti-Smad3, anti-Smad4, or anti-actin Abs (each 0.2 μg/ml) for 2 h at room temperature. The blots were washed three times in TBS-Tween 20 followed by incubation with the secondary Abs (1 μg/ml) for 1 h. After washing, the blots were developed according to the manufacturer's protocol.

DNA affinity assay

Cells (1×10^6 cells/dish) were stimulated with TGF-β (2 ng/ml) and S1P (10 μM) for 30 min, rinsed twice with ice-cold PBS, and harvested into lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 5 mM EDTA) that was supplemented with 1 mM PMSF and 1 μg/ml aprotinin, leupeptin, and pepstatin. Lysate protein (1 mg) was incubated at 4°C for 1 h with a biotinylated dsDNA (30 pM) composed of three tandemly repeated CAGA sequences (CAGA sense, 5′-bio-CGAGAGCCAGACAAGGAGC CAGACAGG AGCCAGACACTCGAG-3′; CAGA antisense, 5′-TCGAGT GTCTGGCTCCTTGTCTG GCTCCTTGTCTGGCTCTCGA-3′) in the presence of 12 μg of poly(dI-dC). DNA-bound proteins were precipitated using streptavidin-agarose and the content of DNA-bound Smad3 protein was detected by Western blot analysis using a mouse monoclonal anti-Smad1/2/3 Ab.

Data evaluation and statistic analysis

The Prism software package (GraphPad) was used for data collection and presentation. Four to six replicates per experiment were measured and, if not stated otherwise, data from one representative of a series of three to five experiments are presented as mean ± SD. Student's *t* test was applied to evaluate statistical significance (*, $p < 0.05$; **, $p < 0.001$).

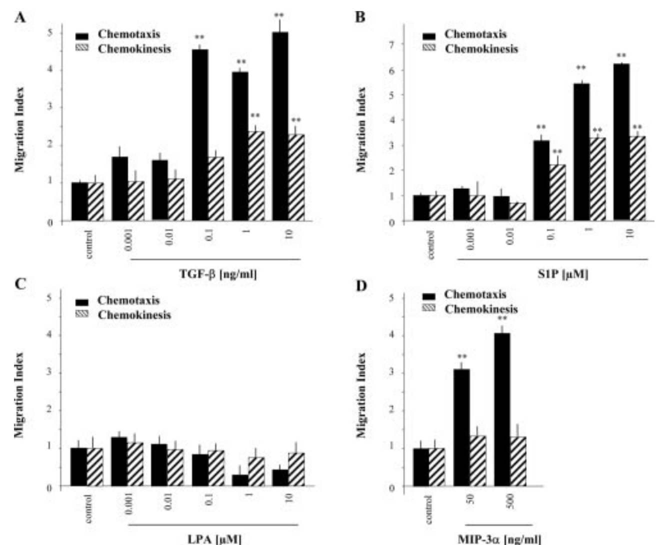

FIGURE 1. Influence of TGF-β (*A*), S1P (*B*), LPA (*C*), and MIP-3α (*D*) on chemotaxis and chemokinesis of XS52 cells. Immature XS52 (5×10^5/well) migration across 10-μm pore-sized filter membranes coated with fibronectin was measured in response to a chemotactic gradient. For chemokinetic actions, cells were treated with equal concentrations of the stimuli on both sites of the filter. TGF-β (*A*) and S1P (*B*) at indicated concentrations induced cell migration and chemokinesis in comparison to unstimulated controls in a dose-dependent manner. In contrast, LPA failed to induce XS52 migration and chemokinesis (*C*). The CCR6 ligand MIP-3α strongly induced chemotactic responses (*D*), thus confirming an immature XS52 differentiation state. Data are expressed as arbitrary migration index after setting random migration of the vehicle controls to 1.0 of representative experiments, each repeated at least three times with triplicate determinations (see *Materials and Methods*).

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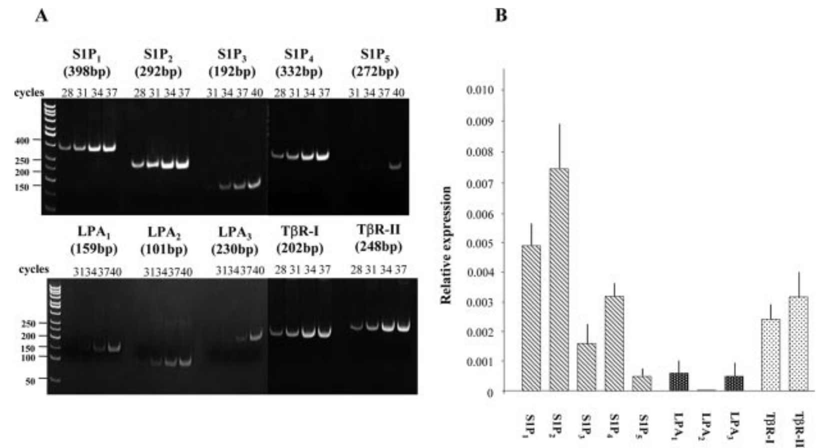

FIGURE 2. Semiquantitative PCR (*A*) and real-time PCR (*B*) analysis of the murine S1P, LPA, and TGF-β receptors in XS52. cDNA from the reverse transcription of 0.2 μg of total RNA isolated from GM-CSF-stimulated XS52 was assessed for expression of S1P, LPA, and TFG-β receptors. One of three experiments with similar results is shown (*A*). Real-time PCR starting with 10 ng of total RNA of three different sets of XS52 was performed with specific primers as indicated and described in *Materials and Methods* using β-actin as a reference gene. Relative mRNA expression was quantified using the comparative threshold cycle method according to the ABI manual (*B*).

Results

S1P and TGF-β but not LPA stimulate migration of the murine LC line XS52

Since the lysophospholipids S1P and LPA as well as the growth factor TGF-β have been implicated as contributors to the regulation of inflammatory processes, we examined their migratory potency on XS52 murine LC. In particular, TGF-β was observed to stimulate XS52 migration in a concentration-dependent and saturable manner. A significant and potent increase of the chemotactic response was visible at a concentration of 0.1 ng/ml, leading to a migration index of 4.5 $\pm$ 0.27. Higher concentrations of TGF-β up to 10 ng/ml did not further enhance the migratory response (Fig. 1*A*). Checkerboard analysis revealed that modest apparent chemokinetic actions occurred after treatment with equal concentrations of the cytokine on both sites of the filter, indicating that chemotaxis is the major response of XS52 toward TGF-β (Fig. 1*A*).

In additional experiments, S1P was also characterized as a chemoattractant for XS52 cells. An increase of migration was observed over a concentration range of S1P of 10^{-7}–10^{-5} M. Indeed, as presented in Fig. 1*B*, the migratory potency of S1P was comparable to that of TGF-β, yielding to a maximal migration index of 6.1 $\pm$ 0.9 in response to 10 μM S1P. The addition of S1P to both sites of the filter revealed that both chemotaxis and, to a minor extent, chemokinesis contribute to the S1P-mediated cell motility (Fig. 1*B*).

Although LPA in analogy to S1P has been identified to promote migration in a variety of cells, this lysophospholipid failed to induce chemotaxis of XS52 in the tested concentration range between 10^{-9} and 10^{-5} M (Fig. 1*C*).

Confirming an immature differentiation status of XS52 LC, migration was examined in response to the CCR6 ligand MIP-3α, the only chemokine produced by the epithelium that selectively attracts LC precursors. Indeed, treatment with MIP-3α led to a potent migration comparable to that of TGF-β and S1P (Fig. 1*D*).

It should be noted that a chemotactic response to S1P in the same concentration range has also been reported in mouse DC, differentiated from freshly isolated monocytes, whereas LPA failed to induce migration, indicating that both XS52 cells and primary DC show similar effects toward these lysophospholipids (42).

Analysis of S1P, LPA, and TGF-β receptor mRNA transcripts in XS52 cells

With respect to the responsiveness of XS52 to TGF-β and S1P, it was of interest to further characterize the involvement of specific receptors. Therefore, we performed semiquantitative PCR analysis, which showed TβRI and TβRII expression nicely correlating to TGF-β-induced XS52 migration. PCR fragments of expected sizes for TβRI (202 bp) and TβRII (248 bp) were obtained in equal amounts after 28 cycles (Fig. 2*A*). Moreover, $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ mRNA were present in XS52 cells as indicated by the specific PCR product sizes 398, 292, 192, and 332 bp, respectively. $S1P_5$ mRNA was detectable but only at very low abundance compared with $S1P_{1-4}$. We also analyzed the mRNA expression of the LPA receptors LPA_{1-3} in XS52 cells. It is of interest that only very faint bands were observed for LPA_1 (159 bp), LPA_2 (101 bp), and LPA_3 (230 bp) appearing first after cycles 34–37, which may explain the lack of LPA to induce XS52 migration (Fig. 2*A*).

To further confirm and quantify these findings, real-time PCR was performed. Indeed, mRNA of all LPA receptor subtypes as well as $S1P_5$ was just marginally expressed; the relative amount of S1P receptor mRNA was $S1P_2 > S1P_1 > S1P_4 > S1P_3 \gg S1P_5$ (Fig. 2*B*).

Involvement of S1P₁ and S1P₃ receptors in XS52 cell migration toward S1P

It has been suggested that S1P mediates its actions either via GPCRs, namely, $S1P_{1-5}$, or as an intracellular second messenger formed by sphingosine kinase (33). Because S1P receptors are known to couple to $G\alpha_i$, we measured the migratory response

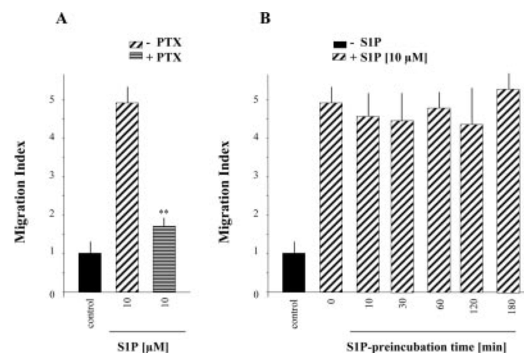

FIGURE 3. Chemotactic activity of XS52 toward S1P in the presence of PTX (*A*) and following homologous desensitization (*B*). XS52 cells were preincubated with PTX (200 ng/ml, 2 h) (*A*) or with 10 μM S1P for the indicated time periods (*B*). Then migration assays were performed as described in *Materials and Methods*. In the presence of PTX, S1P-induced XS52 migration was almost completely abolished, indicating an involvement of $G_{\alpha i}$-coupled S1P receptors in the migration process (*A*). Preincubation of XS52 with S1P for the indicated time periods did not affect the S1P (10 μM)-induced migratory response (*B*). Values are expressed as migratory index of representative experiments, each repeated at least three times with triplicate determinations.

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toward S1P in the presence of PTX to eliminate functional $G\alpha_i$ signaling. Preincubation of XS52 with PTX almost completely abolished the migratory effect of S1P, indicating that it is mainly dependent on GPCR interacting with $G\alpha_i$ proteins (Fig. 3*A*). Since it is known that GPCR may undergo desensitization, resulting in a loss of cellular responsiveness to S1P, we measured chemotaxis toward the lysophospholipid after pretreatment intervals with S1P between 10 min and 3 h. Actually, preincubation with S1P did not affect enhancement of XS52 cell migration by S1P, indicating functionally active and not desensitized S1P receptors (Fig. 3*B*). Using the ASO technique, we further sought to elucidate which of the expressed S1P receptor subtypes are involved in the migration process. Although Western blot analysis revealed that treatment of cells with $S1P_1$-, $S1P_2$-, $S1P_3$-, and $S1P_4$-ASO resulted in a serious reduction of protein levels, only elimination of $S1P_1$ and $S1P_3$ significantly inhibited the migratory response toward S1P (Fig. 4). Thus, $S1P_1$ ASO preincubation resulted in an almost 80% decrease of S1P-mediated migration, whereas treatment with $S1P_3$ ASO was accompanied by a 30% reduction (Fig. 4, *A* and *C*). It is of interest that elimination of $S1P_2$ further enhanced chemotaxis toward the lysophospholipid, indicating that this receptor subtype possesses antimigratory properties (Fig. 4*B*).

Smad expression and Smad3 activation by TGF-β and S1P in XS52 cells

Upon activation, the TGF-β receptor complex induces phosphorylation of Smad2 or Smad3 followed by an oligomerization with the common mediator Smad4. The formed heteromeric complex acts as a transcription factor mediating multiple cellular responses. Because especially Smad3 has been reported to contribute to TGF-β-driven migration in monocytes (43), we investigated whether Smad proteins are present in XS52 cells. RT-PCR analysis revealed the presence of Smad2, 3, and 4 mRNA in XS52, thus implicating the existence of a functional TGF-β signaling machinery (Fig. 5*A*). Moreover, we examined whether TGF-β indeed induces activation of this signaling pathway. For this purpose, oligomerization of Smad3 with Smad4 was measured in response to the growth factor. In a coimmune precipitation experiment with an anti-Smad3 Ab, the appearance of Smad4 was analyzed following TGF-β stimulation over a time period of 90 min. As shown in Fig. 5*B*, TGF-β induced a transient Smad3/4 heteromerization starting at 10 min, which decreased to basal levels after 120 min. We also investigated the dose dependence of Smad activation by TGF-β. As presented in Fig. 5*C*, Smad4 coimmunoprecipitation occurred at 0.1 ng/ml and was further increased up to 2 ng/ml TGF-β. To substantiate that the activated complex contributes to transcriptional activation, a DNA affinity assay was performed. To this end, a biotinylated dsDNA construct composed of three tandemly repeated CAGA sequences, which resembles the Smad3-specific promoter region, was incubated with lysates of TGF-β- or control vehicle-stimulated cells. The proteins bound to DNA were precipitated with streptavidin-agarose followed by Western blot analysis for Smad3. As depicted in Fig. 5*F*, a slight Smad3 band was detectable in control experiments. However, when XS52 were

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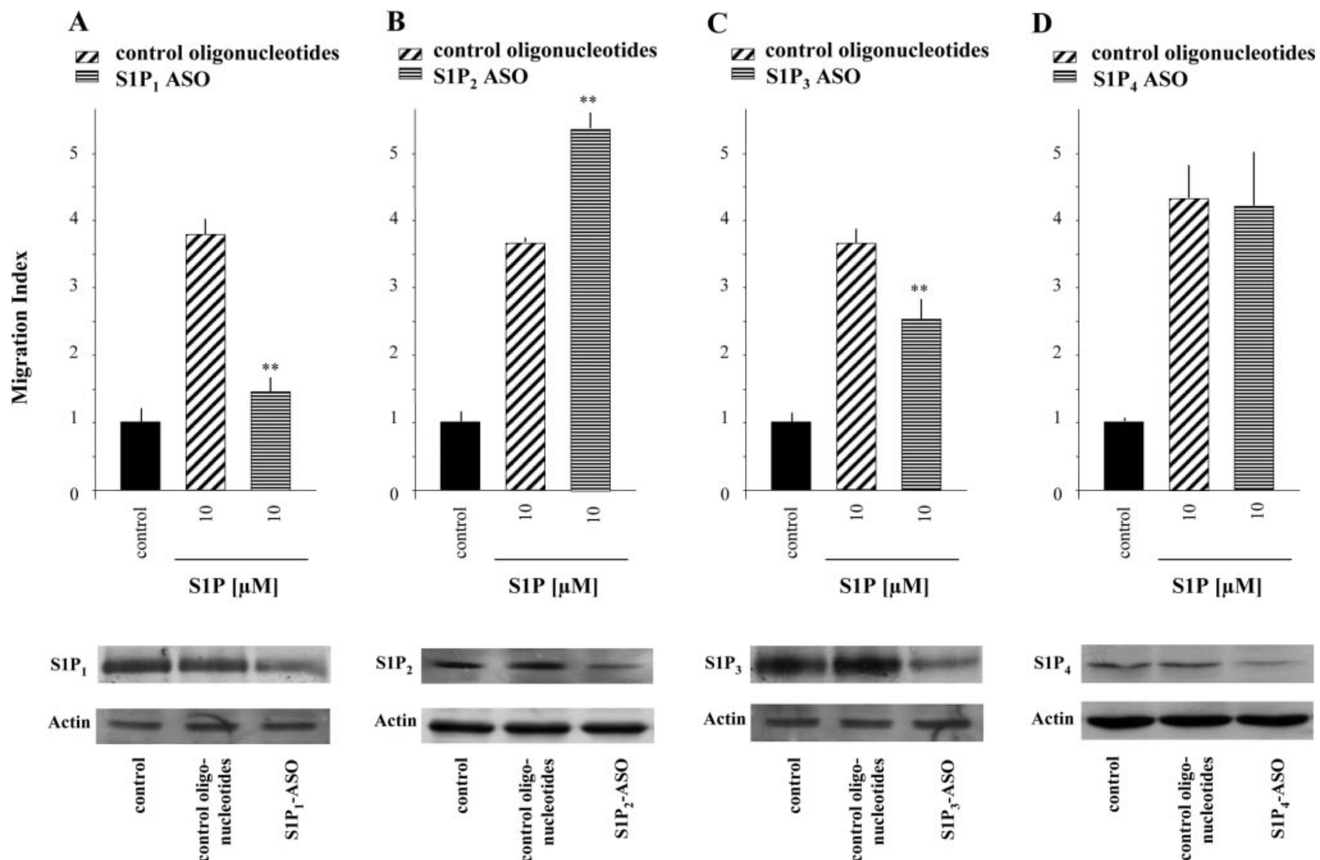

FIGURE 4. Chemotactic activity of XS52 toward S1P in the presence of $S1P_{1-4}$ ASO or control oligonucleotides. XS52 cells were preincubated with $S1P_1$ (*A*)-, $S1P_2$ (*B*)-, $S1P_3$ (*C*)-, or $S1P_4$- (*D*)-specific ASO or control oligonucleotides and then migration assays were performed. Relative abundance of the S1P receptor protein expressed after ASO pretreatment is indicated in the *lower panel* of specific Western blots. ASO directed against $S1P_1$ inhibited the S1P (10 μM)-induced migratory response by almost 90% (*A*), whereas abrogation of $S1P_3$ (*C*) was accompanied by a 30% reduction of S1P-induced XS52 migration. Although $S1P_4$ ASO treatment did not affect S1P-dependent chemotaxis (*D*), reduction of $S1P_2$ protein levels by RNA-specific ASO unleashed an inhibitory effect and enhanced S1P-induced XS52 migration significantly (*B*). Values are expressed as migratory index of representative experiments, each repeated at least three times with triplicate determinations.

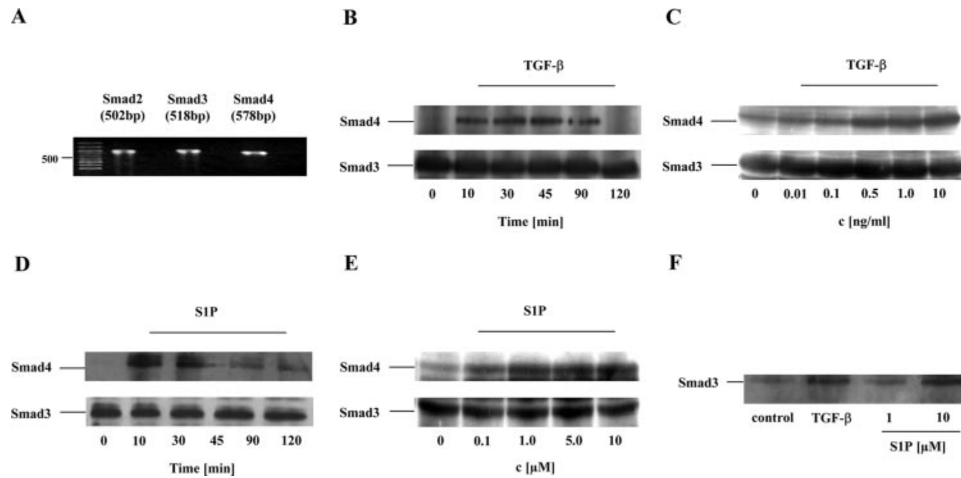

FIGURE 5. Expression of Smad proteins in XS52 cells and their activation in response to TGF-β and S1P. XS52 mRNA was isolated, reverse transcribed, and amplified for Smad2, 3, and 4 mRNA transcripts. PCR products of expected sizes were obtained for Smad2, 3, and 4 (*A*). XS52 were treated with control vehicle, TGF-β (2 ng/ml), or S1P (10 μM) for the indicated time periods (*B* and *D*) or with the indicated concentrations of TGF-β and S1P for a time period of 30 min (*C* and *E*). After immunoprecipitation with an anti-Smad3 Ab, Western blot analysis for either Smad3 or Smad4 was performed. As indicated by the Smad4 band, both TGF-β (*B* and *C*) and S1P (*C* and *D*) induced a Smad3/4 heteromerization. Lysates of TGF-β (2 ng/ml, 30 min)-, S1P (1.0 or 10 μM, 30 min)-, or control vehicle-stimulated cells were incubated with a biotinylated dsDNA containing three tandemly repeated CAGA sequences specific for Smad3 binding (*F*). DNA-bound proteins were precipitated using streptavidin-agarose and the Western blot analysis for Smad3 indicates increased Smad3/DNA binding following S1P and TGF-β stimulation in comparison to unstimulated cells (*F*).

stimulated with TGF-β, there was a significant increase of Smad3, indicating an enhanced DNA binding.

S1P and TGF-β exhibit analogous biological activities in immature LC and it has been recently demonstrated that an activation of Smad proteins is not an exclusive effect of TGF-β (44); therefore, we checked whether S1P might also stimulate oligomerization of Smad3 with Smad4. Indeed, treatment of XS52 cells with S1P resulted in such a complex formation. In analogy to the kinetics of the TGF-β-induced activation, a strong effect occurred at 10 min, whereas at 90 min only a slight stimulation was visible (Fig. 5*D*). The most effective dose to activate Smad signaling was identified with 5–10 μM S1P, which is in agreement with those concentrations to potently induce migration (Fig. 5*E*). Furthermore, the DNA affinity assay revealed an enhanced binding of the complex to the Smad3-responsive oligonucleotide after treatment with 10 μM S1P (Fig. 5*F*). Maximal DNA binding was observed after 30 min, which is consistent with the effect induced by TGF-β.

Participation of Smad3 in TGF-β- and S1P-induced chemotaxis of XS52 cells

Since we found that both TGF-β and S1P activated Smad3 in XS52 cells, it was of great interest to investigate whether this stimulation contributed to their migratory potency. To this end, TGF-β- and S1P-induced XS52 migration was studied following treatment with Smad3 ASO. Western blot analysis revealed that the ASO construct substantially diminished Smad3 expression, whereas control oligonucleotides did not affect its protein levels (Fig. 6*A*). Indeed, TGF-β-mediated XS52 migration was significantly reduced in the presence of ASO compared with a treatment with control oligonucleotides (Fig. 6*B*). Most interesting, S1P failed to induce chemotaxis of Smad3-deficient XS52 cells, indicating that this intracellular signaling molecule is not only central in TGF-β-mediated migratory processes but also necessary for S1P-mediated cell motility (Fig. 6*B*). To conclusively demonstrate that Smad3 abrogation did not generally reduce migration of XS52 cells, chemotaxis was measured in response to MIP-3α (Fig. 6*B*). Indeed, both control and Smad3 ASO-treated cells showed a normal migration index to this chemokine, indicating that Smad3 possessed a specific role in TGF-β- and S1P-induced chemotaxis. Because Smad3 is a specific substrate of activated TβRI, it was of interest to verify whether this receptor subtype is essential for the S1P-induced migration of XS52 cells. To address this, the ATP binding site of TβRI was blocked using the inhibitor SB421543. As expected, TGF-β was not able to increase migration rates in the presence of SB421543 and the migratory potency of MIP-3α was not affected (Fig. 6*C*). Most interesting, the chemotactic properties of S1P were significantly diminished when cells were pretreated with SB421543, indicating the involvement of TβRI in S1P-mediated migration (Fig. 6*C*).

Discussion

The results of the present study clearly demonstrate that TGF-β as well as S1P are potent chemoattractants for skin-derived immature LC. Most interesting, we identified the transcription factor Smad3 as an essential key mediator for transmitting both S1P- and TGF-β-dependent signaling effects in XS52 cells. TGF-β receptor subtypes I and II are expressed by immature XS52 in comparable amounts. Since TGF-β induces XS52 cell migration in concentrations from 0.1 to 10 ng/ml, we concluded that these receptors are functionally active. It is well known that TGF-β induces chemotaxis in monocytes (45) due to the activation of Smad3 (43, 46). Smad2 and Smad3 have been characterized as receptor-mediated proteins in response to TGF-β stimulation, which heteromerize with Smad4 and thus regulate the activation of Smad-sensitive genes (47–49). In accordance with these findings, we detected mRNA transcripts of Smad2, 3, and 4 in XS52. As additional evidence of a functional involvement, treatment of XS52 with TGF-β resulted in a transient Smad3/Smad4 oligomerization and an increased Smad3/DNA binding. Chemotactic responses toward TGF-β were substantially diminished by the treatment of XS52 with Smad3 ASO. These data led us to assume that the multifaceted growth factor TGF-β takes a central role in the appearance and/or differentiation of LC in the skin (5, 6, 9). It would be of interest to investigate whether the cytokine might also fulfill a key role in immature LC physiology, possibly relevant for triggering the differentiation of regulatory T cells.

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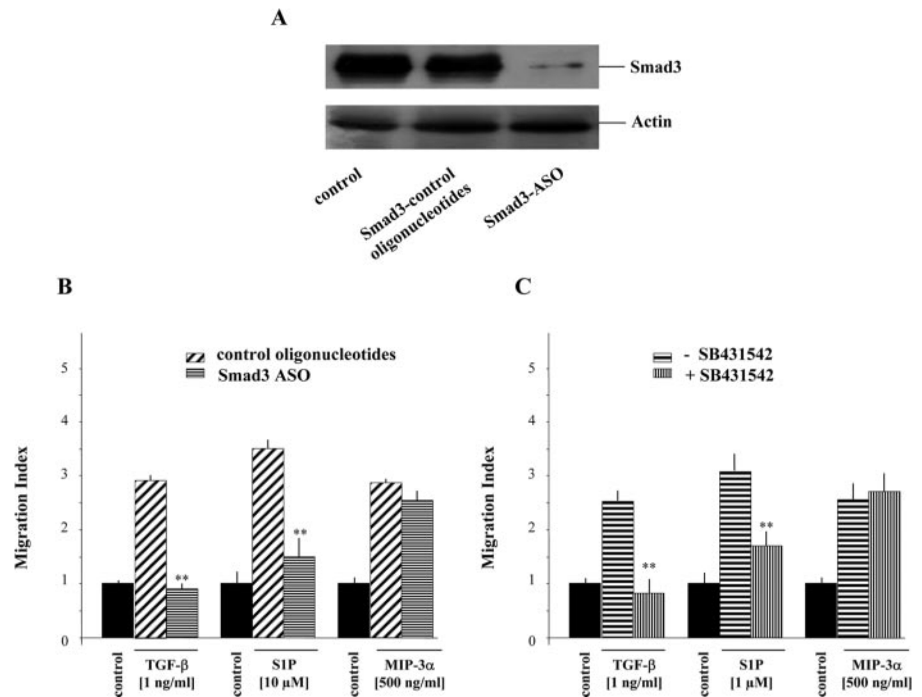

FIGURE 6. Involvement of Smad3 in S1P- and TGF-β-mediated cell migration. XS52 cells were preincubated with Smad3 ASO or control oligonucleotides as described in *Materials and Methods*. Western blot analysis revealed that Smad3 protein expression is decreased in response to Smad3 ASO and that control actin levels were not influenced (*A*). TGF-β-, S1P-, and MIP-3α-induced XS52 migration was measured as described. Compared with control oligonucleotides, chemotaxis in response to TGF-β and S1P but not to MIP-3α could be significantly reduced using ASO directed against the intracellular TGF-β signal transducer Smad3 (*B*). To investigate the involvement of TGF-β receptors, XS52 cells were preincubated with SB431542 (10 μM) for 30 min. After treatment with S1P (1 μM), TGF-β (1 ng/ml), or MIP-3α (500 ng/ml) chemotaxis was measured, indicating that TβRI is necessary for TGF-β- and S1P-induced migration (*C*). Values are expressed as migratory index of representative experiments, each repeated at least three times with triplicate determinations.

However, apart from this latter role in lymph nodes, TGF-β function for LC migration is essential because immune cells have to pass vessels on their way to inflamed tissue. Consequently, endothelial barrier integrity and expression of adhesion molecules on endothelial cells (EC) are important factors. Comprehensive studies regarding S1P-mediated cell migration and motility have been predominantly performed in EC and the receptor $S1P_1$ has already been linked to EC migration processes in the context of angiogenesis and vascular maturation (50–56). In conclusion, S1P has been reported to induce cytoskeletal reorganization through Rho- and $G\alpha_i$-mediated pathways (57). Miura et al. (58) indicated that S1P induces focal adhesion kinase (FAK) phosphorylation that could be abolished using the Rho inactivator C3 transferase while FAK phosphorylation was only partially inhibited by PTX. Ohmori et al. (59) provided evidence that PTX-sensitive migratory processes can be attributed to the receptor $S1P_1$ whereas PTX-insensitive, FAK-mediated migration results from $S1P_3$ receptor activation, as this receptor subtype couples to $G\alpha_q/G\alpha_{13}$ and could be blocked by the GPCR antagonist suramin.

Idzko et al. (60) provided evidence that functional receptors $S1P_{1-4}$ are present in primary immature human DC isolated from whole blood. Indeed, S1P-induced functional cell migration in these immature DC and triggered rapid actin polymerization. These processes were exclusively mediated via $G\alpha_i$-coupled S1P receptors since both could be completely abrogated using PTX. Extending these data, our present study provides clear evidence that S1P induces migration of murine immature LC. S1P significantly augmented chemotaxis already at nanomolar concentrations, which correlates well with previous observations (33). Furthermore, we could confirm PTX sensitivity of the S1P migratory process, indicating that $G\alpha_i$-coupled S1P receptors are involved. RT-PCR analysis demonstrated that four of five subtypes of the S1P receptor are expressed at significant levels in immature XS52 cells. Our data imply that $S1P_1$ and $S1P_3$ are responsible for the S1P-induced migratory response in XS52 cells. With respect to $S1P_2$, it has been observed before that it might represent a counterregulatory receptor for $S1P_{1+3}$ (61). It is abundantly expressed by the LC cell line XS52 and in line with the previous findings specific ASO treatment obviously resulted in a release from its counterregulatory activity, indicating a delicate balance of S1P effects dependent on the signal coupling of its receptor subtypes.

For more than a decade, GPCRs have been regarded as monomeric plasma membrane-bound receptors. However, recently several publications demonstrated that GPCR oligomerization might contribute signaling flexibility. Several GPCRs have been demonstrated to form homodimers and even heterodimers with members of the same or other GPCR families, thus creating signaling complexes with completely new pharmacological and functional characteristics (62–64). With respect to our study, S1P receptors are capable of forming both covalently linked homodimers and heterodimers with other GPCRs (65) as shown by van Brocklyn et al. (66) in systematic coprecipitation studies. Moreover, recent findings support the view that GPCRs, including S1P receptors, also build functional heterodimers with other plasma membrane receptors, e.g., receptor-tyrosine kinases. For instance, Waters et al. (67) provided data that S1P and platelet-derived growth factor (PDGF) act via PDGF-β/$S1P_1$ receptor complexes in airway smooth muscle cells as indicated by coimmune precipitation and $S1P_1$ ASO treatment. In contrast, Kluk et al. (68) reported that PDGF-induced chemotaxis is not affected in both vascular smooth muscle cells and fibroblasts of $S1P_1$ null mice, thus questioning the generality of $S1P_1$ as downstream component of PDGF-induced chemotaxis.

However, it is still open to speculation whether GPCRs can also functionally interact with other plasma membrane receptor types. In this work, we provide functional data concerning an interaction between S1P receptors and the TGF-β signaling. In principle, receptor interactions can exist 1) between the receptor molecules, 2) on any level of their respective signaling pathways, or 3) at gene transcription sites. So far, the data provided in this work analyze the overlap of S1P and TGF-β receptor signaling cascades on the level of Smad protein activation and functional cell migration. Currently, we are examining whether a direct interaction or heterodimerization of S1P and TGF-β receptors is occurring, and in a parallel project we found evidence for an overlapping activation of S1P/TGF-β-regulated genes (69).

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In conclusion, the data presented here raise strong evidence that tissue- or mast cell-derived S1P, apart from its migratory effect, may exert a significant differentiating influence on immature LC exploiting the well-known pathways of TGF-β signaling. Thus, our results constitute a strong and highly interesting basis for both further detailed molecular analysis of the signaling overlaps and for the analysis of the immunological consequences.

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Disclosures

The authors have no financial conflict of interest.

References

1. Shortman, K., and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat. Rev. Immunol. 2:151.*
2. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol. 9:271.*
3. Charbonnier, A. S., N. Kohrgruber, E. Kriehuber, G. Stingl, A. Rot, and D. Maurer. 1999. Macrophage inflammatory protein 3α is involved in the constitutive trafficking of epidermal Langerhans cells. *J. Exp. Med. 190:1755.*
4. Dieu-Nosjean, M. C., C. Massacrier, B. Homey, B. Vanbervliet, J. J. Pin, A. Vicari, S. Lebecque, C. Dezutter-Dambuyant, D. Schmitt, A. Zlotnik, and C. Caux. 2000. Macrophage inflammatory protein 3α is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J. Exp. Med. 192:705.*
5. Borkowski, T. A., J. J. Letterio, A. G. Farr, and M. C. Udey. 1996. A role for endogenous transforming growth factor β1 in Langerhans cell biology: the skin of transforming growth factor β1 null mice is devoid of epidermal Langerhans cells. *J. Exp. Med. 184:2417.*
6. Yang, D., O. M. Howard, Q. Chen, and J. J. Oppenheim. 1999. Cutting edge: immature dendritic cells generated from monocytes in the presence of TGF-β1 express functional C-C chemokine receptor 6. *J. Immunol. 163:1737.*
7. Borkowski, T. A., J. J. Letterio, C. L. Mackall, A. Saitoh, A. G. Farr, X. J. Wang, D. R. Roop, R. E. Gress, and M. C. Udey. 1997. Langerhans cells in the TGF β1 null mouse. *Adv. Exp. Med. Biol. 417:307.*
8. Borkowski, T. A., J. J. Letterio, C. L. Mackall, A. Saitoh, X. J. Wang, D. R. Roop, R. E. Gress, and M. C. Udey. 1997. A role for TGFβ1 in Langerhans cell biology: further characterization of the epidermal Langerhans cell defect in TGFβ1 null mice. *J. Clin. Invest. 100:575.*
9. Hacker, C., R. D. Kirsch, X. S. Ju, T. Hieronymus, T. C. Gust, C. Kuhl, T. Jorgas, S. M. Kurz, S. Rose-John, Y. Yokota, and M. Zenke. 2003. Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat. Immunol. 4:380.*
10. Yamada, N., and S. I. Katz. 1999. Generation of mature dendritic cells from a CD14$^+$ cell line (XS52) by IL-4, TNF-α, IL-1β, and agonistic anti-CD40 monoclonal antibody. *J. Immunol. 163:5331.*
11. Xu, S., P. R. Bergstresser, and A. Takashima. 1995. Phenotypic and functional heterogeneity among murine epidermal-derived dendritic cell clones. *J. Invest. Dermatol. 105:831.*
12. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
13. Belz, G. T., W. R. Heath, and F. R. Carbone. 2002. The role of dendritic cell subsets in selection between tolerance and immunity. *Immunol. Cell Biol. 80:463.*
14. Lutz, M. B., and G. Schuler. 2002. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol. 23:445.*
15. Mahnke, K., E. Schmitt, L. Bonifaz, A. H. Enk, and H. Jonuleit. 2002. Immature, but not inactive: the tolerogenic function of immature dendritic cells. *Immunol. Cell Biol. 80:477.*
16. Geissmann, F., P. Revy, A. Regnault, Y. Lepelletier, M. Dy, N. Brousse, S. Amigorena, O. Hermine, and A. Durandy. 1999. TGF-β1 prevents the noncognate maturation of human dendritic Langerhans cells. *J. Immunol. 162:4567.*
17. Geissmann, F., M. C. Dieu-Nosjean, C. Dezutter, J. Valladeau, S. Kayal, M. Leborgne, N. Brousse, S. Saeland, and J. Davoust. 2002. Accumulation of immature Langerhans cells in human lymph nodes draining chronically inflamed skin. *J. Exp. Med. 196:417.*
18. Dhodapkar, M. V., R. M. Steinman, J. Krasovsky, C. Munz, and N. Bhardwaj. 2001. Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J. Exp. Med. 193:233.*
19. Dhodapkar, M. V., and R. M. Steinman. 2002. Antigen-bearing immature dendritic cells induce peptide-specific CD8$^+$ regulatory T cells in vivo in humans. *Blood 100:174.*
20. Wotton, D., and J. Massague. 2001. Smad transcriptional corepressors in TGFβ family signaling. *Curr. Top. Microbiol. Immunol. 254:145.*
21. Tsukazaki, T., T. A. Chiang, A. F. Davison, L. Attisano, and J. L. Wrana. 1998. SARA, a FYVE domain protein that recruits Smad2 to the TGFβ receptor. *Cell 95:779.*
22. Massague, J. 1998. TGF-β signal transduction. *Annu. Rev. Biochem. 67:753.*
23. Massague, J., and Y. G. Chen. 2000. Controlling TGF-β signaling. *Genes Dev. 14:627.*
24. Spiegel, S., and S. Milstien. 2003. Sphingosine-1-phosphate: an enigmatic signalling lipid. *Nat. Rev. Mol. Cell Biol. 4:397.*
25. Graeler, M., and E. J. Goetzl. 2002. Activation-regulated expression and chemotactic function of sphingosine 1-phosphate receptors in mouse splenic T cells. *FASEB J. 16:1874.*
26. Graeler, M., G. Shankar, and E. J. Goetzl. 2002. Cutting edge: suppression of T cell chemotaxis by sphingosine 1-phosphate. *J. Immunol. 169:4084.*
27. Cinque, B., L. Di Marzio, C. Centi, C. Di Rocco, C. Riccardi, and C. M. Grazia. 2003. Sphingolipids and the immune system. *Pharmacol. Res. 47:421.*
28. Baumruker, T., and E. E. Prieschl. 2002. Sphingolipids and the regulation of the immune response. *Semin. Immunol. 14:57.*
29. Lee, M. J., J. R. Van Brocklyn, S. Thangada, C. H. Liu, A. R. Hand, R. Menzeleev, S. Spiegel, and T. Hla. 1998. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science 279:1552.*
30. Fukushima, N., I. Ishii, J. J. Contos, J. A. Weiner, and J. Chun. 2001. Lysophospholipid receptors. *Annu. Rev. Pharmacol. Toxicol. 41:507.*
31. Spiegel, S., and S. Milstien. 2003. Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. *Biochem. Soc. Trans. 31:1216.*
32. McIntyre, T. M., A. V. Pontsler, A. R. Silva, A. St Hilaire, Y. Xu, J. C. Hinshaw, G. A. Zimmerman, K. Hama, J. Aoki, H. Arai, and G. D. Prestwich. 2003. Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARγ agonist. *Proc. Natl. Acad. Sci. USA 100:131.*
33. Mandala, S., R. Hajdu, J. Bergstrom, E. Quackenbush, J. Xie, J. Milligan, R. Thornton, G. J. Shei, D. Card, C. Keohane, et al. 2002. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science 296: 346.*
34. Brinkmann, V., M. D. Davis, C. E. Heise, R. Albert, S. Cottens, R. Hof, C. Bruns, E. Prieschl, T. Baumruker, P. Hiestand, et al. 2002. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J. Biol. Chem. 277:21453.*
35. Brinkmann, V., and K. R. Lynch. 2002. FTY720: targeting G-protein-coupled receptors for sphingosine 1-phosphate in transplantation and autoimmunity. *Curr. Opin. Immunol. 14:569.*
36. Im, D. S. 2003. Linking Chinese medicine and G-protein-coupled receptors. *Trends Pharmacol. Sci. 24:2.*
37. Xu, S., P. R. Bergstresser, and A. Takashima. 1995. Phenotypic and functional heterogeneity among murine epidermal-derived dendritic cell clones. *J. Invest. Dermatol. 105:831.*
38. Yokota, K., K. Ariizumi, T. Kitajima, P. R. Bergstresser, N. E. Street, and A. Takashima. 1996. Cytokine-mediated communication between dendritic epidermal T cells and Langerhans cells: in vitro studies using cell lines. *J. Immunol. 157:1529.*
39. Wirth, M., E. Berthold, M. Grashoff, H. Pfutzner, U. Schubert, and H. Hauser. 1994. Detection of *Mycoplasma* contaminations by the polymerase chain reaction. *Cytotechnology 16:67.*
40. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem. 162: 156.*
41. Poncelet, A. C., M. P. de Caestecker, and H. W. Schnaper. 1999. The transforming growth factor-β/SMAD signaling pathway is present and functional in human mesangial cells. *Kidney Int. 56:1354.*
42. Renkl, A., L. Berod, M. Mockenhaupt, M. Idzko, E. Panther, C. Termeer, P. Elsner, M. Huber, and J. Norgauer. 2004. Distinct effects of sphingosine-1-phosphate, lysophosphatidic acid and histamine in human and mouse dendritic cells. *Int. J. Mol. Med. 13:203.*
43. Ashcroft, G. S., X. Yang, A. B. Glick, M. Weinstein, J. L. Letterio, D. E. Mizel, M. Anzano, T. Greenwell-Wild, S. M. Wahl, C. Deng, and A. B. Roberts. 1999. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat. Cell Biol. 1:260.*
44. Firth, S. M., and R. C. Baxter. 2002. Cellular actions of the insulin-like growth factor binding proteins. *Endocr. Rev. 23:824.*
45. Wiseman, D. M., P. J. Polverini, D. W. Kamp, and S. J. Leibovich. 1988. Transforming growth factor-β (TGFβ) is chemotactic for human monocytes and induces their expression of angiogenic activity. *Biochem. Biophys. Res. Commun. 157:793.*
46. Ashcroft, G. S. 1999. Bidirectional regulation of macrophage function by TGF-β. *Microbes Infect. 1:1275.*
47. Di Guglielmo, G. M., C. Le Roy, A. F. Goodfellow, and J. L. Wrana. 2003. Distinct endocytic pathways regulate TGF-β receptor signalling and turnover. *Nat. Cell Biol. 5:410.*
48. Itoh, S., M. Thorikay, M. Kowanetz, A. Moustakas, F. Itoh, C. H. Heldin, and P. Ten Dijke. 2003. Elucidation of Smad requirement in transforming growth factor-β type I receptor-induced responses. *J. Biol. Chem. 278:3751.*
49. Moustakas, A., K. Pardali, A. Gaal, and C. H. Heldin. 2002. Mechanisms of TGF-β signaling in regulation of cell growth and differentiation. *Immunol. Lett. 82:85.*
50. English, D., D. N. Brindley, S. Spiegel, and J. G. Garcia. 2002. Lipid mediators of angiogenesis and the signalling pathways they initiate. *Biochim. Biophys. Acta 1582:228.*
51. Paik, J. H., S. Chae, M. J. Lee, S. Thangada, and T. Hla. 2001. Sphingosine 1-phosphate-induced endothelial cell migration requires the expression of EDG-1 and EDG-3 receptors and Rho-dependent activation of α vβ3- and β1-containing integrins. *J. Biol. Chem. 276:11830.*

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52. Pyne, S., and N. Pyne. 2000. Sphingosine 1-phosphate signalling via the endothelial differentiation gene family of G-protein-coupled receptors. *Pharmacol. Ther. 88:115.*
53. Daniel, T. O., and D. Abrahamson. 2000. Endothelial signal integration in vascular assembly. *Annu. Rev. Physiol. 62:649.*
54. English, D., A. T. Kovala, Z. Welch, K. A. Harvey, R. A. Siddiqui, D. N. Brindley, and J. G. Garcia. 1999. Induction of endothelial cell chemotaxis by sphingosine 1-phosphate and stabilization of endothelial monolayer barrier function by lysophosphatidic acid, potential mediators of hematopoietic angiogenesis. *J. Hematother. Stem Cell Res. 8:627.*
55. Lee, M. J., S. Thangada, K. P. Claffey, N. Ancellin, C. H. Liu, M. Kluk, M. Volpi, R. I. Sha'afi, and T. Hla. 1999. Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell 99:301.*
56. Liu, C. H., S. Thangada, M. J. Lee, J. R. Van Brocklyn, S. Spiegel, and T. Hla. 1999. Ligand-induced trafficking of the sphingosine-1-phosphate receptor EDG-1. *Mol. Biol. Cell 10:1179.*
57. Takuwa, Y. 2002. Subtype-specific differential regulation of Rho family G proteins and cell migration by the Edg family sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta 1582:112.*
58. Miura, Y., Y. Yatomi, G. Rile, T. Ohmori, K. Satoh, and Y. Ozaki. 2000. Rho-mediated phosphorylation of focal adhesion kinase and myosin light chain in human endothelial cells stimulated with sphingosine 1-phosphate, a bioactive lysophospholipid released from activated platelets. *J. Biochem. 127:909.*
59. Ohmori, T., Y. Yatomi, H. Okamoto, Y. Miura, G. Rile, K. Satoh, and Y. Ozaki. 2001. G_i-mediated Cas tyrosine phosphorylation in vascular endothelial cells stimulated with sphingosine 1-phosphate: possible involvement in cell motility enhancement in cooperation with Rho-mediated pathways. *J. Biol. Chem. 276:5274.*
60. Idzko, M., E. Panther, S. Corinti, A. Morelli, D. Ferrari, Y. Herouy, S. Dichmann, M. Mockenhaupt, P. Gebicke-Haerter, F. Di Virgilio, G. Girolomoni, and J. Norgauer. 2002. Sphingosine 1-phosphate induces chemotaxis of immature and modulates cytokine-release in mature human dendritic cells for emergence of Th2 immune responses. *FASEB J. 16:625.*
61. Sugimoto, N., N. Takuwa, H. Okamoto, S. Sakurada, and Y. Takuwa. 2003. Inhibitory and stimulatory regulation of Rac and cell motility by the $G_{12/13}$-Rho and G_i pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. *Mol. Cell. Biol. 23:1534.*
62. Benkirane, M., D. Y. Jin, R. F. Chun, R. A. Koup, and K. T. Jeang. 1997. Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5δ32. *J. Biol. Chem. 272:30603.*
63. Nimchinsky, E. A., P. R. Hof, W. G. Janssen, J. H. Morrison, and C. Schmauss. 1997. Expression of dopamine D3 receptor dimers and tetramers in brain and in transfected cells. *J. Biol. Chem. 272:29229.*
64. Scandella, E., Y. Men, S. Gillessen, R. Forster, and M. Groettrup. 2002. Prostaglandin E_2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood 100:1354.*
65. Salim, K., T. Fenton, J. Bacha, H. Urien-Rodriguez, T. Bonnert, H. A. Skynner, E. Watts, J. Kerby, A. Heald, M. Beer, et al. 2002. Oligomerization of G-protein-coupled receptors shown by selective co-immunoprecipitation. *J. Biol. Chem. 277:15482.*
66. Van Brocklyn, J. R., B. Behbahani, and N. H. Lee. 2002. Homodimerization and heterodimerization of S1P/EDG sphingosine-1-phosphate receptors. *Biochim. Biophys. Acta 1582:89.*
67. Waters, C., B. Sambi, K. C. Kong, D. Thompson, S. M. Pitson, S. Pyne, and N. J. Pyne. 2003. Sphingosine 1-phosphate and platelet-derived growth factor (PDGF) act via PDGF β receptor-sphingosine 1-phosphate receptor complexes in airway smooth muscle cells. *J. Biol. Chem. 278:6282.*
68. Kluk, M. J., C. Colmont, M. T. Wu, and T. Hla. 2003. Platelet-derived growth factor (PDGF)-induced chemotaxis does not require the G protein-coupled receptor S1P1 in murine embryonic fibroblasts and vascular smooth muscle cells. *FEBS Lett. 533:25.*
69. Xin, C., S. Ren, B. Kleuser, M. Schäfer-Korting, S. Shabahang, W. Eberhardt, H. H. Radeke, J. M. Pfeilschifter, and A. Huwiler. 2004. Sphingosine-1-phosphate cross-activates the Smad signaling cascade and mimics TGFβ-induced cell responses in renal mesangial cells. *J. Biol. Chem. 279:35255.*

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