The sphingosine 1-phosphate receptor $S1P_2$ triggers hepatic wound healing

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ABSTRACT **Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid produced by sphingosine kinase (SphK1 and 2). We previously showed that S1P receptors ($S1P_1$, $S1P_2$, and $S1P_3$) are expressed in hepatic myofibroblasts (hMF), a population of cells that triggers matrix remodeling during liver injury. Here we investigated the function of these receptors in the wound healing response to acute liver injury elicited by carbon tetrachloride, a process that associates hepatocyte proliferation and matrix remodeling. Acute liver injury was associated with the induction of $S1P_2$, $S1P_3$, SphK1, and SphK2 mRNAs and increased SphK activity, with no change in $S1P_1$ expression. Necrosis, inflammation, and hepatocyte regeneration were similar in $S1P_2^{-/-}$ and wild-type (WT) mice. However, compared with WT mice, $S1P_2^{-/-}$ mice displayed reduced accumulation of hMF, as shown by lower induction of smooth muscle α-actin mRNA and lower induction of TIMP-1, TGF-β1, and PDGF-BB mRNAs, overall reflecting reduced activation of remodeling in response to liver injury. The wound healing response was similar in $S1P_3^{-/-}$ and WT mice. *In vitro*, S1P enhanced proliferation of cultured WT hMF, and PDGF-BB further enhanced the mitogenic effect of S1P. In keeping with these findings, PDGF-BB up-regulated $S1P_2$ and SphK1 mRNAs, increased SphK activity, and $S1P_2$ induced PDGF-BB mRNA. These effects were blunted in $S1P_2^{-/-}$ cells, and $S1P_2^{-/-}$ hMF exhibited reduced mitogenic and comitogenic responses to S1P. These results unravel a novel major role of $S1P_2$ in the wound healing response to acute liver injury by a mechanism involving enhanced proliferation of hMF.—Serriere-Lanneau, V., Teixeira-Clerc, F., Li, L., Schippers, M., de Wries, W., Julien, B., Tran-Van-Nhieu, J., Manin, S., Poelstra, K., Chun, J., Carpentier, S., Levade, T., Mallat, A., Lotersztajn, S. The sphingosine 1-phosphate receptor $S1P_2$ triggers hepatic wound healing. *FASEB J.* 21, 2005–2013 (2007)**

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SPHINGOSINE 1-PHOSPHATE (S1P) IS A BIOACTIVE sphingolipid metabolite of ceramide that is produced after phosphorylation of sphingosine by sphingosine kinase (SphK) types 1 and 2 (1). The lipid was originally identified as an intracellular messenger that regulates cell survival and differentiation, but its actions are also mediated by a family of G-protein-coupled receptors referred to as $S1P_{1–5}$ (1–7). Delineation of specific intracellular or receptor-dependent functions of S1P is still limited by the lack of selective receptor agonists and antagonists. Nevertheless, some specific S1P receptor-dependent functions have emerged, owing to the recent availability of $S1P_1$, $S1P_2$, and $S1P_3$ receptor knockout mice. Thus, $S1P_1$-dependent functions include regulation of S1P signaling, fertility, angiogenesis and vascular maturation, and lymphocyte development and trafficking (1–5, 8–10). $S1P_2$ promotes myogenic differentiation whereas $S1P_3$ regulates angiogenesis, heart rate, triggers vasoactive effects, and compromises lung barrier integrity (9, 11–13). Finally, $S1P_1$, $S1P_2$, and $S1P_3$ are implicated in cytoskeletal reorganization, cell migration, proliferation, differentiation, and survival (1–4, 14).

Only a few recent studies investigated the function of S1P in liver pathophysiology (15–20). Thus, it has been shown that S1P inhibits proliferation of cultured hepatocytes *via* S1P2 (18). We recently identified S1P receptors ($S1P_1$, $S1P_2$, and $S1P_3$) in cultured human hepatic myofibroblasts, a population of fibrogenic cells that plays a key role in the wound healing response associ-

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ated with liver injury (15). We also demonstrated that activation of S1P receptors triggers survival of hepatic myofibroblasts (16). These results therefore suggested that specific S1P receptors might participate in the wound healing response to liver injury. We explored this hypothesis by using $S1P_2$- and $S1P_3$-deficient mice that were subjected to acute carbon tetrachloride (CCl_4) intoxication, an established model of liver injury characterized by acute hepatitis, followed by a wound healing response that associates hepatocyte proliferation and a matrix remodeling process driven by hepatic myofibroblasts.

MATERIALS AND METHODS

Materials

Collagenase was from Roche (Nutley, NJ, USA), culture media and reagents were from Life Technologies, Inc. (Carlsbad, CA, USA), fetal calf serum (FCS) was from JBio Laboratories (Les Ulis, France), platelet-derived growth factor-BB came from PreproTech Inc. (Rocky Hill, NJ, USA), Nicodenz was from Abcys (Paris, France), and S1P was from Biomol (Hamburg, Germany). A stock solution of S1P was prepared in methanol and stored at −80°C. Freshly made dilutions were prepared in 0.4% fatty acid-free bovine serum albumin after evaporation of methanol, resuspension in 0.4% fatty acid-free bovine serum albumin, and sonication. [*methyl*-^{3}H]Thymidine (25 Ci/mmol) was purchased from ICN (Irvine, CA, USA).

Animals and experimental design

Mice invalidated for $S1P_2$ or $S1P_3$ receptors ($S1P_2^{-/-}$ or $S1P_3^{-/-}$) were generated as described previously (21, 22). $S1P_2^{-/-}$ or $S1P_3^{-/-}$ and their wild-type (WT) littermates were generated from heterozygous mice bred for seven generations on a C57Bl/6J background. Animals were housed in temperature- and humidity-controlled rooms, kept on a 12 h light/dark cycle, and provided unrestricted amounts of food and water. Male and female mice aged 8–12 wk were used. Animal procedures were conducted in accordance with French government policies (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture).

Acute liver injury was induced by an intraperitoneal injection of CCl_4 [Sigma, St. Louis, MO, USA; 0.5 ml/kg body weight, 1:5 dilution in mineral oil (MO)], as described previously (23). Animals were sacrificed 24, 48, or 72 h after treatment. WT (n=22) and $S1P_2^{-/-}$ (n=14) animals were divided into the following groups: WT/sham (MO, n=5); WT/CCl_4 sacrificed either 24 h (n=3), 48 h (n=6), or 72 h (n=8) after CCl_4 injection; $S1P_2^{-/-}$/sham (MO, n=3); $S1P_2^{-/-}$/CCl_4 sacrificed either 24 h (n=3), 48 h (n=3), or 72 h (n=5) after injection of CCl_4. WT (n=17) and $S1P_3^{-/-}$ (n=11) animals were divided into the following groups: WT/sham (MO, n=4); WT/CCl_4 sacrificed 24 h (n=5) or 72 h (n=8) after CCl_4 injection; $S1P_3^{-/-}$/sham (MO, n=2); $S1P_3^{-/-}$/CCl_4 sacrificed 24 h (n=4) or 72 h (n=5) after CCl_4 injection. No mortality was observed throughout treatment. Liver samples were taken from several lobes, fixed in buffered formalin or snap frozen in liquid nitrogen, and stored at −80°C until use.

Liver function tests

Alanine aminotransferase activity (ALT) was measured on an automated analyzer in the Biochemistry Department of Henri Mondor Hospital in Creteil.

Liver histology

Liver specimens were fixed in formalin and embedded in paraffin. Tissue sections (4 μm-thick) were stained with hematoxylin-eosin for routine examination. Histological grading (necrosis and inflammation) was blindly assessed on at least four fragments from different areas of each liver, as described previously (24). All samples were scored simultaneously.

Immunostaining

Immunohistochemical staining for smooth muscle α-actin (α-SMA) was performed as described previously (23) in liver tissue fixed in formalin and embedded in paraffin using a Vector M.O.M. immunodetection kit in accordance with the protocol specified by the manufacturer (Vector Laboratories, Burlingame, CA, USA), with a 1:1000 dilution of a monoclonal antibody to α-SMA (Sigma). The area of positive staining was scored blindly and measured on four or five liver fragments per animal, as described previously (24). No staining was observed when omitting the primary antibody.

Western blot analysis of PCNA expression

Western blot analysis of PCNA was performed with 30 μg of lysates, obtained as described previously (25), using mouse monoclonal antibody to PCNA (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and peroxidase-conjugated goat anti-mouse IgG antibody (1:5000, Jackson Immunoresearch, West Grove, PA, USA) as a secondary antibody. Protein expression was visualized by using an enhanced chemiluminescence (ECL Plus) assay kit according to the manufacturer's instructions (Amersham Biosciences, Arlington Heights, IL, USA). Signals were normalized to the β-actin signals (mouse monoclonal anti-β-actin antibody, 1:5000, Sigma).

RNA isolation and RT-PCR

Total RNA was extracted from cells using an RNeasy kit (Qiagen, Valencia, CA, USA) and from frozen liver specimens using Tri-Reagent. Real-time PCR was carried out on a LightCycler (Roche Diagnostics), as described previously (24), using a Quantitech SYBR Green PCR kit (Qiagen). Oligonucleotide primers (MWG Biotech) for the following mouse genes were $S1P_1$ receptor: $S1P_1$ sense, 5′-ACTTTGCGAGTGAGCTG-3′ and $S1P_1$ antisense 5′-AGTGAGCCTTCAGTTACAGC-3′; $S1P_2$ receptor: $S1P_2$ sense, 5′-TTCTGGAGGGTAACACAGTGGT-3′ and $S1P_2$ antisense, 5′-ACACCCTTTGTATCAAGTGGCA-3′; $S1P_3$ receptor: $S1P_3$ sense 5′-TGGTGTGCGGCTGTCTAGTCAA-3′ and $S1P_3$ antisense, 5′-CACAGCAAGCAGACCTCCAGA-3′. SphK1: SphK1 sense 5′-TGTCACCCATGAACCTGCTGTCCCTGCACA-3′ and SphK1 antisense 5′-AGAAGGCACTGGCTCCAGAGGAACAAG-3′; SphK2: SphK2 sense 5′-ACAGAACCATGCCCGTGAG-3′ and SphK2 antisense 5′-AGGTCAACACCGACAACCTG-3′; α-SMA sense 5′-TCCTCCCTGGAGAAGAGCTAC and α-SMA antisense 5′-TATAGGTGGTTTCGTGGATGC; TGF-β1 sense 5′-TGCGCTTGCAGAGATTAAAA-3′up and TGF-β1 antisense 5′-TCACTGGAGTTGTACGGCAG-3′;

procollagen α1(I): Col α1(I) sense 5′-GAAACCCGAGGTATGCTTGA-3′ and Col α1(I) antisense 5′-GACCAGGAGGACCAGGAAGT-3′; PDGF-BB: PDGF-BB sense 5′-GGTGAGAAAGATTGAGATTGT-3′ and PDGF-BB antisense 5′-GAGCTTGAGGCGTCTTGGCT-3′; TIMP-1: TIMP-1 sense 5′-GCATCTCTGGCATCTGGCATC-3′ and TIMP-1 antisense 5′-GCGGTTCTGGGACTTGTGGGC-3′, TNF-α: TNF-α sense: AATGGCCTCCCTCTCATCAGTT and TNF-α antisense: CCACTTGGTGGTTTGCTACGA; β2-microglobulin was used as the reference gene for quantification of data from *in vivo* experiments, and 18S for quantification of cell culture experiments: β2-microglobulin sense 5′-ATGCTGAAGAACGGGAAAAA-3′ and β2-microglobulin antisense 5′-CGGCCATACTGTCATGCTTA-3′; 18S sense 5′-GTAACCCGTTGAACCCCATT-3′ and 18S antisense 5′-CCATCCAATCGGTAGTAGCG-3′. The PCR-amplified products were analyzed on a 2% agarose gel and sequenced.

Isolation and culture of murine hepatic myofibroblasts

Murine hepatic myofibroblasts were isolated from WT (n=3) and $S1P_2^{-/-}$ (n=3) animals by collagenase perfusion and purified by density gradient in Nicodenz, as described previously (23). After isolation, cells were cultured in DMEM medium containing 20% FCS. One day after isolation, cell debris and nonadherent cells were removed by washing and the cells were further cultured in DMEM medium containing 10% FCS. Cells were used between the fourth and ninth passage and expressed characteristic markers of hepatic myofibroblasts were found *in situ* in the fibrotic liver, α-SMA, fibulin-2, and interleukin-6 (26).

DNA synthesis and cell proliferation assays

DNA synthesis was measured in triplicate wells by incorporation of [^{3}H]-thymidine, as described previously (25). Confluent serum-starved mouse myofibroblasts were starved for 24 h in the presence of 0.02% BSA, and further stimulated for 24 h with 20 ng/ml PDGF-BB and 0.02% BSA. S1P (0 to 10 μM) was added to cells 30 min before PDGF-BB incubations. [^{3}H]-Thymidine (0.5 μCi/well) was added during the last 20 h of incubation. Cell proliferation was assessed as described previously (27) in 60 mm plates. Cells (200 000 cells/plate) were allowed to attach overnight in DMEM medium containing 10% FCS, washed in serum-free DMEM, and further incubated with or without 20 ng/ml PDGF-BB in the presence of 5 μM S1P or vehicle. After 3 days, cells were trypsinized and counted with a hemocytometer.

Sphingosine kinase assay

Sphingosine kinase activity was determined essentially as described in ref. 28, with minor modifications. Confluent hepatic myofibroblasts were made quiescent in serum-free medium for 24 h. Cells were further incubated for the indicated periods with the effectors, washed twice in ice-cold PBS, harvested, and cell pellets were frozen at −80°C. Cells were lysed in 500 μl of sphingosine kinase buffer (20 mM Tris buffer pH 7.4 containing 20% glycerol, 1 mM β-mercaptoethanol, 1 mM EDTA, 1 mM sodium orthovanadate, 15 mM NaF, 10 μg/ml leupeptin and aprotinin, 1 mM PMSF, 0.5 mM 4-deoxypyridoxine, and 40 mM β-glycerophosphate) and the lysates were centrifuged for 1 h at 100,000 *g* at 4°C. Lysates from liver samples were prepared under the same conditions. The kinase assay was performed by mixing protein extracts (50–100 μg) with 10 μl of 1 mM sphingosine (dissolved in 5% Triton X-100) and 10 μl of [γ^{32}P]ATP (10 μCi, 20 mM) containing 200 mM $MgCl_2$. After incubation for 30 min at 37°C, the reaction was stopped by addition of 20 μl of 1 N HCl and the [γ^{32}P]ATP-labeled S1P was extracted, isolated by thin-layer chromatography, and quantified after scraping by liquid scintillation.

Statistics

Results are expressed as mean ± SE of n experiments. Results were analyzed by Mann Whitney test or 1-way analysis of variance (ANOVA), followed by Bonferroni's post test, when appropriate. Statistical analysis of the quantitative RT-PCR data was performed using the REST© program (29). $P < 0.05$ was taken as the minimum level of significance.

RESULTS

Acute liver injury promotes activation of the hepatic S1P system

We first investigated whether acute CCl_4 injury is associated with up-regulation of the hepatic S1P

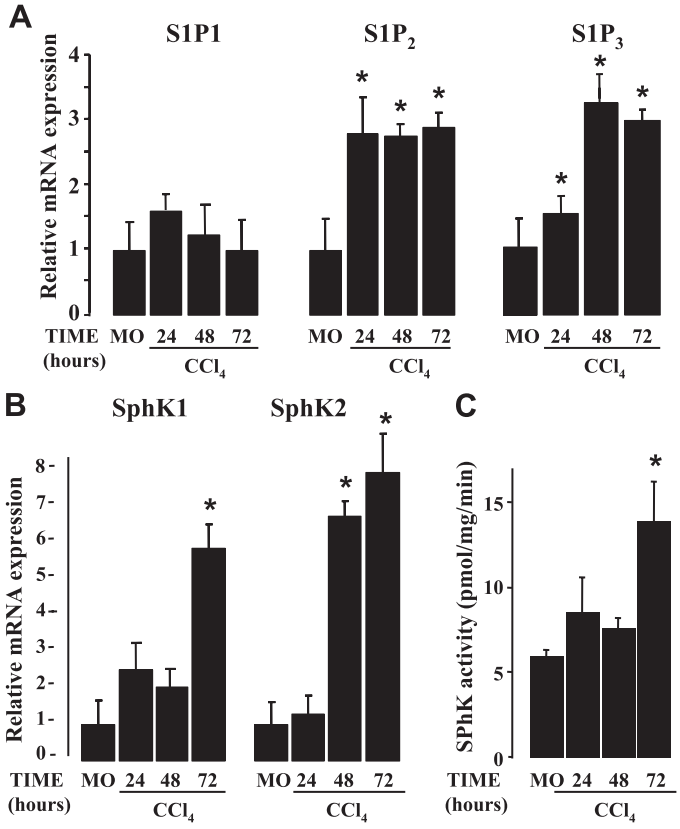

Figure 1. Expression of S1P receptors and sphingosine kinase after acute hepatic injury. Mice injected with a single dose of CCl_4 or mineral oil (MO) were sacrificed after 24 h [CCl_4-treated mice (n=3) and MO-treated mice (n=3)], 48 h [CCl_4-treated mice (n=3) and MO-treated mice (n=3)], or 72 h [CCl_4-treated mice (n=8) and MO-treated mice (n=3)]. Relative hepatic levels of *A)* $S1P_1$, $S1P_2$, $S1P_3$ mRNAs, and *B)* SphK1 and SphK2 mRNAs were measured by quantitative RT-PCR, as described in Materials and Methods. *C)* Sphingosine kinase activity was assayed on liver extracts prepared as described in Materials and Methods. *$P < 0.05$ *vs.* MO-treated mice.

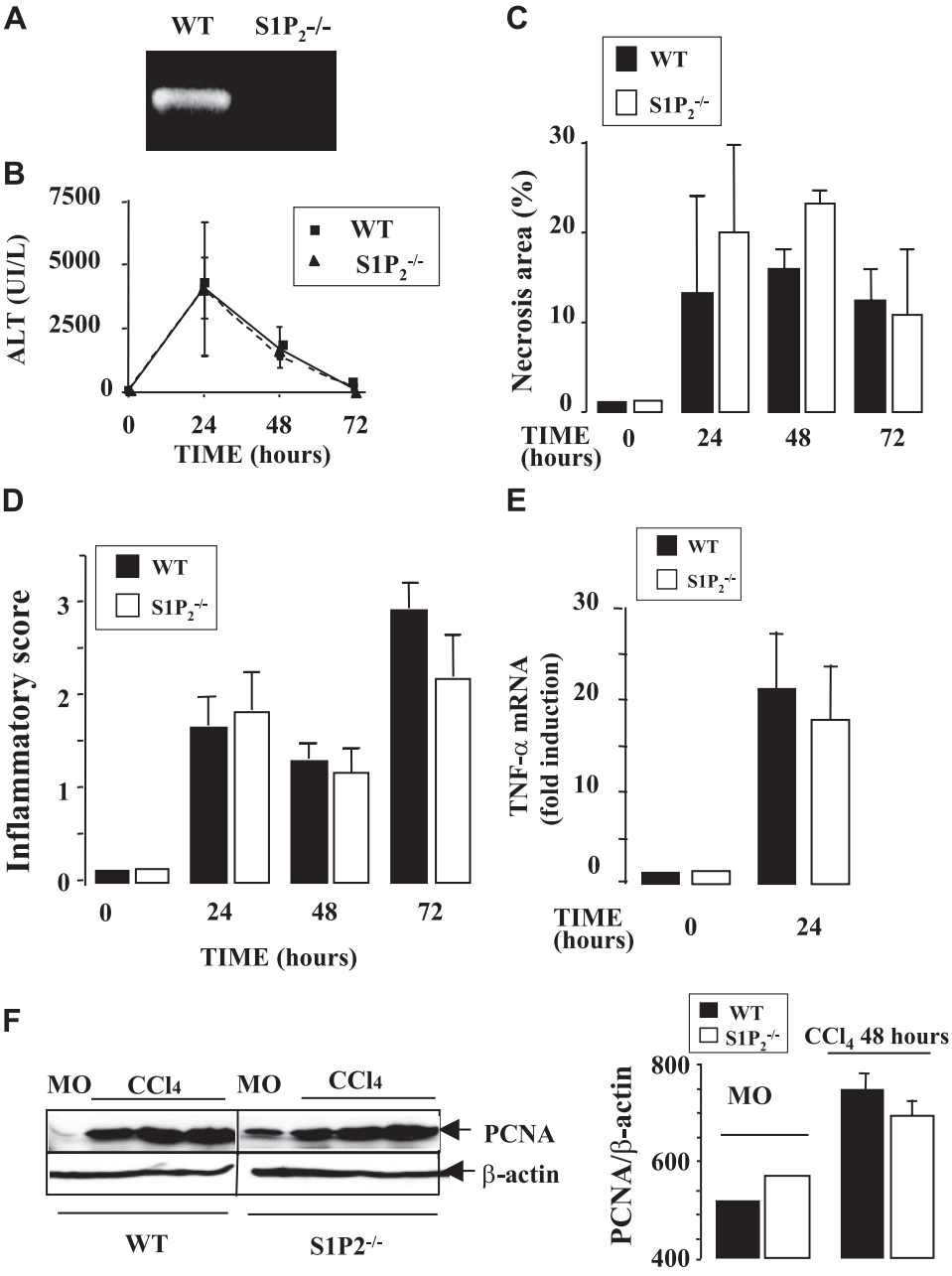

Figure 2. Genetic inactivation of $S1P_2$ does not affect liver injury and hepatocyte proliferation in response to carbon tetrachloride. *A)* $S1P_2$ is not expressed in $S1P_2^{-/-}$ mice. A band of 271 bp corresponding to the expected size of the $S1P_2$ receptor PCR product was identified in the liver of WT mice but not in the liver of $S1P_2^{-/-}$ mice. *B, C)* Similar liver injury in WT and $S1P2^{-/-}$ mice after CCl_4 treatment. *B)* Alanine aminotransferase (ALT) and *C)* histological estimation of hepatic necrosis area were assessed 24 h (n=3 WT, n=3 $S1P_2^{-/-}$), 48 h (n=6 WT, n=3 $S1P_2^{-/-}$), and 72 h (n=8 WT, n=5 $S1P_2^{-/-}$) after CCl_4 or MO treatment, as described in Materials and Methods. $P < 0.05$ for WT and $S1P_2^{-/-}$ *vs.* MO-treated mice; n.s for WT *vs.* $S1P_2^{-/-}$ CCl_4-treated mice. *D, E)* Similar inflammation in WT and $S1P_2^{-/-}$ mice after CCl_4 treatment. *D)* Histological grading of liver inflammation and *E)* hepatic TNF-α mRNA were assessed as described in Materials and Methods. $P < 0.05$ *vs.* MO-treated mice; n.s for WT *vs.* $S1P_2^{-/-}$ CCl_4-treated mice. *F)* WT and $S1P_2^{-/-}$ mice show similar hepatic regeneration after CCl_4 treatment. Western blot analysis of PCNA in liver extracts from WT and $S1P_2^{-/-}$ mice 48 h after injection of CCl_4 or MO (left panel). Results were normalized to β-actin expression (right panel). $P < 0.05$ *vs.* MO-treated mice; n.s for WT *vs.* $S1P_2^{-/-}$ CCl_4-treated mice.

system. This liver injury model is characterized by development of acute hepatitis after 24 h, followed by a wound healing response that associates both hepatocyte proliferation after 48 h and matrix remodeling triggered by hepatic myofibroblasts expressing α-SMA after 72 h (30). $S1P_1$ receptor mRNA expression was not modified by CCl_4-induced acute liver injury. In contrast, hepatic $S1P_2$ and $S1P_3$ receptor mRNAs were markedly induced from 24 h through 72 h (**Fig. 1*A***). In addition, there was a strong hepatic induction of mRNAs encoding SphK1 and SphK2 (Fig. 1*B*), the rate-limiting enzymes for S1P biosynthesis, and an increase in SphK enzyme activity (Fig. 1*C*). This coordinated up-regulation of the hepatic S1P system strongly suggested a role for S1P in the wound healing response to acute liver injury.

Reduced hepatic wound healing response in $S1P_2^{-/-}$ but not in $S1P_3^{-/-}$ mice

We next assessed the impact of $S1P_2$ receptors on liver injury and wound healing response owing to the use of $S1P_2^{-/-}$ animals. As expected, $S1P_2^{-/-}$ mice did not express $S1P_2$ mRNA (**Fig. 2*A***). After acute CCl_4 injury, WT and $S1P_2^{-/-}$ mice showed no differences in either the kinetics of transaminase (alanine aminotransferase, ALT) elevation (Fig. 2*B*), histological staging of hepatic necrosis and inflammation (Fig. 2*C, D*), or hepatic induction of TNF-α, a marker of inflammation (Fig. 2*E*). We also assessed hepatocyte proliferation by proliferating cell nuclear antigen (PCNA) expression. PCNA expression was maximally induced 48 h after CCl_4 administration in hepatocytes of WT mice, as demonstrated by immunohistochemistry (not shown).

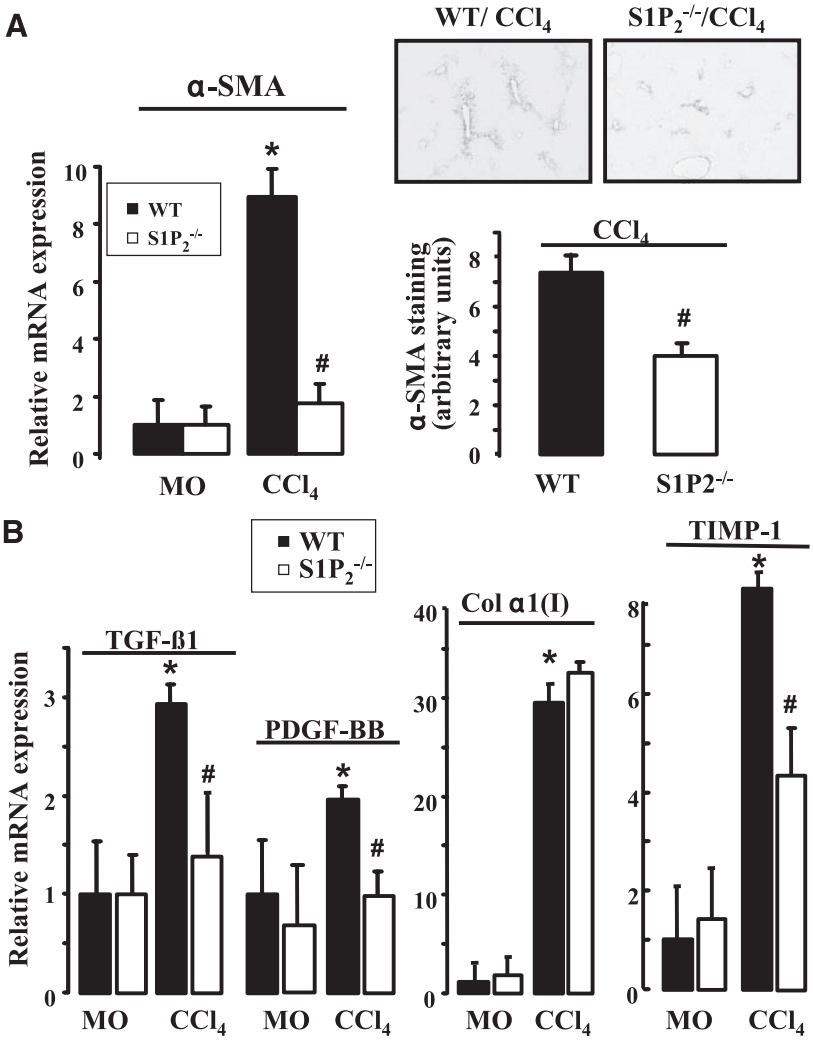

Figure 3. Reduced wound healing in $S1P_2^{-/-}$ mice. WT or $S1P_2^{-/-}$ mice injected with a single dose of CCl_4 or mineral oil were sacrificed 72 h after injection [CCl_4-treated WT mice (n=8) and MO-treated WT mice (n=5); CCl_4-treated $S1P_2^{-/-}$ mice (n=5) and MO-treated $S1P_2^{-/-}$ mice (n=3)]. *A)* Hepatic expression of α-SMA in WT and $S1P_2^{-/-}$ mice during wound healing. Expression of α-SMA mRNA was determined by quantitative RT-PCR, as described in Materials and Methods. $^*P < 0.05$ *vs.* MO-treated mice; $^{\#}P < 0.05$ *vs.* CCl_4-treated WT mice. α-SMA protein was detected by immunohistochemical staining, and representative hepatic staining of α-SMA from CCl_4-treated WT and $S1P_2^{-/-}$ mice is shown. Quantification was performed on 4–5 liver tissue sections per animal as described in Materials and Methods. $^{\#}P < 0.05$ *vs.* CCl_4-treated WT mice. *B)* Hepatic expression of TGFβ-1, PDGF-BB, procollagen α1(I) (Col α1(I)), and TIMP-1 mRNA expression during wound healing. Expression of mRNAs was determined by quantitative RT-PCR. $^*P < 0.05$ *vs.* MO-treated mice. $^{\#}P < 0.05$ *vs.* CCl_4-treated WT mice.

By Western blot analysis, PCNA expression was similarly enhanced in $S1P_2^{-/-}$ and WT mice, indicating that $S1P_2$ does not affect hepatic regeneration after acute liver injury (Fig. 2*F*).

The matrix remodeling process was subsequently evaluated by several parameters. As expected, CCl_4 administration triggered an increase in hepatic myofibroblast accumulation in WT mice after 72 h, as shown by an 8.4-fold up-regulation of α-SMA mRNA and a 7.4 increase in α-SMA immunostaining (**Fig. 3*A***). Accordingly, accumulation of hepatic myofibroblasts was associated with a strong induction of hepatic TGF-β1, PDGF-BB, procollagen α1(I), and TIMP-1 mRNAs (Fig. 3*B*). In contrast, CCl_4-treated $S1P_2^{-/-}$ animals showed a significantly lower induction of α-SMA mRNA expression and α-SMA immunostaining, indicating that $S1P_2$ inactivation decreases hepatic myofibroblast accumulation after acute liver injury (Fig. 3*A*). Inductions of TGF-β1, PDGF-BB, and TIMP-1 were also significantly reduced in $S1P_2^{-/-}$ animals compared with WT counterparts (Fig. 3*B*), whereas there were no differences in α1(I) (Fig. 3*B*) and α1(III) (not shown) procollagen mRNA inductions between groups, suggesting that $S1P_2$ decreases hepatic matrix remodeling by shutting down matrix degradation rather than stimulating matrix synthesis.

We also investigated the function of $S1P_3$ receptors owing to the use of $S1P_3^{-/-}$ animals. As expected, $S1P_3$ mRNA was undetectable in the liver of $S1P_3^{-/-}$ mice (**Fig. 4*A***). The extent and time course of transaminase elevation were similar in WT and $S1P_3^{-/-}$ animals, peaking after 24 h (not shown), while the severity of hepatitis at 24 h was similar in both groups of animals, as assessed by histological staging of necrosis and inflammation (not shown). Finally, there were no significant differences in the wound healing response in $S1P_3^{-/-}$ and WT animals, as shown by similar inductions of α-SMA and TGF-β1 mRNAs 72 h after CCl_4 treatment (Fig. 4*B*).

Cultured $S1P2^{-/-}$ hepatic myofibroblasts show reduced mitogenic properties

Further experiments were conducted to characterize the mechanisms whereby genetic invalidation of $S1P_2$ reduces accumulation of hepatic myofibroblasts. We focused on the regulation of their proliferation, using cultured hepatic myofibroblasts isolated from WT and

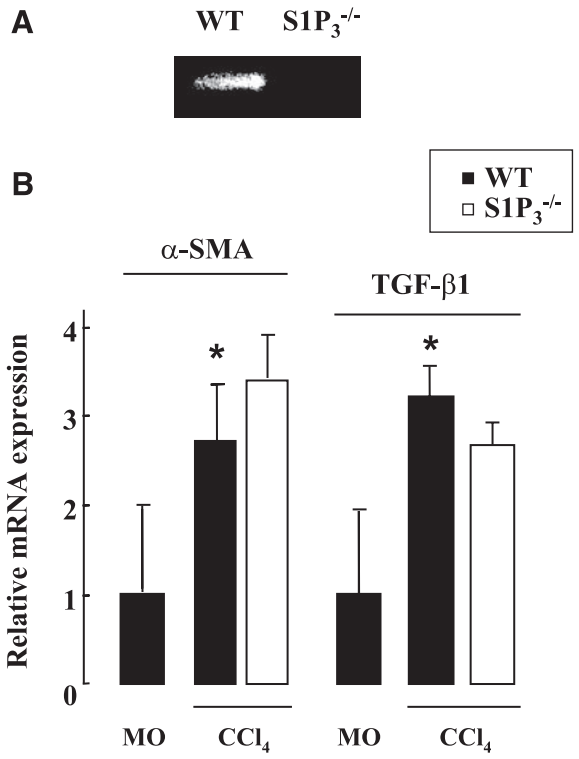

Figure 4. $S1P_3^{-/-}$ and WT mice elicit similar responses to acute liver injury. *A)* $S1P_3$ is expressed in WT mice but not in $S1P_3^{-/-}$ mice. A band of 305 bp corresponding to the expected size of the $S1P_3$ receptor PCR product was identified in the liver of WT mice, but not in the liver of $S1P_3^{-/-}$ mice. *B)* Expression of hepatic α-SMA and TGF-β1 in WT and $S1P_3^{-/-}$ after acute liver injury. Mice injected with a single dose of CCl_4 or mineral oil were sacrificed 72 h after injection [CCl_4-treated mice (n=5) and MO-treated mice (n=3)]. Expression of mRNAs was determined by quantitative RT-PCR, as described in Materials and Methods. *$P < 0.05$ *vs.* MO-treated mice; n.s. for WT *vs.* $S1P_3^{-/-}$ CCl_4-treated mice.

$S1P_2^{-/-}$ mice. As expected, $S1P_2$ mRNA was undetectable in cells isolated from $S1P_2^{-/-}$ livers, in contrast to hepatic myofibroblasts obtained from WT livers (**Fig. 5*A***). S1P stimulated DNA synthesis and cell proliferation of hepatic myofibroblasts from WT mice, but did not affect proliferation of $S1P_2^{-/-}$ cells (Fig. 5*B*). Since PDGF-BB expression was down-regulated in the liver of CCl_4-treated $S1P_2^{-/-}$ animals (see Fig. 3*B*), we also examined the effects of this potent mitogen for hepatic myofibroblasts (30). In keeping with *in vivo* data, PDGF-BB mRNA expression was lowered in $S1P_2^{-/-}$ cells compared with WT cells (Fig. 5*C*). In WT cells, PDGF-BB strongly up-regulated the S1P system, eliciting a 3.8-fold and 2.7-fold increase in S1P2 mRNA and SphK1 mRNA expression without affecting SphK2 mRNA expression (Fig. 5*C*). Induction of SphK1 mRNA by PDGF-BB was associated with an increase in SphK activity (Fig. 5*C*, inset). In contrast, $S1P_2^{-/-}$ hepatic myofibroblasts exhibited a marked down-regulation of basal and PDGF-BB-stimulated SphK1 mRNA levels whereas SphK2 was unaffected (Fig. 5*C*). Accordingly, activation of SphK activity by PDGF-BB was blunted in $S1P_2^{-/-}$ cells; however, there was no significant change in total (*i.e.*, contributed by SphK1 and 2) basal SphK activity of $S1P_2^{-/-}$ cells compared with WT counterparts (Fig. 5*C*, inset). Functional studies showed that in addition to its mitogenic properties (Fig. 5*D, E*), PDGF-BB potentiated the mitogenic effect of S1P in WT cells (Fig. 5*D, E*). Hepatic myofibroblasts isolated from $S1P_2^{-/-}$ mice displayed enhanced proliferation in response to PDGF-BB alone (Fig. 5*D, E*), but its comitogenic effect with S1P was blunted (Fig. 5*D, E*). Reduction in SphK1 and PDGF-BB expression was associated with a decrease in basal and PDGF-BB stimulated DNA synthesis in $S1P_2^{-/-}$ hepatic myofibroblasts compared with WT cells (Fig. 5*B, D*).

DISCUSSION

The present study unravels a major role of $S1P_2$ in the wound healing response to acute liver injury. Indeed, we show herein that $S1P_2^{-/-}$ mice display decreased activation of hepatic matrix remodeling by a mechanism involving reduced proliferation of hepatic myofibroblasts.

It is well known that acute liver injury triggers a wound healing process that is associated with hepatic regeneration after the proliferation of hepatocytes and matrix remodeling. The matrix remodeling process results from both the hepatic induction of fibrogenic cytokines such as TGF-β1 and PDGF-BB, and the proliferation of hepatic myofibroblasts that overexpress matrix genes and inhibitors of matrix metalloproteinases (TIMPs) (30). Here we show a strong up-regulation of the S1P system during hepatic wound healing, as reflected by a rise in $S1P_2$, $S1P_3$, SphK1, and SphK2 mRNAs after acute administration of CCl_4. We also demonstrate that $S1P_2$ is a key mediator of the wound healing process that triggers matrix remodeling without affecting hepatocyte regeneration. Indeed, WT and $S1P_2^{-/-}$ mice display similar regenerative capacities, in contrast to a previous report showing that S1P inhibits both proliferation of cultured rat hepatocytes and liver regeneration after partial hepatectomy (18). The authors suggested that the growth inhibitory effect of S1P in hepatocytes relies on $S1P_2$ based on the use of a selective $S1P_2$ agonist *in vitro*; however, the study did not include experiments demonstrating $S1P_2$ receptor dependency on the growth inhibitory effect of S1P *in vivo*. In contrast, we unravel a major selective role of $S1P_2$ in matrix remodeling after acute liver injury. Thus, $S1P_2^{-/-}$ mice show reduced accumulation of hepatic myofibroblasts and decreased hepatic induction of TGF-β1, PDGF-BB, and TIMP-1, all of which are synthesized, albeit not exclusively, by hepatic myofibroblasts (30). In contrast, $S1P_3^{-/-}$ mice show similar induction of matrix remodeling in response to acute liver injury. Culture experiments of hepatic myofibroblasts shed light on the mechanisms mediating the regulatory effects of $S1P_2$ on hepatic myofibroblast accumulation. Thus, we demonstrate that WT myofibroblasts display enhanced proliferation in response to

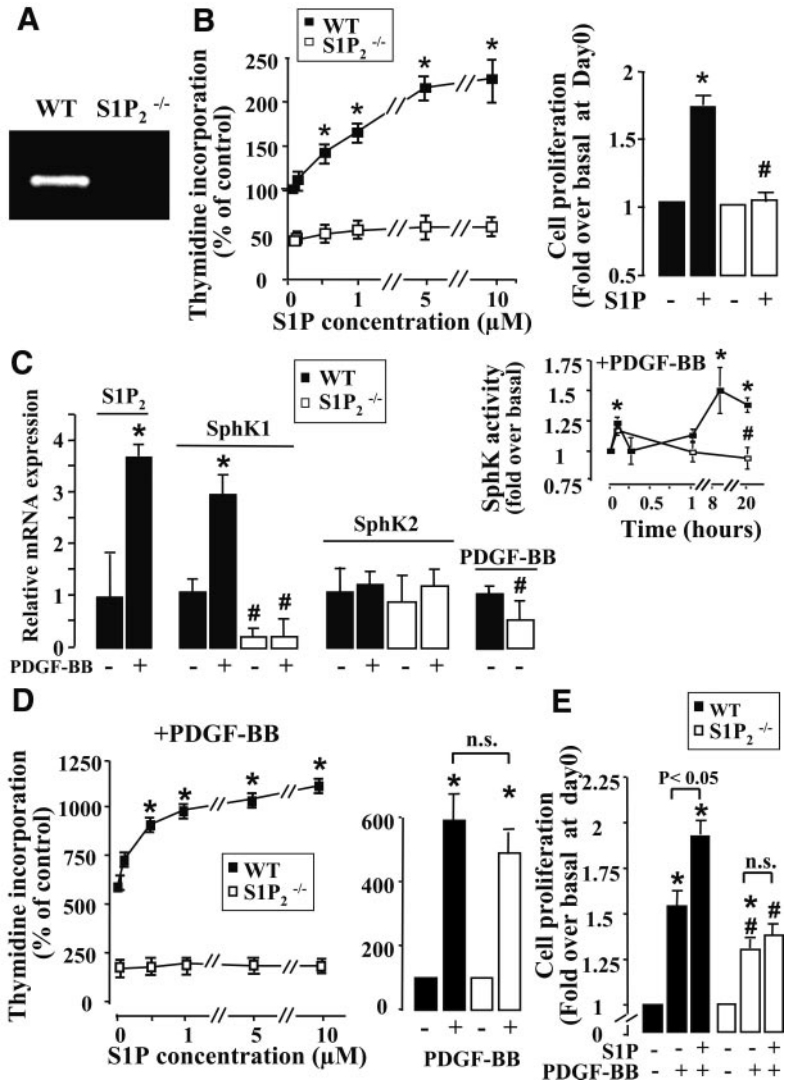

Figure 5. S1P stimulates DNA synthesis in mice hepatic myofibroblasts *via* an $S1P_2$-dependent pathway. *A)* $S1P_2$ is expressed in WT but not in $S1P2^{-/-}$ hepatic myofibroblasts. A band of 271 bp corresponding to the expected size of the $S1P_2$ receptor PCR product was identified in WT but not in $S1P_2{}^{-/-}$ hepatic myofibroblasts. *B)* Effects of S1P on DNA synthesis and cell proliferation in WT and $S1P_2{}^{-/-}$ mice. Left panel: DNA synthesis was measured after stimulation of cells for 30 h with varying concentrations of S1P (see Materials and Methods). Results (mean ± SE, $n=10$) are expressed as percent of control, and were obtained from three WT and three $S1P_2{}^{-/-}$ cell preparations. $P < 0.05$ for S1P *vs.* control in WT myofibroblasts; *significant effects of S1P were obtained for concentrations of > 0.1 μM. $P < 0.05$ for $S1P2^{-/-}$ *vs.* WT myofibroblasts. Right panel: effects of S1P on proliferation of WT and $S1P_2{}^{-/-}$ hepatic myofibroblasts. Cells were incubated over a period of 3 days with 5 μM S1P or vehicle. Cell number was determined at day 0 and day 3. Results are expressed as fold increase in cell number at day 0; $*P < 0.05$ for S1P *vs.* control in WT cells. $\#P < 0.05$ *vs.* hepatic myofibroblasts. *C)* Regulation of the S1P system and PDGF-BB expression in WT and $S1P_2{}^{-/-}$ hepatic myofibroblasts. WT and $S1P_2{}^{-/-}$ hepatic myofibroblasts were treated for 8 h with 20 ng/ml of PDGF-BB or vehicle as indicated, and $S1P_2$, SphK1, SphK2, and PDGF-BB mRNA expressions were measured by quantitative RT-PCR analysis. Data are the mean ± SE of three determinations obtained in three WT and three $S1P2^{-/-}$ cell preparations. $*P < 0.05$ *vs.* untreated cells. $^{\#}P < 0.05$ for $S1P_2{}^{-/-}$ *vs.* WT myofibroblasts. Inset: time course of the effect of PDGF-BB on SphK activity. WT and $S1P_2{}^{-/-}$ hepatic myofibroblasts were incubated for various periods with 20 ng/ml PDGF-BB. Sphingosine kinase activity was measured in cell lysates, as described in Materials and Methods. $*P < 0.05$ *vs.* untreated cells. $^{\#}P < 0.05$ for $S1P_2{}^{-/-}$ *vs.* WT myofibroblasts. Data are the mean ± SE of six determinations obtained in two WT and two $S1P2^{-/-}$ cell preparations. *D)* Effects of S1P on PDGF-BB-stimulated DNA synthesis in WT and $S1P_2{}^{-/-}$ mice. Cells were stimulated for 30 h with varying concentrations of S1P with 20 ng/ml of PDGF-BB. Results (mean ± SE, $n=10$) are expressed as percent of control and were obtained from three WT and three $S1P_2{}^{-/-}$ cell preparations. $*P < 0.05$ for S1P *vs.* control in WT myofibroblasts; significant effects of S1P were obtained for concentrations of > 0.1 μM. $P < 0.05$ for $S1P2^{-/-}$ *vs.* WT myofibroblasts. Inset: effect of PDGF-BB on DNA synthesis in WT and $S1P_2{}^{-/-}$ hepatic myofibroblasts. Results are expressed as percent above respective control levels in WT and $S1P_2{}^{-/-}$ cells. $P < 0.05$ for PDGF-BB *vs.* basal in WT and $S1P2^{-/-}$ cells. *E)* Effects of S1P on PDGF-BB-stimulated proliferation WT and $S1P_2{}^{-/-}$ hepatic myofibroblasts. Cells were incubated over a period of 3 days with 5 μM S1P or vehicle. Cell number was determined on day 0 and day 3. Results are expressed as fold increase in cell number on day 0; $*P < 0.05$ for PDGF-BB *vs.* control in WT and $S1P2^{-/-}$ cells. $^{\#}P < 0.05$ *vs.* WT.

S1P, whereas the mitogenic effect is blunted in $S1P_2{}^{-/-}$ cells. Taken together, these data demonstrate that S1P promotes proliferation of hepatic myofibroblasts *via* $S1P_2$ *in vivo* and *in vitro*. It should be noted that two recent studies showed opposite $S1P_2$-dependent growth inhibitory effects of S1P *in vitro* in cultured myoblasts and embryonic fibroblasts (14, 31). Therefore, these data suggest that the overall effect of $S1P_2$ on cell proliferation may result from the balance between simultaneous positive and negative signals within a given cell.

As SphK1 has been shown to promote cell proliferation and survival (1, 32), we investigated whether Sphk1 may also participate in the $S1P_2$-dependent mitogenic response of hepatic myofibroblasts found *in vivo*. We found that SphK1 mRNA is down-regulated in $S1P_2{}^{-/-}$ hepatic myofibroblasts whereas SphK2 mRNA, an isoform with growth inhibitory and apoptotic properties (33, 34), is unchanged. These results suggest the presence of a regulatory loop whereby $S1P_2$ up-regulates SphK1, the latter in turn leading to constitutive activation of $S1P_2$ by endogenous production of S1P (**Fig. 6**). The mechanism by which intracellularly generated S1P may be released from hepatic myofibroblasts remains to be elucidated, but could involve ABCC1, a protein recently identified as an S1P exporter (35). Finally, as shown in dermal fibroblasts (19, 36), SphK1 could also contribute to the mitogenic effect of $S1P_2$ by up-

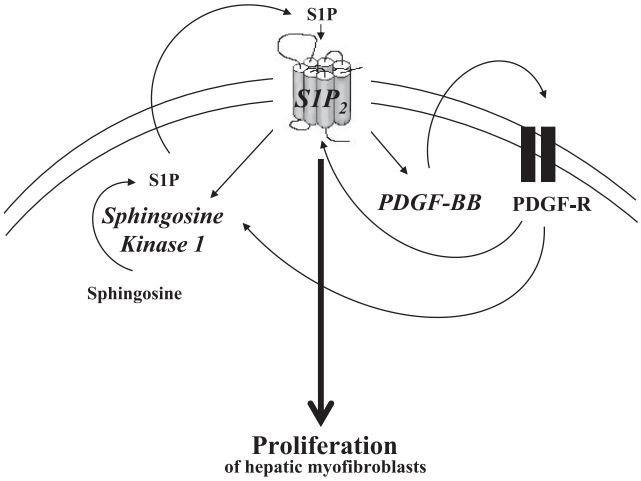

Figure 6. Proposed mechanism for $S1P_2$-mediated mitogenic effects of S1P in hepatic myofibroblasts. Two regulatory loops contribute to the mitogenic effect of $S1P_2$. On the one hand, $S1P_2$ up-regulates SphK1, the latter in turn leading to constitutive activation of $S1P_2$ by endogenous production of S1P. On the other hand, $S1P_2$ up-regulates PDGF-BB and PDGF-BB up-regulates $S1P_2$ as well as Sphk1, thereby enhancing the mitogenic effect of S1P.

regulating TIMP-1, an inhibitor of metalloproteinase with mitogenic effects in hepatic myofibroblasts (19, 36) that shows reduced expression in $S1P_2^{-/-}$ mice. However, this hypothesis is unlikely, since TIMP-1 mRNA levels were similar in $S1P_2^{-/-}$ and WT hepatic myofibroblasts (not shown). Therefore, down-regulation of TIMP-1 in $S1P_2^{-/-}$ mice after acute liver injury probably reflects the reduced number of hepatic myofibroblasts rather than direct regulation of TIMP-1 expression *via* $S1P_2$/SphK1.

In addition to its mitogenic effect, $S1P_2$ also triggers an autocrine positive feedback loop responsible for a comitogenic effect of S1P and PDGF-BB. Indeed, $S1P_2$ up-regulates PDGF-BB and PDGF-BB up-regulates $S1P_2$ as well as Sphk1, in keeping with previous studies (14), thereby enhancing the mitogenic effect of S1P (Fig. 6). Cross-talk between PDGF receptors and $S1P_2$ could account for this comitogenic effect, as reported in several instances for tyrosine kinase and G-protein-coupled receptors, such as S1P receptors (14, 37).

In summary, acute liver injury is associated with a marked hepatic induction of $S1P_2$ and $S1P_3$ and with a parallel up-regulation of SphK1 and SphK2 and of sphingosine kinase activity, strongly suggesting an increase in S1P production. Our data demonstrate that $S1P_2$ up-regulates SphK1 expression and mediates the mitogenic effect of S1P in hepatic myofibroblasts. They also reveal a multistep positive feedback loop initiated by an $S1P_2$-dependent increase in SphK1 and PDGF-BB expression, followed by a PDGF-BB-dependent up-regulation of $S1P_2$ expression, ultimately leading to an enhanced mitogenic effect of S1P (Fig. 6). Our study also unravels a novel specific role of $S1P_2$ in matrix remodeling during acute hepatic wound healing and illustrates the emerging concept of specific functions triggered by distinct S1P receptors. A recent study pointed out another $S1P_2$-dependent specific liver function of S1P, namely, regulation of intrahepatic resistance (20). Altogether, these results invite investigation into whether $S1P_2$ may be a mediator of liver fibrogenesis and portal hypertension during chronic liver injury. FJ

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