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Sphingosine-1-Phosphate Stimulates the Functional Capacity of Progenitor Cells by Activation of the $CXCR_4$-Dependent Signaling Pathway via the $S1P_3$ Receptor

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Objective—Sphingosine-1-phosphate (S1P) is a bioactive lipid, which influences migration and proliferation of endothelial cells through activation of S1P receptors and has been shown to support SDF-1 induced migration and bone marrow homing of $CD34^+$ progenitors.

Methods and Results—Here, we show that incubation of patient-derived endothelial progenitor cells (EPCs) with S1P or its synthetic analog FTY720 improved blood flow recovery in ischemic hind limbs. Likewise, recovery of blood flow was dramatically reduced after induction of hindlimb ischemia in mice deficient for the S1P receptor 3 ($S1P_3$). $S1P_3^{-/-}$ bone marrow–derived mononuclear cells (BMCs) failed to augment neovascularization after hind limb ischemia. Of note, treatment of BMCs derived from $S1P_3^{-/-}$ mice with S1P did not rescue blood flow recovery. Mechanistically, S1P and FTY720 induced phosphorylation of $CXCR_4$, activated the Src kinase, and stimulated phosphorylation of JAK2. The contribution of $CXCR_4$ for S1P-mediated effects was further supported by the findings that S1P preincubation failed to stimulate invasion capacity and in vivo blood flow recovery of BMCs from $CXCR_4^{+/-}$ mice. The activation of $CXCR_4$ was dependent on the Src kinase family as demonstrated by preincubation with the Src inhibitor PP2. The activation of the $CXCR_4$ signaling by S1P is mediated via the $S1P_3$ receptor, since S1P-induced Src phosphorylation was abrogated in EPC from $S1P_3^{-/-}$ mice.

Conclusions—S1P agonists might serve as sensitizers of $CXCR_4$-mediated signaling and may be applied in clinical progenitor cell therapy to improve EPC or BMC function in patients with coronary artery disease. (***Arterioscler Thromb Vasc Biol*. 2007;27:275-282.**)

Key Words: S1P ■ receptor cross-talk ■ progenitor cells

Sphingosine-1-phosphate (S1P) is a bioactive lipid, which influences proliferation, differentiation, migration, and survival of endothelial cells, but also of smooth muscle cells or bone marrow cells through activation of the G protein–coupled S1P receptors.[1–6] In addition, S1P has been shown to play a critical role in vascular development, determine vessel maturation,[7,8] and promote HDL-induced vascular reactivity.[9] Migratory responses or angiogenic activities of endothelial cells have been shown to be promoted via the receptors, $S1P_1$ or $S1P_3$.[1,10–12] A synthetic analog, FTY720, originally developed as an immunosuppressive agent for kidney transplantation, activates 4 of the 5 S1P receptors[13] and induces lymphocyte migration and homing in secondary lymphatic organs by regulating egress from lymph nodes.[14,15] FTY720 was recently shown to support SDF-1–induced migration and bone marrow homing of $CD34^+$ progenitors.[16]

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Transplantation of culture-expanded progenitor cells or selected bone marrow mononuclear cells successfully promotes therapeutic neovascularization in both ischemic hind limbs as well as acute myocardial infarction models.[17–21] Mechanistically, these cells can either induce angiogenesis by incorporation into vascular structures depicting phenotypes of endothelial cells or may induce angiogenesis by production of growth factors acting in a paracrine manner (for review see[22]).

Moreover, recent clinical studies suggest that restoration of blood flow in peripheral artery disease and recovery of left ventricular function can be enhanced after autologous transplantation of bone marrow–derived cells or cultured EPCs in patients with coronary artery disease.[23–26] However, EPCs or

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BMCs derived from patients are functionally impaired[27–29] compared with EPCs from healthy donors. Recent data indicate that the therapeutic success is determined by functional properties of transplanted cells,[30,31] providing the basis for improvement of functional activities, eg, by pharmacological stimulation of surface receptors in order to enhance homing of progenitor or stem cells. One important family of surface receptors is the family of the S1P receptors. Seitz et al demonstrated that sustained activation of the S1P1 receptor by an agonist during the homing process resulted in increased engraftment in vivo.[32] Furthermore, S1P plays a crucial role in the cardiovascular system: it acts as a proangiogenic molecule,[12] improves endothelial function,[9] myocardial perfusion,[33] and is involved in cardiovascular development.[6,34] Additional studies demonstrate that S1P modulates homing and trafficking of cells.[14,35]

Therefore, we investigated whether S1P or the synthetic analog, FTY720, enhances the efficiency of transplanted EPCs or BMCs for therapeutic neovascularization. Furthermore, we attempted to elucidate the underlying mechanisms.

Materials and Methods

For detailed descriptions of the materials and methods, please see supplemental material (available online at http://atvb.ahajournals.org).

Study Population and Patient Characteristics

Peripheral blood mononuclear cells or bone marrow mononuclear cells were isolated from peripheral blood of healthy volunteers or patients with coronary artery disease as documented by angiographic evidence of coronary lesions. Patients with signs of acute myocardial ischemia documented by classical symptoms of chest pain, ECG alterations, elevation of creatine kinase or Troponin T were excluded. Further exclusion criteria were the presence of active or chronic infection, surgical procedures, stroke or trauma within the last three months, or evidence for malignant diseases (for details see supplementary Table I). The ethics review board of the Johann Wolfgang Goethe University of Frankfurt, Germany approved the protocol, and the study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each patient.

Pharmacological Agents: S1P and FTY720

S1P (Sigma) and a synthetic analog FTY720 (Novartis Institute for Biomedical Research, Basel, Switzerland, kindly provided by V.Brinkmann) were used.

Mice Strain

$CXCR_4^{+/-}$ mice (B6.129X-*Cxcr4^{tm1Qma}*/J) and C57Bl6/J background mice were purchased from Jackson Laboratories (Charles River, Germany). $S1P_3^{-/-}$ mice were kindly provided by J. Chun (La Jolla, Calif).[36]

Results

S1P Receptor Agonists Promote EPC-Mediated Neovascularization in Hindlimb Ischemia

Patient-derived EPCs and BMCs showed significantly impaired capacity for neovascularization in a mouse model of hind limb ischemia.[37,38] Because the S1P receptor family has been shown to play an important role in homing and trafficking of cells, we wanted to investigate whether S1P or FTY720 enhance neovascularization.[35] Therefore, we preincubated patient-derived EPCs for 2 hours with S1P or FTY720 before intravenous infusion in a mouse model of hindlimb ischemia. Preincubation of human EPCs with S1P and FTY720 followed by i.v. transplantation into the ischemic nude mice model resulted in significantly improved blood flow recovery in ischemic hind limbs as measured by Laser Doppler at 2 weeks after ligation of the femoral artery and almost completely restored blood flow recovery compared with EPCs from healthy volunteers (Figure 1a and 1b).

Impaired Angiogenesis in $S1P_3^{-/-}$ Mice

To investigate the role of $S1P_{1-4}$ receptors in blood flow recovery after cell transplantation into ischemic hind limbs, we first assessed the expression of S1P receptors on cultured human EPC by fluorescence-activated-cell sorter (FACS)-analysis. The predominantly expressed receptors in endothelial cells, $S1P_1$ and $S1P_3$, were also expressed on EPCs (Figure 2a). Therefore, we made use of mice deficient in the $S1P_3$ (knock down of the other major receptor, $S1P_1$, is embryonically lethal) and characterized the phenotype of isolated and cultivated EPCs derived from spleen of $S1P_3^{-/-}$ mice. EPC numbers as counted by Dil-acetylated LDL positive cells were lower in $S1P_3^{-/-}$ mice compared with wild-type mice. In addition, colony forming units were also reduced in EPC cultures derived from $S1P_3^{-/-}$ mice (data not shown). Furthermore, S1P-induced migration was abolished in EPC derived from $S1P_3^{-/-}$ mice (Figure 2b). Having demonstrated that EPCs derived from $S1P_3^{-/-}$ mice were functionally impaired, we investigated the recovery of blood flow in these mice after hindlimb ischemia. Indeed, recovery of blood flow was dramatically reduced after induction of hindlimb ischemia in $S1P_3^{-/-}$ mice, compared with ischemic wild-type litter mates (Figure 2c). Likewise, the capacity to restore blood flow recovery in the ischemic hindlimb model of nude mice was also significantly impaired using BMCs derived from $S1P_3^{-/-}$mice (Figure 2d). Of note, only treatment of wild-type BMCs with S1P enhanced blood flow recovery in nude mice after hindlimb ischemia (Figure 2d). Accordingly, capillary density was induced by S1P-stimulated BMC derived from wild-type mice (capillary/myocyte ratio increased with S1P to 122±13%, P=0.032, N=4). In contrast, capillary density in ischemic hindlimb muscles of nude mice was not different after transplantation of BMCs derived from $S1P_3^{-/-}$mice with or without S1P incubation (99±10% of capillary/myocyte ratio from $S1P_3^{-/-}$ without S1P).

Activation of the $CXCR_4$-Receptor and the Downstream Signaling Pathway by S1P

Next, we attempted to investigate the mechanisms underlying the effects of S1P or FTY720 on EPC function. S1P agonists have been shown to stimulate SDF-induced migration[16] and $CXCR_4$ importantly contribute to EPC migration and neovascularization.[38] Moreover, CXCR4-driven transendothelial migration of peripheral lymphnode T cells is stimulated by S1P and FTY720.[39] Thus, we postulated that S1P stimulates the activation of $CXCR_4$ and/or its downstream signaling. To assess whether stimulation of S1P receptors activates tyrosine phosphorylation of $CXCR_4$, we immunoprecipitated $CXCR_4$. Tyrosine phosphorylation of $CXCR_4$ by S1P agonists occurred rapidly reaching a 2-fold induction after 30 minutes

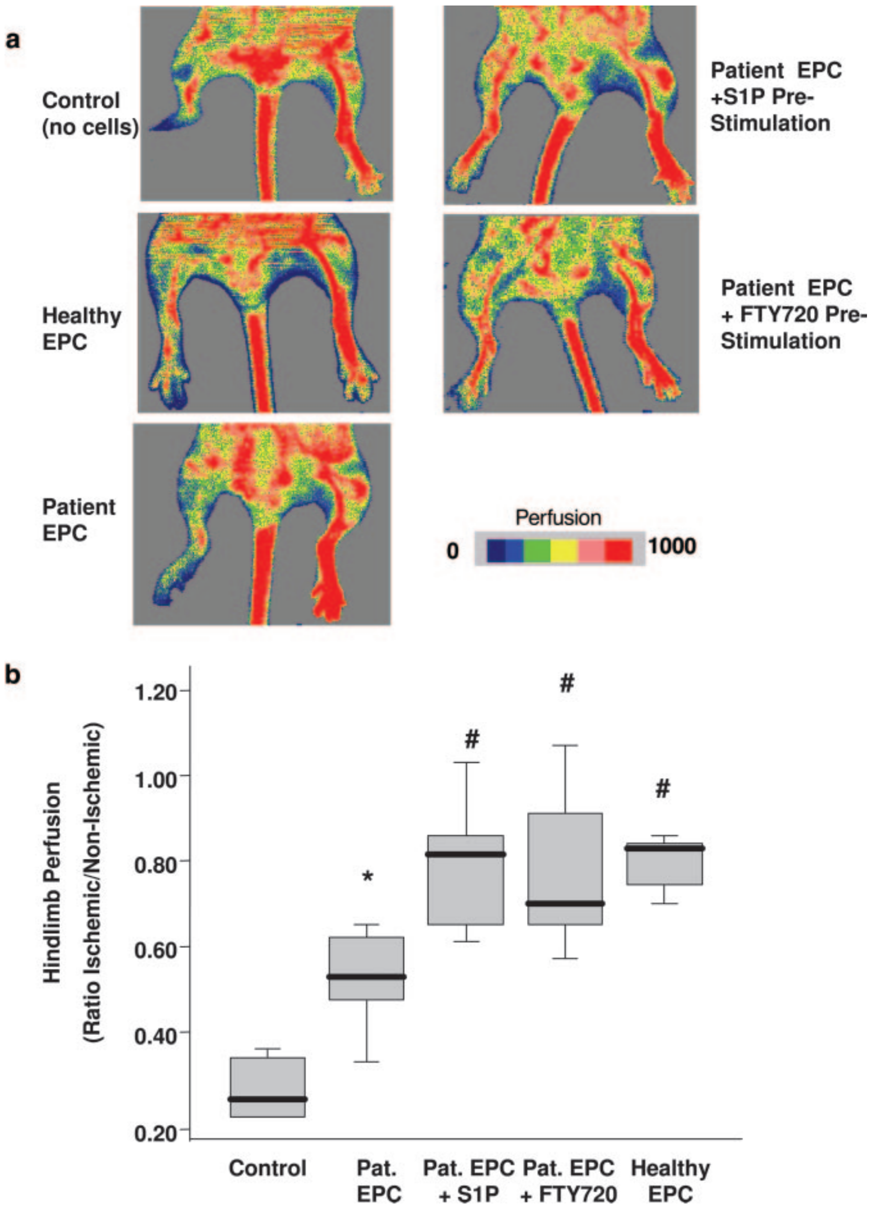

Figure 1. S1P and FTY720 promote in vivo neovascularization after EPC transplantation. a, Representative photographs from laser Doppler perfusion imaging at day 14 of ischemic (right) and nonischemic limbs (left) in nude mice after ligation of femoral artery in mice without EPC transplantation (control) and in mice receiving injections of 5×10^5 EPC pre-incubated with or without S1P (200 nM) or FTY720 (10 nM), N=6 to 7 per group. (b) Quantification of laser Doppler-derived blood flow at day 14. Hindlimb perfusion was significantly improved at day 14 in animals receiving EPC incubated with S1P or FTY720 compared with untreated EPC. *, $P<0.01$ versus control animals without cell transplantation; #, $P<0.01$ versus untreated EPC from patients.

incubation (1.96±0.57, $P<0.01$; Figure 3a). Because JAK2 is a known downstream target of the $CXCR_4$ receptor,[38] we investigated whether JAK2 phosphorylation was induced by S1P. Indeed, immunoblotting revealed that JAK2 phosphorylation was significantly increased after preincubation of EPCs with S1P or FTY720 (Figure 3b). Taken together, these data suggest that S1P and FTY720 induce $CXCR_4$-mediated signaling.

To further strengthen this hypothesis, we next investigated the effects of S1P in $CXCR_4^{+/-}$mice. We previously demonstrated that BMCs and EPCs from $CXCR_4^{+/-}$ mice showed impaired angiogenic activities.[38] In line with these findings, S1P-mediated JAK2 phosphorylation was significantly reduced in BMCs derived from heterozygous $CXCR_4^{+/-}$ mice (Figure 4a and 4b). Likewise, S1P incubation failed to stimulate the invasion capacity of BMCs of heterozygous $CXCR_4^{+/-}$ mice (Figure 4c). Finally, S1P preincubation of transplanted BMCs from $CXCR_4^{+/-}$ mice failed to improve in vivo blood flow recovery in the ischemic hindlimb model of nude mice (blood flow ratio 26±11% versus 30±18%), further supporting the concept that the activation of the $CXCR_4$ receptor signaling by S1P is required to improve the functional capacities of EPCs or BMCs. Similar findings were observed with FTY720 (data not shown).

Involvement of the Src Kinase Family in the Transactivation of $CXCR_4$ Signaling

Src-family tyrosine kinases have been implicated in the S1P-induced activation of several growth factor receptors like the VEGF-receptor 2 and the PDGF receptor.[40,41] Therefore, we investigated whether the Src kinase family is involved in S1P-stimulated activation of $CXCR_4$ receptor signaling in EPCs. To elucidate the mechanism by which S1P or FTY720 activate $CXCR_4$, we preincubated EPCs with the Src kinase family inhibitor PP2. Basal and S1P-induced JAK2 phosphorylation was reduced by PP2 in human EPCs (Figure 5a and 5b). Likewise, JAK2 phosphorylation was inhibited by coin-

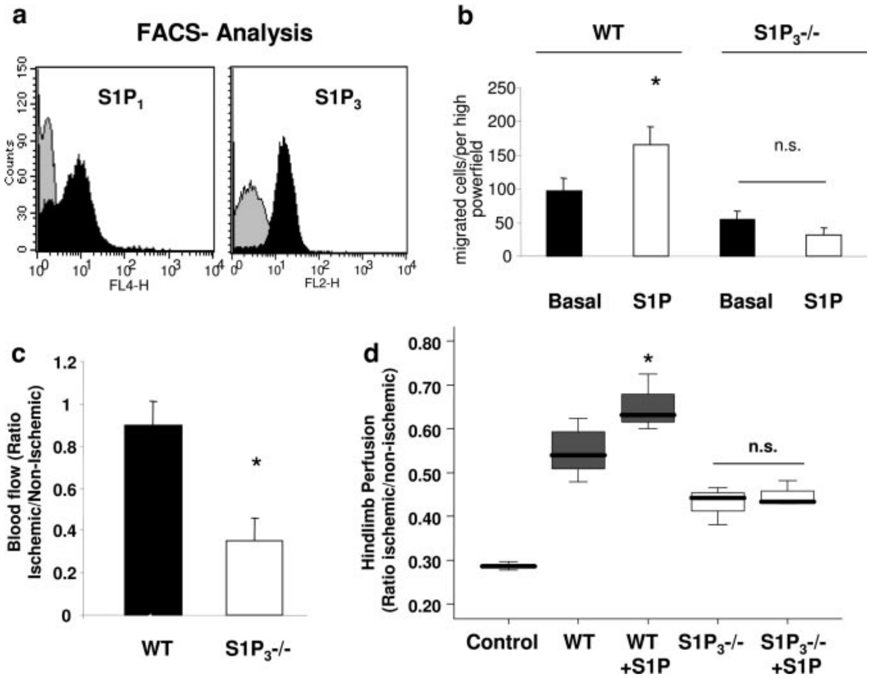

Figure 2. Impaired angiogenic capacities in murine EPCs derived from $S1P_3^{-/-}$ mice. a, Sphingosine 1 phosphate receptor expression levels of $S1P_1$ and $S1P_3$ in human EPCs from healthy volunteers (n>4 per group). b, Migration capacity toward SDF-1 of EPCs derived from spleen. S1P stimulates migratory capacity in wild-type mice, but failed to stimulate migration in EPCs derived from $S1P_3^{-/-}$ mice, n=3 to 4. c, Laser doppler perfusion at 14 days after induction of endogenous hindlimb ischemia in wild-type vs $S1P_3^{-/-}$ mice (n=6 to 7) indicating dramatically reduced recovery of blood flow in $S1P_3^{-/-}$mice, $P<0.001$. d, Infusion of BMCs derived from wild-type mice or $S1P_3^{-/-}$ mice into nude mice after induction of hindlimb ischemia. S1P incubation induced blood flow recovery (*$P<0.001$ vs control and wild-type cells and $S1P_3^{-/-}$ cell groups, n≥3).

cubation of PP2 with S1P in murine BMCs (Figure 5c and 5d). PP2 incubation also reduced S1P-or FTY720-induced invasion of BMC toward SDF-1 (Figure 5e).

S1P Induced Activation of Src Kinases Is Dependent on $S1P_3$

Next, we investigated whether S1P induced the activation of Src. As demonstrated in Figure 6a, concomitantly with the time-dependent phosphorylation of active Src at tyrosine 416 in human EPCs, phosphorylation of the inactive form of Src at tyrosine 527 was reduced after S1P incubation. Finally, we evaluated whether Src kinase activation is detectable in EPC from $S1P_3^{-/-}$ mice. Src phosphorylation at tyrosine 416 was induced by S1P in EPC derived from wildtype mice, whereas Src phosphorylation and activation were abrogated in EPC derived from $S1P_3^{-/-}$ mice (Figure 6b).

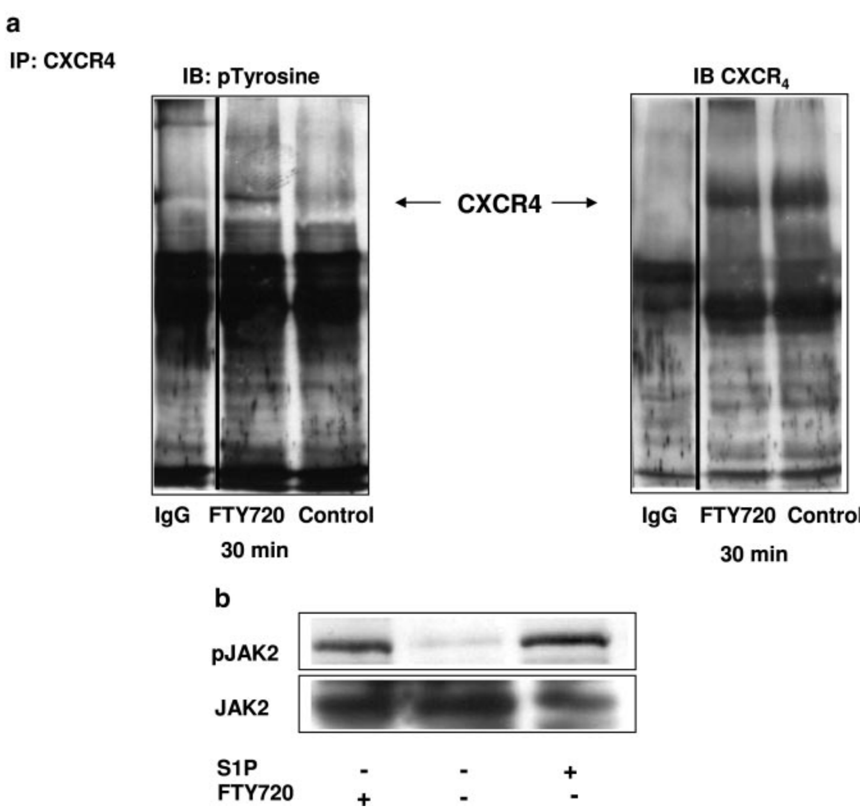

Figure 3. Activation of $CXCR_4$ receptor signaling by S1P. a, EPCs were serum depleted for 12 hours. Immunoprecipitation of $CXCR_4$: Representative immunoblot shows tyrosine receptor phosphorylation which was induced by preincubation of EPCs with S1P agonists, n=5. b, Immunoblotting of JAK2 phosphorylation (upper panel) and JAK2 (lower panel) after preincubation of EPCs with S1P (200 nmol/L) or FTY720 (10 nmol/L), n=4 to 6. For quantification see also Figure 5.

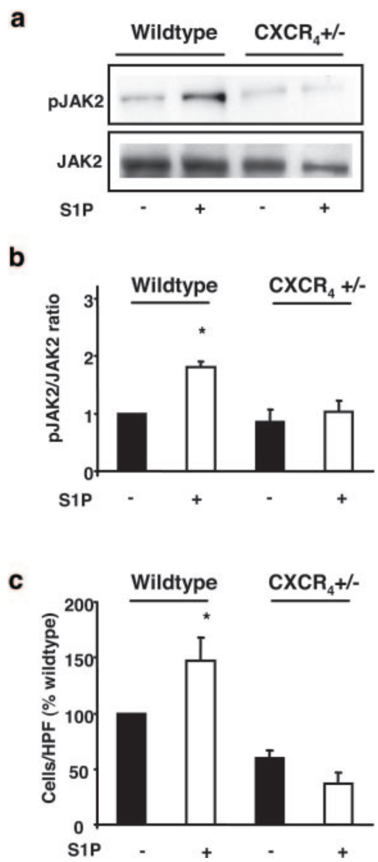

Figure 4. S1P does not activate JAK2 phosphorylation in $CXCR_4^{+/-}$ mice. a and b, JAK2 phosphorylation in BMCs from wild-type and $CXCR_4^{+/-}$ mice. BM cells were serum depleted for 6 hours and treated with S1P agonists for the indicated time points. Quantification expressed as pJAK2/JAK2 ratio. *$P<0.01$ vs basal, n=4. c, Quantification of SDF-1–induced invasion in BM-MNCs from background and $CXCR_4^{+/-}$ mice with or without S1P stimulation, n=7. *$P<0.05$.

Discussion

The results of the present study demonstrate that S1P and FTY720 improved EPC-mediated blood flow recovery in ischemic hind limbs. Specific deletion of $S1P_3$ blocked the S1P effects on progenitor cells and lead to severe impairment of neovascularization. Mechanistically, S1P stimulates the transactivation of the $CXCR_4$ receptor leading to the activation of Src and JAK2 signaling.

S1P plays a crucial role in the cardiovascular system: it acts as a proangiogenic molecule,[12] improves endothelial function,[9] myocardial perfusion,[33] and is involved in cardiovascular development.[6,34] Additional studies demonstrate that S1P modulates homing and trafficking of cells.[14,35] In accordance, the present study documents that S1P influences the functional activity of endothelial progenitor cells to improve neovascularization after ischemia. S1P exerts its effects by activating the G protein–coupled receptors $S1P_{1-5}$. $S1P_{1-3}$ are widely expressed and have been shown to be involved in the development of a mature vascular system during embryonic development. $S1P_1$ and $S1P_3$ are considered to be the major receptors in endothelial cells contributing to angiogenic processes. Using $S1P_3^{-/-}$ cells, we demonstrated that specifically the $S1P_3$ receptor is required for progenitor cell-mediated migration in vitro, neovascularization improvement in vivo, and is essential for S1P-mediated activation of down stream signaling pathway. Although $S1P_1$ and $S1P_3$ have been shown to act cooperatively,[42] distinct functions can be attributed to both receptor subtypes. Previous studies revealed that selective $S1P_1$ agonists control lymphocyte recirculation, whereas $S1P_3$, and not $S1P_1$, regulates heart rate.[11] The specific role of $S1P_3$ in proangiogenic signaling was underscored by the finding that vascular sprouting can also be induced by a synthetic peptide derived from the second intracellular loop of $S1P_3$.[12] This is in line with the data of the present study demonstrating that $S1P_3$-deficient mice show a severe impairment of neovascularization after ischemia. However, other studies using knockdown strategies by RNA interference clearly identified $S1P_1$ as a critical component of tumor angiogenic responses.[43] Because $S1P_1$ is also expressed in EPC and $S1P_1$ knock out mice are embryonically lethal, one may speculate that the combined deletion of both receptors may even more severely affect EPC function.

The signaling downstream of the $S1P_3$ receptor to mediate the S1P effects on EPCs involves the transactivation of the $CXCR_4$ receptor. Previous studies demonstrated that S1P transactivates several receptors involved in angiogenesis such as the PDGF receptor, VEGF receptor-2 and EGF receptor, which mediate migratory activities in endothelial cells or smooth muscle cells.[40,44–46] The present study now extends these findings by demonstrating that tyrosine phosphorylation of the $CXCR_4$ receptor is stimulated by S1P in EPCs. $CXCR_4$ plays a crucial role for endothelial cell migration and is essential for homing and functional integration of EPCs to ischemic tissues.[38,47,48] Moreover, $CXCR_4^{+/-}$ EPCs failed to augment neovascularization in a previous experimental study.[38] Consistently, the S1P-mediated stimulation of EPC function and signaling was abolished in $CXCR_4^{+/-}$ mice. S1P-mediated activation of the $CXCR_4$ receptor signaling was sensitive to the Src inhibitor PP2, implicating that Src family kinases mediate the signaling between the $S1P_3$ receptor and the $CXCR_4$ receptor. Because nothing is known about an intrinsic tyrosine kinase activity of the S1P receptors, it is tempting to speculate that Src is required for $CXCR_4$ tyrosine phosphorylation. Indeed, Src-family tyrosine kinases are activated by S1P stimulation and have previously been implicated in S1P-mediated activation of several growth factor receptors.[40,41,49,50] Although our data showed a Src-dependent activation of the $CXCR_4$ receptor signaling and a requirement of $CXCR_4$ expression for S1P responses, we cannot rule out that Src activation improves EPC function by additional pathways. Thus, Src may activate the VEGF receptor 2, which plays an important role for endothelial progenitor cell mobilization, survival, and function (for review see[51,52]). Moreover, Src has been shown to activate the PI3K/Akt/eNOS-signaling pathway[53] and by this means may exert beneficial effects on endothelial progenitor cell function and homing.

The stimulation of progenitor cells with S1P or its analog FTY720 may also be an interesting therapeutic tool to augment EPC function. Risk factors for coronary artery disease and severe heart failure have been shown to impair

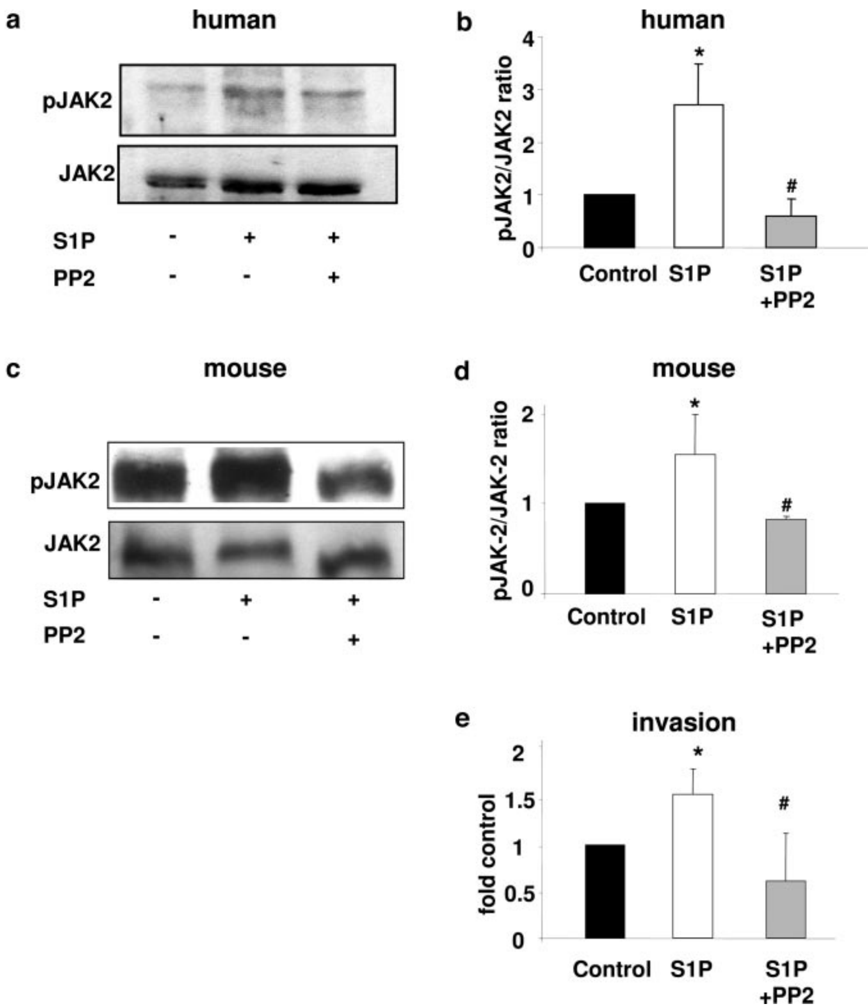

Figure 5. Involvement of Src kinases in S1P mediated JAK2 phosphorylation of cultured EPCs. a, Representative JAK2 protein phosphorylation by immunoblotting in cultured EPCs with or without stimulation by S1P. EPCs were serum depleted for 12 hours before stimulation. b, Equal loading was confirmed by JAK2 protein expression and quantification expressed as pJAK2/JAK2 ratio, n=4. **P*<0.001 vs control, #*P*<0.01 vs control or S1P stimulation. c, Representative JAK2 protein phosphorylation by immunoblotting in BMCs from wild-type mice with or without stimulation by S1P and with or without coincubation with PP2; PP2 (Calbiochem) was used at 1 μmol/L for 30 minutes preincubation. d, Equal loading was confirmed by JAK2 protein and quantification expressed as pJAK2/JAK2 ratio. **P*<0.001 vs control, #*P*=ns vs control. e, Invasion of wild-type BMCs, stimulated with S1P or S1P and PP2, n=3 to 4. **P*<0.05 vs control, #*P*<0.01 vs S1P.

circulating blood-derived EPCs,[27,54,55] limiting the functional capacity of the isolated cells to augment blood flow recovery after infusion in experimental animal models.[38] Likewise, bone marrow–derived cells isolated from patients with chronic ischemic heart disease showed a significantly impaired homing and neovascularization improvement capacity.[29] Although risk factor for coronary artery disease may affect multiple signaling pathways,[37,56] recent data demonstrate that $CXCR_4$ signaling and the response toward SDF-1 is significantly impaired in patient-derived cells compared with healthy controls.[38] The transactivation of the $CXCR_4$ receptor signaling cascade by S1P in patient-derived cells may compensate for the reduced activity of this important signaling pathway.

The compound FTY720 is currently in clinical trials for renal transplantation rejection. However, its precise mechanism of action is not entirely clear. After being metabolized by cellular kinase(s), the compound FTY720 bears structural similarities with S1P and was shown to act as S1P analog. Consistently, FTY720 stimulated $CD34^+$ cell migration.[16] However, other studies have demonstrated that FTY720 also can act as antagonist of S1P-induced responses. When given systemically, FTY720 blocked S1P-angiogenesis and VEGF-induced tumor vascularization.[57] The reason for the different responses may be explained by the pretreatment of the animals and cells with proangiogenic stimuli such as S1P or VEGF. Thus, it is tempting to speculate that further incubation of pretreated cells with FTY720 may lead to internalization of S1P receptors and, thereby, inhibiting S1P responses. In the present study, FTY720 was only used to pretreat the unstimulated cells for 2 hours before reinfusion, whereas the antitumor activity was detected in mice which were prestimulated.

In summary, S1P and its analog FTY720 profoundly stimulate the angiogenic activity and neovascularization capacity of cultured EPCs or BMCs. Mechanistically, S1P activates the $CXCR_4$ dependent JAK2 signaling, involving in part the activity of Src kinases via the $S1P_3$ receptor. S1P agonists might serve as ideal sensitizers of $CXCR_4$-mediated signaling and may be applied in clinical progenitor cell therapy to improve EPC or BMC function in patients with coronary artery disease.

Acknowledgments

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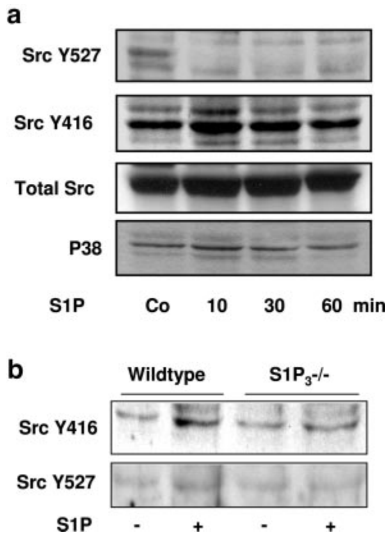

Figure 6. S1P activates Src kinase via $S1P_3$. a, Immunoblot showing active (tyrosine 416) or inactive (tyrosine 527) form of Src. S1P incubation-induced Src phosphorylation at tyrosine 416 in human EPCs as early as 10 minutes. Concomitantly, the inactive form of Src was reduced. b, Immunoblot detecting the active (tyrosine 416) or inactive (tyrosine 527) form of Src. After stimulation of cultured EPCs derived from spleen of each of 15 mice, protein extracts were analyzed for Src activation. Src phosphorylation was induced by S1P in EPCs derived from wild-type mice, whereas no induction of Src phosphorylation was detectable in EPC derived from $S1P_3^{-/-}$ mice.

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Disclosures

None.

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Expanded Material and Methods

Cell Isolation:

Human EPC culture

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density gradient centrifugation with Biocoll ®-1077 (Biochrom AG, Berlin, Germany) and cultured for 4 days on human fibronectin in EBM (Clonetics) supplemented with EGM SingleQuots (Clonetics) and 20% FCS as previously described [1]. EPC were characterized by flow cytometry as previously described [2]. In brief, EPC were characterized as adherent cells after four days of cultivation that were double-positive for both lectin and Dil-acetylated-LDL uptake. Additionally, the endothelial phenotype was confirmed by demonstrating the expression of the endothelial marker proteins KDR, vascular endothelium-cadherin and von Willebrand factor by flow cytometry [2, 3].

Murine EPC

EPC derived from spleen were isolated and cultured as previously described [1].

Murine bone marrow mononuclear cells

Bone marrow mononuclear cells were isolated as previously described after ficoll density centrifugation of bone marrow-aspirates [4].

EPC Migration Assay

A total of 2 x 10^4 murine EPC were isolated, resuspended in 250 µl EBM-medium and pipetted at day 4 in the upper chamber of a modified Boyden chamber (Costar Transwell ® assay, 8 µm pore size, Corning, NY). The chamber was placed in a 24-well culture dish containing 500 µl EBM supplemented with either PBS, or 100 ng/ml stromal cell-derived factor 1 (SDF-1). After 24 hours incubation at 37°C, transmigrated cells were counted by independent investigators blinded to treatment.

Invasion Assay

A total of 5 x 10^5 BMC was resuspended in 250 µl X-Vivo medium and placed in the upper chamber of a modified Boyden chamber filled with matrigel (BioCoat® invasion assay, 8 µm pore size, Becton Dickinson Labware, Massachusetts). Then, the chamber was placed in a 24-well culture dish containing 500 µl EBM supplemented with either PBS, or 100 ng/ml stromal cell-derived factor 1 (SDF-1). After 24 hours incubation at 37°C, transmigrated cells were counted by independent investigators.

Murine hindlimb ischemia model

The incorporation of human EPC and murine BMC or EPC, respectively, and their contribution to neovascularization was investigated in a murine model of hind limb ischemia, using 8–10 wk old athymic NMRI nude mice (The Jackson Laboratory, Bar Harbor, Maine) weighing 18–22 g. Ischemia was induced by occluding the proximal femoral artery including the superficial and the deep branch and all arterial side branches using an electrical coagulator (Erbe, Tübingen, Germany). The overlying skin was closed using surgical staples. After 24 hours, mice received an intravenous injection of 5×10^5 human EPC or 5×10^5 murine BMC.

Murine hindlimb perfusion

Blood flow to ischemic and non-ischemic limbs was measured at 2 weeks using a laser Doppler blood flow meter (Laser Doppler Perfusion Imager System, moorLDI™-Mark 2, Moor Instruments, Wilmington, Delaware).

FACS-Analysis

Fluorescence activated cell sorting (FACS) was used to detect the expression of cell surface markers and endothelial lineage antigens on EPC as previously characterized [2]. S1P-receptor expression (S1P1-4) was determined by anti-human antibodies (Ex-alpha, Watertown, USA) after permeabilization. Isotype-identical directly conjugated antibodies served as a negative control. Immunofluorescence labeled cells were fixed with 2% para-formaldehyde and analyzed by quantitative flow cytometry using FACStar flow cytometer (Becton Dickinson) and Cell Quest Software counting 10,000 events/sample.

Histological Analysis

Capillary density is expressed as number of capillaries/myocyte ratio (40X). At least 5 randomly selected sections from N=4 muscles per group were analyzed and counted by blinded investigators.

Immunoblotting

Protein extracts from EPC derived from healthy volunteers or CAD patients were directly lysed in 62.5 mM Tris-HCl (pH 6.8, 2 % SDS, 10 % glycerol, 50 mM DTT, 02 & bromphenolblue). JAK2 and Src phosphorylation were determined by anti-phospho-JAK2 or anti-phospho Src antibodies (Y416 or Y527) (Upstate, 1:500). Equal loading was confirmed by JAK2, Src or p38 kinase antibodies (Upstate 1:500). Densitometric analysis was performed with the NIH imaging program.

Immunoprecipitation

EPC were lysed in RIPA buffer containing 20mM NaF, 1mM Na_3VO_4, and 20mM sodiumpyruvate. Lysates containing 1000 µg of protein were precleared with G-sepharose and immunoprecipitated with $CXCR_4$ antibody (Santa Cruz). After addition of G-sepharose, immunoprecipitates were washed and loaded onto 10% SDS-Page and tyrosine phosphorylation of the $CXCR_4$ receptor was detected (Upstate 1:1000).

Statistical Analysis

All data are presented as mean ± SEM or SD as indicated. Continuous variables were compared by means of Student´s t test or Mann-Whitney U Test. Multiple comparisons were performed by Kruskal-Wallis test or ANOVA with Bonferroni`s correction using SPSS 11.0. A *P* value of <0.05 was considered significant.

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Table 1: Characteristics of study population

	Patients w/ CAD	Healthy controls	P value
	N = 26	N = 22	
Age (yrs)	63 ± 10	32 ± 4.6	<0.001
Male gender (%)	84.6	95.4	ns
Hypertension (%)	60.4	0	<0.001
Hyperlipidemia (%)	73	n.a	
Diabetes (%)	18.8	0	<0.001
Current Smoking (%)	46.1	13.6	<0.001
History of MI	30.7	0	<0.001
Ejection fraction (visually estimated)	50 ± 10	n.a	
C-reactive protein (mg/dl)	0.4 ± 0.25	n.a	