

Syk and Slp-76 Mutant Mice Reveal a Cell-Autonomous Hematopoietic Cell Contribution to Vascular Development

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Summary

Developmental studies support a common origin for blood and endothelial cells, while studies of adult angiogenic responses suggest that the hematopoietic system can be a source of endothelial cells later in life. Whether hematopoietic tissue is a source of endothelial cells during normal vascular development is unknown. Mouse embryos lacking the signaling proteins Syk and Slp-76 develop abnormal blood-lymphatic endothelial connections. Here we demonstrate that expression of GFPSlp-76 in a subset of hematopoietic cells rescues this phenotype, and that deficient cells confer focal vascular phenotypes in chimeric embryos consistent with a cell-autonomous mechanism. Endogenous Syk and Slp-76, as well as transgenic GFPSlp-76, are expressed in circulating cells previously proposed to be endothelial precursors, supporting a causal role for these cells. These studies provide genetic evidence for hematopoietic contribution to vascular development and suggest that hematopoietic tissue can provide a source of vascular endothelial progenitor cells throughout life.

Introduction

Close spatial and temporal association (Sabin, 1920), genetic studies (Shalaby et al., 1995; Stainier et al., 1995), and studies of cultured embryonic stem cells (Kennedy et al., 1997) support a common origin for blood and endothelial cells during embryonic development. Recent studies using adult animals have extended this relationship with evidence that mature hematopoietic stem cells can give rise to circulating cells that par-

ticipate in adult vessel growth in response to angiogenic stimuli (reviewed in Rafii and Lyden, 2003). This work has revealed the possibility of a novel angiogenic mechanism with great therapeutic potential, but whether and how hematopoietic cells contribute to normal vascular growth and development remain unknown.

A primary difficulty in defining the hematopoietic cell contribution to new vessel growth is accurately discriminating between vascular endothelial cells of different origins. In developing animals, lineage tracing experiments using Cre recombinase suggest that the extent of hematopoietic contribution to embryonic endothelium may be low (Stadtfeld and Graf, 2005), but such studies are descriptive and limited both by the choice of the Cre driver (which may be either too restrictive and not express in the appropriate hematopoietic progenitor cells, or not restrictive enough and have “leaky” expression in mature endothelial cells regardless of their origin) and by the ability to detect a potentially rare population of hematopoietic-derived endothelial cells in an extensive vascular network. In mature animals, definitive identification of hematopoietic origin has been achieved through reconstitution of lethally irradiated animals with hematopoietic tissue that stably expresses a reporter gene to mark all cell progeny. Because mature animals are not in an angiogenic state, however, this tagging system must be coupled to the application of potent but nonphysiologic angiogenic stimuli such as the sudden cessation of blood flow (Asahara et al., 1997), injection of tumor cells (Lyden et al., 2001), or direct administration of angiogenic growth factors (Asahara et al., 1999).

Such studies have yielded controversial and even conflicting results regarding both the hematopoietic cell requirement for different angiogenic responses and the identity of the hematopoietic cell type required. Bone marrow-derived progenitor cells that carry endothelial cell markers have been identified in the blood of mice and humans (Asahara et al., 1997) and experimental angiogenic responses have been shown to require the recruitment of hematopoietic cells (Aicher et al., 2003; Lyden et al., 2001), but whether such requirements reflect a causal role for these rare precursor cells or for another hematopoietic cell type is not established. In particular, adult angiogenic models are associated with, and may even require, recruitment of inflammatory hematopoietic cell types such as tissue macrophages that may be difficult to distinguish from endothelial cells due to their infiltration into the vessel wall and/or expression of endothelial molecular markers (Grunewald et al., 2006; Maruyama et al., 2005; Rabelink et al., 2004; Rehman et al., 2003; Ziegelhoeffer et al., 2004). Thus whether and how hematopoietic cell precursors contribute to physiologic vascular growth is not yet clear.

In the present study we have used functional, genetic approaches to investigate the cellular mechanism for the vascular endothelial phenotype that arises in mouse embryos lacking Syk and Slp-76. Transgenic rescue demonstrates a hematopoietic cell requirement for Slp-76 signaling, while chimeric embryo studies reveal

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a cell-autonomous mechanism in which deficient cell contribution to new lymphatic vessels confers blood-lymphatic vascular mixing. These findings point to a defect that arises from loss of signaling in hematopoietic cells that function as endothelial precursors during embryonic development, a conclusion supported by a defect in a cognate cell population, circulating endothelial precursor cells (CEPs), in surviving *Slp-76*-deficient animals. These findings provide new evidence for hematopoietic cell contribution to normal vessel growth and development.

Results

Generation of GATA1-GFPSlp-76 Transgenic Animals that Express GFPSlp-76 in a Restricted Number of Hematopoietic Cell Types

Studies of *Slp-76* and *Syk* gene expression using *in situ* hybridization failed to detect endothelial expression of either gene in E11.5 or E14.5 embryos, time points at which deficient embryos exhibit lymphatic vascular phenotypes (Abtahian et al., 2003). Flow cytometric analysis of neonatal skin endothelial cells similarly failed to demonstrate endothelial expression of these proteins (see Figure S1A in the Supplemental Data available with this article online). Thus, at the level of both mRNA and protein, *Syk* and *Slp-76* gene expression is detected in hematopoietic cells but not in mature endothelial cells *in vivo*.

To functionally discriminate between a requirement for *Slp-76* in hematopoietic and endothelial cells, we adopted a complementation strategy to rescue *Slp-76* expression in hematopoietic but not endothelial cells using a GATA1 minigene previously shown to restrict transgene expression to a subset of hematopoietic lineages (McDevitt et al., 1997). Transgenic gain of function is superior to conditional loss of function for this purpose because coexpression of genes such as *Tie-2* and *Vav* in hematopoietic stem cells and endothelial cells drives gene excision in the mature cells of both lineages in Cre-expressing transgenic mice (Constien et al., 2001; Georgiades et al., 2002). To precisely follow transgene expression, we generated transgenic mice that expressed a GFPSlp-76 fusion protein (Figure S1B). GATA1 is a transcription factor expressed in hematopoietic stem cells (Sposi et al., 1992) and in a limited repertoire of differentiated hematopoietic cells including erythrocytes, platelets, eosinophils, and mast cells (Migliaccio et al., 2003; Shivdasani et al., 1997; Whitelaw et al., 1990; Yu et al., 2002), but not in lymphocytes, macrophages, or endothelial cells (McDevitt et al., 1997). The GATA1 minigene has been used previously to distinguish between hematopoietic and endothelial requirements for specific gene products such as the *Scf*/*Tal-1* transcription factor and the erythropoietin receptor using a similar rescue strategy (Suzuki et al., 2002; Visvader et al., 1998). Two GATA1-GFPSlp-76 transgenic lines were characterized (Figure S1C). The studies reported below utilized the higher expressing transgenic line to optimize detection of GFPSlp-76, but identical results were obtained using both transgenic lines.

Transgene expression was first assayed using flow cytometry of live cells obtained from postnatal animals to detect GFPSlp-76. Among the hematopoietic line-

ages, GFPSlp-76 was detected in platelets, erythrocytes, eosinophils, and a subset of *lin*⁻, *Sca1*⁺, *c-kit*⁺ stem cells, but not in T or B lymphocytes, macrophages, neutrophils, NK cells, or dendritic cells (Figure 1A and data not shown). GFPSlp-76 was also not detected in peritoneal mast cells, a finding consistent with the reported expression of a lacZ transgene using the same GATA1 minigene (McDevitt et al., 1997). To determine whether GATA1-GFPSlp-76 transgenic mice express GFPSlp-76 in endothelial cells, neonatal skin cells (known to contain both blood and lymphatic endothelial cells) from wild-type, *Tie2*-GFP transgenic, and GATA1-GFPSlp-76 transgenic animals were analyzed using flow cytometry for expression of VE-cadherin (CD144), an endothelial cell marker, and CD45, a cell surface protein found on blood but not endothelial cells. *Tie2*-GFP transgenic mice express GFP in a subset of vascular endothelial cells but not in mature circulating cells (Motoike et al., 2000). GFP⁺, CD144⁺ endothelial cells were identified in the skin of *Tie2*-GFP but not GATA1-GFPSlp-76 transgenic animals (Figure 1B). Conversely GFP⁺, CD45⁺ hematopoietic cells were identified in the skin of GATA1-GFPSlp-76 but not *Tie2*-GFP transgenic animals (Figure 1B). Thus, GATA1-GFPSlp-76 transgenic animals express the GFPSlp-76 fusion protein exclusively in stem cells, erythrocytes, platelets, and eosinophils, and not in vascular endothelial cells postnatally.

To determine the cellular sites of GATA1-GFPSlp-76 transgene expression in the developing embryo, flow cytometry and antibody staining of embryo sections were performed. Flow cytometry revealed GFP expression in CD45⁺, CD144⁻ blood cells but not in CD144⁺, CD45⁻ endothelial cells derived from E14.5 embryos (Figure 1C). Immunohistochemical staining for GFP identified GFP⁺ cells in the lumen of blood vessels and in the fetal liver, the source of hematopoietic cells at E14.5, but not in vascular endothelial cells (Figure 1D). To better determine whether GFP⁺ cells might participate directly or indirectly in the formation of the early lymphatic vessels, immunofluorescent studies were performed to detect GFP⁺ cells in the region of the LYVE1⁺ endothelial cells of the primitive lymph sacs in E12.5 embryos. LYVE1⁺ endothelial cells are known to line the primitive lymph sac and the dorsal aspect of the cardinal vein, but not the aorta (Wigle et al., 2002). While GFP⁺ cells were detected in the lumen of the cardinal vein and aorta, none were detected either in or next to LYVE1⁺ endothelial cells (Figure 1E). Thus, GATA1-GFPSlp-76 transgene expression is restricted to a subset of circulating blood cells and is not detected in endothelial or perivascular cells during early lymphatic development, the time point at which embryos lacking *Syk* or *Slp-76* exhibit lymphatic vascular defects.

The GATA1-GFPSlp-76 Transgene Rescues *Slp-76* Function in Platelets but Not in Neutrophils or T Cells

To test GATA1-GFPSlp-76 transgene function, we generated *Slp-76*-deficient animals that carried the GATA1-GFPSlp-76 transgene (G1 rescue animals). *Slp-76* is required for signaling through the platelet collagen receptor glycoprotein VI (GPVI) and *Slp-76*-deficient platelets fail to secrete P-selectin (CD62) following exposure to the strong GPVI agonist convulxin (CVX)

(Figure 2A; Judd et al., 2002). G1 rescue animals exhibited complete rescue of Slp-76-dependent platelet CVX activation, with no change in platelet activation by the G protein-coupled receptor agonist AYPGKF (an activating peptide for protease-activated receptor 4; Figure 2A).

Slp-76 is required for neutrophil release of superoxide in response to integrin ligand binding (Newbrough et al., 2003), as well as for T cell maturation in the thymus (Clements et al., 1998). Transgenic GFPSlp-76 expression could not be detected in either of these cell types, predicting a persistent loss of function in G1 rescue animals. Indeed, neutrophil superoxide release in response to fibrinogen binding in G1 rescue animals was not detectable (Figure 2B), while CD4⁺ CD8⁺ T cells failed to develop in the thymus of G1 rescue animals due to a block in the transition from a CD44⁻ CD25⁺ to a CD44⁻ CD25⁻ phenotype identical to that observed in Slp-76-deficient animals (Figure 2C and data not shown; Clements et al., 1998; Pivniouk et al., 1998). Because expression of GFPSlp-76 in even a small number of T cell progenitors would permit selective repopulation of the thymus with mature T cells, these studies indicate that detection of GFPSlp-76 expression by flow cytometry in GATA1-GFPSlp-76 transgenic animals accurately predicts functional rescue of Slp-76 deficiency.

GATA1-GFPSlp-76 Transgene Expression Rescues Vascular Mixing in Slp-76-Deficient Mice

Lymphatic vessels first arise in the skin in midgestation, and a striking pattern of blood-filled cutaneous lymphatics is a hallmark of midgestation embryos lacking Slp-76 or Syk (Figure 3A; Abtahian et al., 2003). To determine whether GATA1-GFPSlp-76 transgene expression rescued the vascular phenotype of Slp-76-deficient animals, we therefore examined E14.5 G1 rescue and nontransgenic, Slp-76-deficient littermates. G1 rescue animals appeared indistinguishable from wild-type embryos and, in contrast to nontransgenic Slp-76-deficient littermates, had no gross or histologic evidence of blood-filled lymphatic vessels (Figure 3A).

The intestines are a site of dense lymphatic growth due to their role in transporting absorbed dietary fats. As a consequence of blood-lymphatic connections, postnatal Slp-76-deficient animals develop intestinal vascular malformations that mediate arterio-venous shunting of blood (Abtahian et al., 2003). Most Slp-76-deficient animals die by age 3 weeks as a result of this vascular defect, and surviving animals exhibit marked cardiac hypertrophy due to a chronic high-cardiac output state (Abtahian et al., 2003). Postnatal G1 rescue animals exhibited normal gut vasculature, normal survival, and no evidence of cardiac hypertrophy (Figure 3C and data not shown). Thus, all the vascular abnormalities characteristic of Slp-76-deficient animals are corrected by the GATA1-GFPSlp-76 transgene despite restriction of GFPSlp-76 expression to a small number of hematopoietic cell types.

Syk^{-/-}; Syk^{+/+} and Slp-76^{-/-}; Slp-76^{+/+} Chimeric Animals Develop Focal Phenotypes Consistent with a Cell-Autonomous Mechanism of Vascular Mixing

The ability of the GATA1-GFPSlp-76 transgene to rescue development of the vascular phenotype is consistent

with either a cell-nonautonomous mechanism in which circulating cells utilize Slp-76 signals to indirectly influence vascular development, or a cell-autonomous mechanism in which Slp-76 is required in hematopoietic cells that contribute directly to vascular development as endothelial precursors. To distinguish between these two possibilities, we generated chimeric animals by injecting embryonic stem cells deficient in either Syk (Syk^{-/-}) or Slp-76 (Slp-76^{-/-}) into wild-type blastocysts derived from ROSA26 β -galactosidase-expressing animals. In the case of a cell-nonautonomous mechanism, the presence of wild-type circulating cells, that is, erythrocytes, platelets, or eosinophils, is predicted to rescue the vascular phenotype throughout the developing embryo. In contrast, with a cell-autonomous mechanism, the vascular phenotype is predicted to still develop to a variable extent due to the persistent participation of deficient endothelial precursors. Chimeric embryos generated with either Syk-deficient or Slp-76-deficient embryonic stem cells developed striking vascular phenotypes in which some regions of the embryo (e.g., the skin overlying the upper extremity; Figure 4A) developed blood-filled lymphatics associated with local edema, while adjacent regions (e.g., the periorbital skin; Figure 4A) were unaffected. Histologic analysis revealed blood-filled LYVE1⁺ lymphatic vessels characteristic of deficient embryos (Figure 4B and data not shown). Chimeric embryo vascular phenotypes varied widely both between and within individual embryos but were not observed in chimeric embryos generated with wild-type ES cells (Figure S2). The presence of wild-type cells in the tissues of animals with vascular phenotypes was confirmed by X-gal staining and the presence of wild-type hematopoietic cells was confirmed using the fluorescent β -galactosidase substrate FDG (Figures 4C and 4D).

Previous studies of embryos lacking Syk or Slp-76 revealed heterotypic vessels containing endothelial cells that were both positive and negative for the lymphatic molecular marker LYVE1 at sites likely to mediate connections between the blood and lymphatic circulations (Abtahian et al., 2003). This endothelial cell phenotype was also detected in chimeric embryos with visible vascular phenotypes (Figure 4B). If deficient cells conferred vascular mixing in a cell-autonomous manner, we reasoned that deficient cells should be incorporated at such sites at a higher frequency than in apparently unaffected, homotypic lymphatic vessels elsewhere in the embryo. To positively follow deficient cell contribution at the single-cell level, Syk-deficient ES cells were GFP tagged using a lentiviral vector and deficient cells were identified by immunofluorescent detection of GFP in chimeric embryos. GFP immunofluorescent staining revealed deficient cells throughout the embryo (e.g., the trachea; Figure 4E), and flow cytometry demonstrated contribution to the hematopoietic cells of the fetal liver (Figure 4F). Analysis of endothelial cells in heterotypic vessels virtually always revealed a significant number of deficient cells, while deficient cells were only rarely observed in homotypic LYVE1⁺ vessels (29/29 versus 2/13 vessels; $p < .025$; see Table S1; Figure 4G). These findings are consistent with endothelial cell-autonomous conferral of blood-lymphatic vascular mixing by deficient cells.

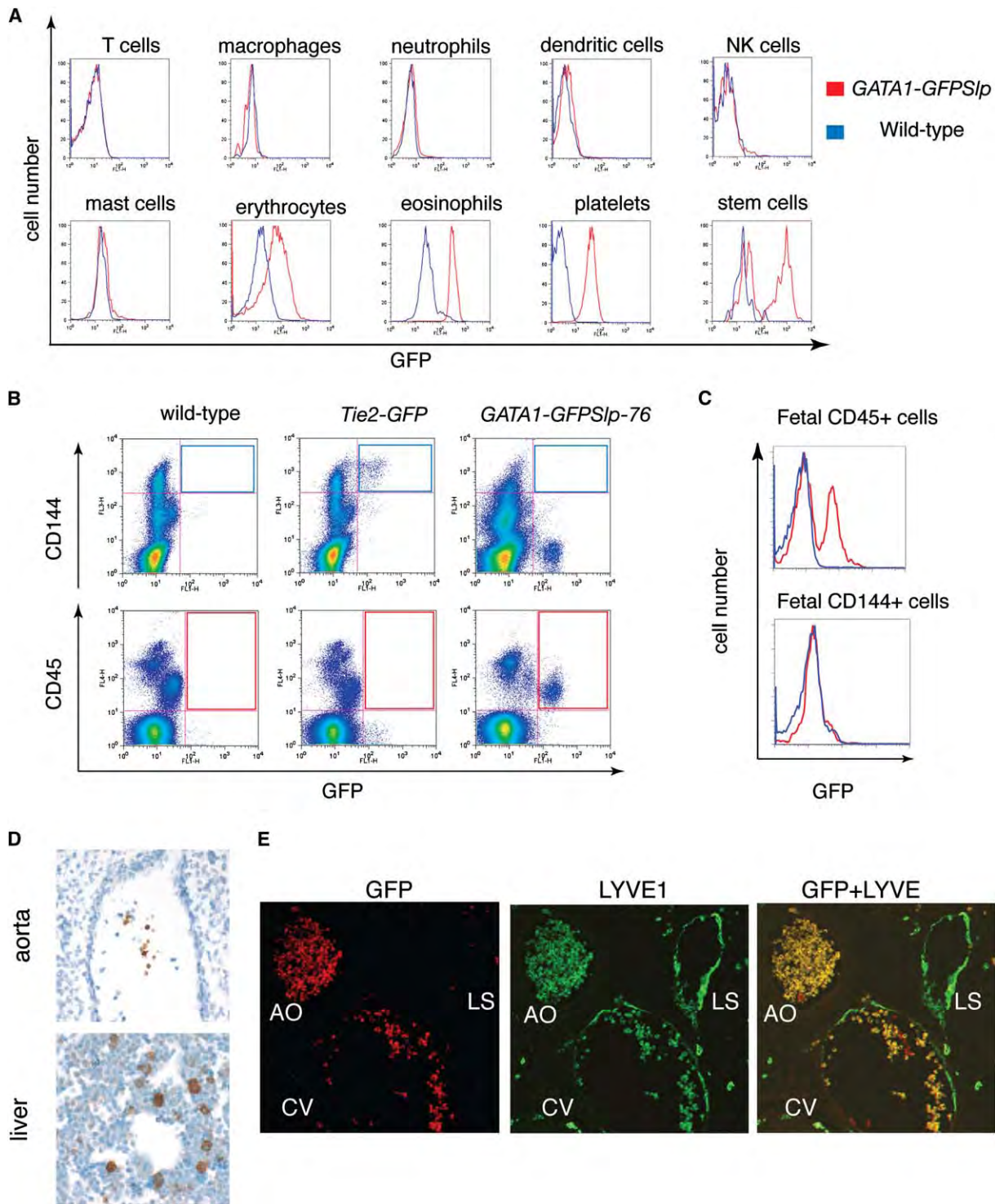


Figure 1. Expression of GFPSlp-76 in GATA1-GFPSlp-76 Transgenic Mice

(A) Expression of GFPSlp-76 in hematopoietic cell lineages. GFP was assayed in bone marrow or circulating cells using the following cell surface markers: T cells (CD90⁺), B cells (CD45R⁺), monocytes (CD11b⁺ and FSC/SSC profile), neutrophils (Ly-6G⁺ and FSC/SSC profile), natural killer (NK) cells (NK1.1⁺ DX5⁺), dendritic cells (Ly-6G⁻ CD11b^{low} CD11c^{high}), erythrocytes (TER119⁺), bone marrow stem cells (CD117⁺ Ly-6A/E⁺ CD3ε⁻ CD45R⁻ CD11b⁻ Ly-6G⁻ TER-119⁻), platelets (FSC/SSC), and eosinophils (CD23⁺ CCR3⁺). Mast cells were collected from the peritoneal cavity of naïve mice via saline lavage and defined as CD23⁺ high, CCR3⁻ to distinguish them from eosinophils and macrophages. Red curves indicate cells derived from GATA1-GFPSlp-76 transgenic mice and blue curves indicate wild-type cells.

(B) GFPSlp-76 is not expressed in mature endothelial cells. GFP was assayed in hematopoietic (CD45⁺ CD144⁻) and endothelial (CD45⁻ CD144⁺) cells harvested from the skin of neonatal wild-type, *Tie2-GFP*, and *GATA1-GFPSlp-76* transgenic mice. Blue boxes indicate GFP⁺ CD144⁺ endothelial cells and red boxes indicate GFP⁺ CD45⁺ hematopoietic cells.

Syk and Slp-76 Are Expressed in Circulating Endothelial Precursor Cells and Are Required to Generate CEPs with Lymphatic Markers

Rescue of the vascular phenotype by the GATA1-GFPSlp-76 transgene and the evidence that Syk and Slp-76 are required cell autonomously during vascular development suggested that Syk and Slp-76 might signal in a hematopoietic cell that functions as an endothelial precursor. Rare cells termed circulating endothelial precursor cells (CEPs) have been described on the basis of molecular markers that define them as lacking CD45, a common blood cell marker, and expressing ckit, a stem cell marker, and VEGFR2, a receptor for vascular endothelial growth factor required for endothelial differentiation (Bertolini et al., 2003; Rafii and Lyden, 2003; Shaked et al., 2005; Figure 5A). To determine whether Syk and Slp-76 are expressed in CEPs, we used flow cytometric detection of intracellular protein. Consistent with previous expression studies (Chan et al., 1994; Jackman et al., 1995), Syk was detected in B but not T lymphocytes, while Slp-76 was detected in T but not B lymphocytes (Figure 5B). In contrast, both Syk and Slp-76 were detected in CEPs, but not in cells that were CD45⁻, VEGFR2⁺, and ckit⁻ (commonly defined as circulating endothelial cells or CECs) (Figure 5B). Lack of Syk and Slp-76 expression in CECs could reflect loss of expression in more mature hematopoietic endothelial precursors or could indicate that CECs are nonhematopoietic endothelial cells sloughed from the vessel wall. To distinguish between these two possibilities, we analyzed CEPs and CECs derived from lethally irradiated wild-type mice that had been reconstituted with GFP-expressing bone marrow. GFP expression was detected exclusively in CEPs (Figure 5C), indicating that CEPs but not CECs are hematopoietic in origin, a result consistent with the lack of detectable Syk and Slp-76 expression in endothelial cells in neonatal skin and embryonic cardinal vein (Figure S1A; Abtahian et al., 2003). Significantly, consistent with the correction of vascular mixing in G1 rescue animals, the GATA1-GFPSlp-76 transgene was expressed in CEPs (Figure 5D).

We next assessed CEPs in a cohort of the rare Slp-76-deficient mice that survive to adulthood. CEPs were detected in the circulating blood of 12-week-old Slp-76-deficient animals at similar levels to that of wild-type animals (Figure 5E). Studies have recently suggested the presence of circulating endothelial precursors that acquire lymphatic fate (Religa et al., 2005; Salven et al., 2003). We therefore examined the CEP population of wild-type and Slp-76-deficient mice for cells expressing the lymphatic molecular markers LYVE1 and VEGFR3. A small but reproducible fraction

of LYVE1⁺ and VEGFR3⁺ CEPs was detected in wild-type mice (Figure 5A; Figure S3). Despite preserved levels of LYVE1⁻ and VEGFR3⁻ CEPs, virtually no LYVE1⁺ or VEGFR3⁺ CEPs could be detected in the blood of Slp-76-deficient mice (Figure 5E; Figure S3B). Consistent with a requirement for Slp-76 in these cells, normal or supranormal levels of LYVE1⁺ and VEGFR3⁺ CEPs were detected in the blood of G1 rescue mice (Figure 5E; Figure S3C). CEPs are believed to arise from a population of progenitor cells in the bone marrow that carry the same molecular markers, termed endothelial progenitor cells (EPCs). In contrast to peripheral blood CEPs, the numbers of LYVE1⁺ and VEGFR3⁺ EPCs in the bone marrow of wild-type and Slp-76-deficient animals were equivalent (Figure S4). Thus, development and rescue of the vascular phenotype follow Syk and Slp-76 expression in CEPs and Slp-76-deficient animals exhibit a defect in peripheral blood CEPs, results consistent with a causal role for these cells.

Discussion

In the present study, we have investigated the cellular basis for a vascular endothelial defect that arises during lymphatic vascular development in mouse embryos lacking the signaling proteins Syk and Slp-76. Our findings provide functional evidence that hematopoietic cells are a source of endothelial precursors during development.

Our conclusions are based on transgenic rescue of gene function in knockout mice, conferral of the vascular phenotype by deficient cells in chimeric embryos, and studies of cognate circulating endothelial precursors in adult animals. Functionally, we have defined the causal cell type as a GATA1-GFPSlp-76-expressing cell that confers blood-lymphatic vascular mixing despite the presence of wild-type cells in chimeric embryos. Among the differentiated hematopoietic cell lineages, GATA1-GFPSlp-76 expression is restricted to platelets, erythrocytes, and eosinophils. Platelets and erythrocytes circulate in the blood where wild-type counterparts would be expected to readily rescue a paracrine signal, and embryos lacking platelets entirely do not develop a vascular phenotype (Shivdasani and Orkin, 1995). In theory, a collection of deficient, extravasated hematopoietic cells could confer a "pseudotransgenic" phenotype in chimeric embryos if such cells played an essential local or perivascular support role during lymphatic vascular development. Such a role has been defined for perivascular macrophages and dendritic cells in induced angiogenesis assays in adult animals (Grunewald et al., 2006), but GATA1-GFPSlp-76 is not expressed in the macrophage or dendritic lineages, similar vascular

(C) GFPSlp-76 is expressed in embryonic blood but not endothelial cells. Single cells isolated from E14.5 GATA1-GFPSlp-76 embryos were analyzed using flow cytometry as described above.

(D) Detection of GFPSlp-76 expression in embryo blood but not endothelial cells using immunohistochemistry. GFPSlp-76 was identified using anti-GFP antibody staining of a transverse section through the descending aorta and fetal liver of an E12.5 GATA1-GFPSlp-76 transgenic embryo. GFPSlp-76 expression is seen as brown staining of individual luminal blood cells (upper panel) and a subset of fetal liver cells (lower panel).

(E) GFPSlp-76 is not expressed in or around the endothelial cells of developing lymphatic vessels. Costaining for GFP (red) and the lymphatic endothelial cell marker LYVE1 (green) was performed on sections of an E12 GATA1-GFPSlp-76 transgenic embryo. Shown is a transverse section above the heart through the dorsal aorta (AO), cardinal vein (CV), and primitive lymph sac (LS). No GFP staining was detected in LYVE1⁺ endothelial cells, and no GFP⁺ cells were detected in a perivascular location adjacent to the developing lymph sacs. The blood cells in the aorta and cardinal vein exhibit high autofluorescence but some GFP⁺ luminal blood cells can be identified as strongly red-staining cells in the overlay (right panel). The results shown are representative of three to seven individual experiments performed.

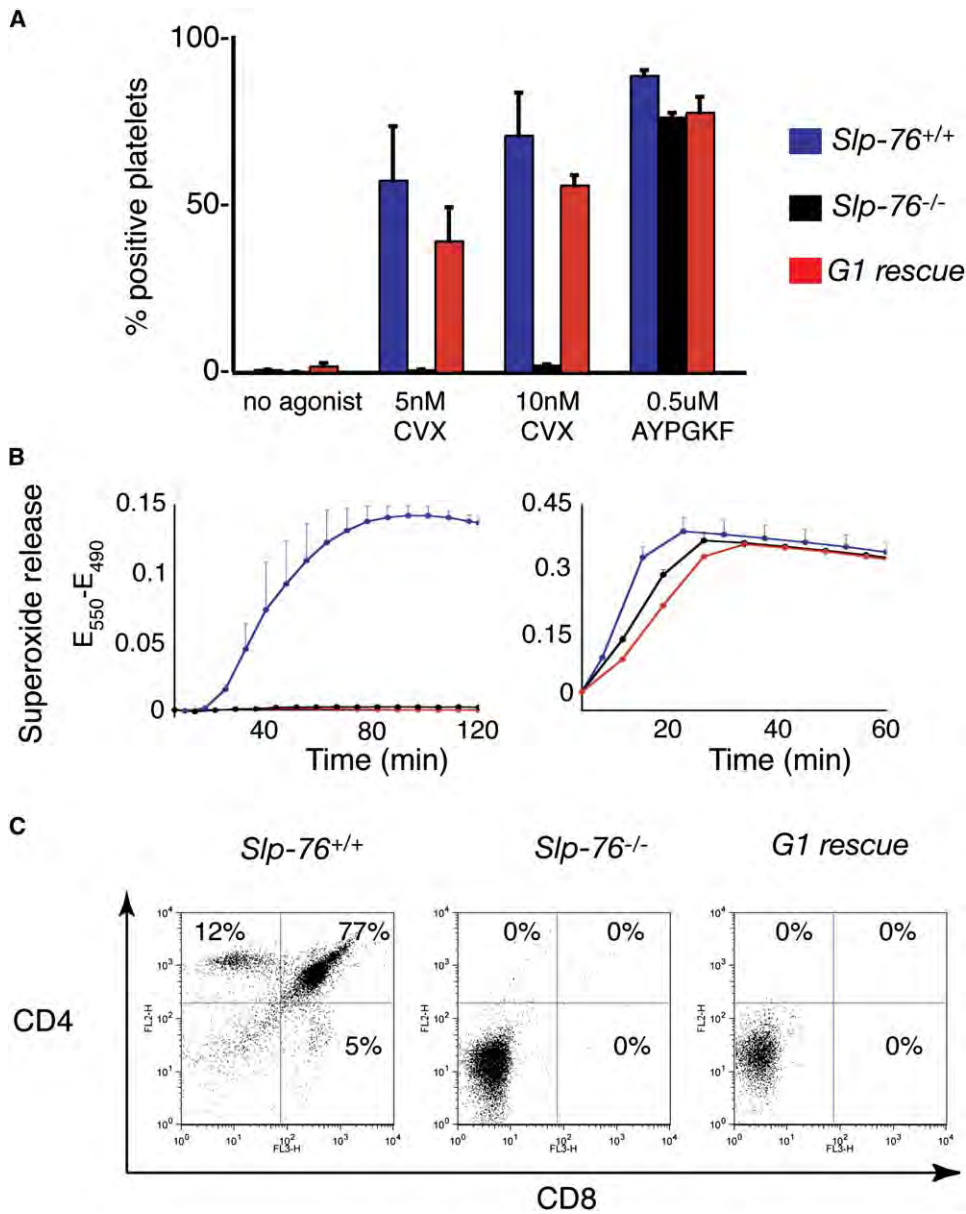


Figure 2. The GATA1-GFPSlp-76 Transgene Rescues Platelet Responses but Not Neutrophil Responses or T Cell Development in *Slp-76*-Deficient Mice

(A) Deficient glycoprotein VI (GPVI) responses in *Slp-76*^{-/-} platelets are restored by the GATA1-GFPSlp-76 transgene. Platelets from wild-type (blue bars), *Slp-76*-deficient (black bars), and *G1 rescue* (red bars) mice were exposed to convulxin (CVX), a GPVI receptor agonist, or AYPGKF (AYP), an agonist for the G protein-coupled PAR4 receptor. Platelet activation was measured by surface expression of P-selectin after activation-dependent granule release. Mean and standard deviation are shown, N = 4–5 for each group.

(B) Deficient *Slp-76*^{-/-} neutrophil responses are not rescued by GATA1-GFPSlp-76. Neutrophils obtained from wild-type (blue), *Slp-76*-deficient (black), or *G1 rescue* animals (red) were plated on fibrinogen after stimulation with either tumor necrosis factor α (left graph) or phorbol myristate acetate (right graph) and release of superoxide was detected through cytochrome C oxidation. Mean and standard deviation are shown, N = 5 for each group.

(C) Defective T cell maturation in *Slp-76*-deficient mice is not rescued by GATA1-GFPSlp-76. Thy1.2⁺ thymocytes were harvested from 10- to 12-week-old wild-type (*Slp-76*^{+/+}), *Slp-76*-deficient (*Slp-76*^{-/-}), and *G1 rescue* mice and stained for CD4 and CD8 as indicated. The percentage of cells in each quadrant is shown. The results shown are representative of six independent experiments.

defects do not arise in mice that lack eosinophils (Yu et al., 2002), and, most significantly, expression analysis of GATA1-GFPSlp-76 during early lymphatic development failed to identify any extravascular GFP⁺ cells in association with either blood or lymphatic vessels at the time point when deficient embryos develop vascular phenotypes. Instead, several lines of evidence support

a truly cell-autonomous mechanism. First, chimeric embryos with vascular phenotypes form heterotypic vessels in which LYVE1⁻ endothelial cells are fused to LYVE1⁺ endothelial cells in vessels identical to those observed in deficient embryos (Abtahian et al., 2003). Such vessels demonstrate a disproportionate incorporation of Syk-deficient cells compared to unaffected, homotypic

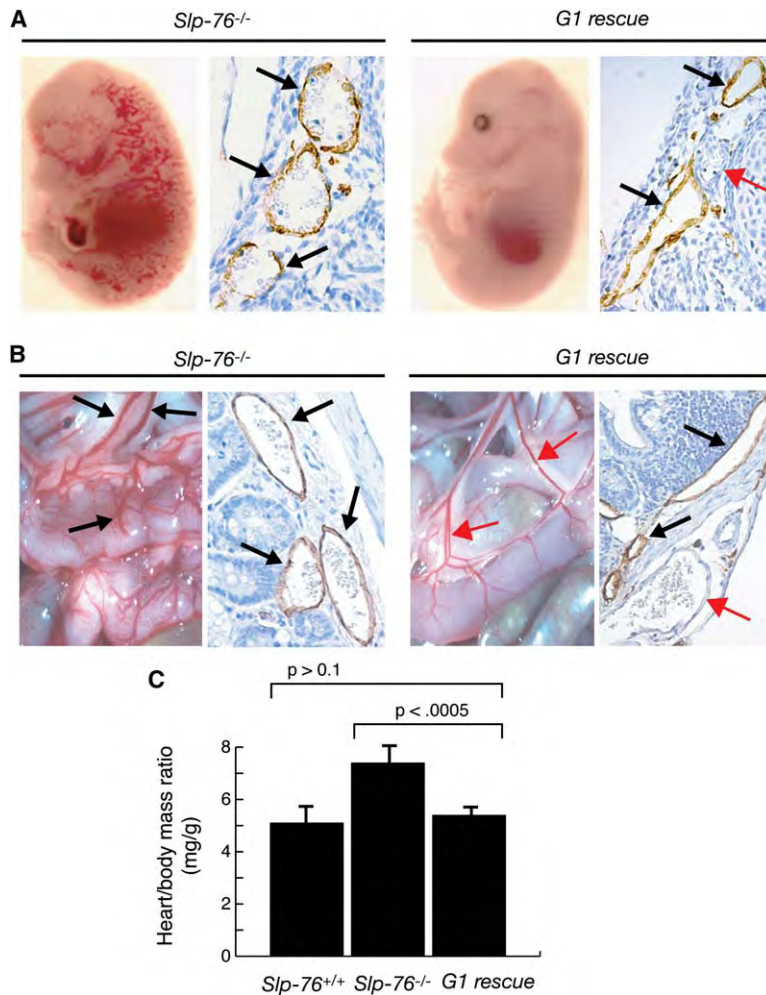


Figure 3. The Vascular Phenotype of *Slp-76^{-/-}* Mice Is Rescued by GATA1-GFPSlp-76

(A) Rescue of midgestation blood-lymphatic mixing in *G1 rescue* embryos. E14.5 *Slp-76*-deficient embryos with (*G1 rescue*) or without (*Slp-76^{-/-}*) the GATA1-GFPSlp-76 transgene are shown. Correction of vascular mixing is demonstrated by gross and histologic absence of blood in the lymphatic vessels of *G1 rescue* embryos. Black arrows, lymphatic vessels indicated by positive immunostaining with anti-LYVE1 antibody. Red arrows, blood vessels indicated by negative LYVE1 immunostaining. The histologic sections are of skin at the level of the neck and chest.

(B) Rescue of mesenteric and intestinal vascular mixing in *G1 rescue* adult mice. The mesenteric and intestinal vasculature of 10-week-old *Slp-76*-deficient (left panels) and *G1 rescue* (right panels) mice are shown. Lymphatic vessels (black arrows) are not blood filled in *G1 rescue* animals. The histologic sections are of intestine just below the submucosal layers.

(C) Correction of cardiac hypertrophy in *G1 rescue* animals. Heart/body mass ratios were measured at age 12 weeks. Mean and standard deviation are shown, $N = 6-9$ animals per group. p values are shown for comparison of values obtained in *G1 rescue* mice and wild-type and *Slp-76*-deficient mice.

vessels, a result consistent with an endothelial cell-autonomous defect. Second, endogenous Syk and *Slp-76* as well as transgenic GATA1-GFPSlp-76 are expressed in CEPs, cognate cells believed to function as hematopoietic endothelial precursors in adult animals. Finally, surviving *Slp-76*-deficient mice exhibit a defect in their CEP population that is corrected by the GATA1-GFPSlp-76 transgene. These findings support the conclusion that the vascular phenotypes in deficient and chimeric embryos arise due to a cell-autonomous mechanism in which Syk and *Slp-76* signals are required in hematopoietic cells that become endothelial cells.

An outstanding question remains the nature of the functional defect in hematopoietic endothelial progenitors lacking Syk or *Slp-76*. Surviving adult *Slp-76*-deficient animals have circulating endothelial progenitor cells, indicating that blood-lymphatic mixing does not arise due to simple loss of these cells. A clue to the functional defect may be found in an apparent discrepancy between the ability of wild-type cells to rescue the vascular phenotype conferred by deficient cells in two different experimental systems. In a previous report, we demonstrated that cotransplanted wild-type bone marrow rescues vascular mixing conferred by deficient bone marrow in adult radiation chimeras (Abtahian et al., 2003). In contrast, here we show that wild-type

cells are unable to rescue vascular mixing conferred by deficient cells during embryonic development. This apparent difference in cell autonomy does not appear to reflect a functional difference between adult and embryonic hematopoietic cells, as chimeric fetal liver harvested from embryos with vascular phenotypes does not confer a vascular phenotype when transplanted into lethally irradiated adult animals (Figure S5). Instead, these results are likely to reflect differences in either the hematopoietic environment (i.e., fetal liver versus adult bone marrow) or the nature of the angiogenic response (i.e., vessel development versus vessel repair). A proposed mechanism by which wild-type cells prevent development of the vascular phenotype in adult radiation chimeras but not embryo chimeras is through competitive exclusion of KO cells, at the level of release into the circulating blood and/or incorporation into peripheral vessels (see diagram, Figure 6B). Our finding that *Slp-76*-deficient animals have an abnormal peripheral blood CEP population despite a normal bone marrow EPC population is suggestive of a block in progenitor cell recruitment/exit from the bone marrow. Syk and *Slp-76* are required for transduction of integrin outside-in signals (Law et al., 1999; Mocsai et al., 2002; Newbrough et al., 2003; Oberfell et al., 2001), and integrins mediate both progenitor cell recruitment from the bone marrow

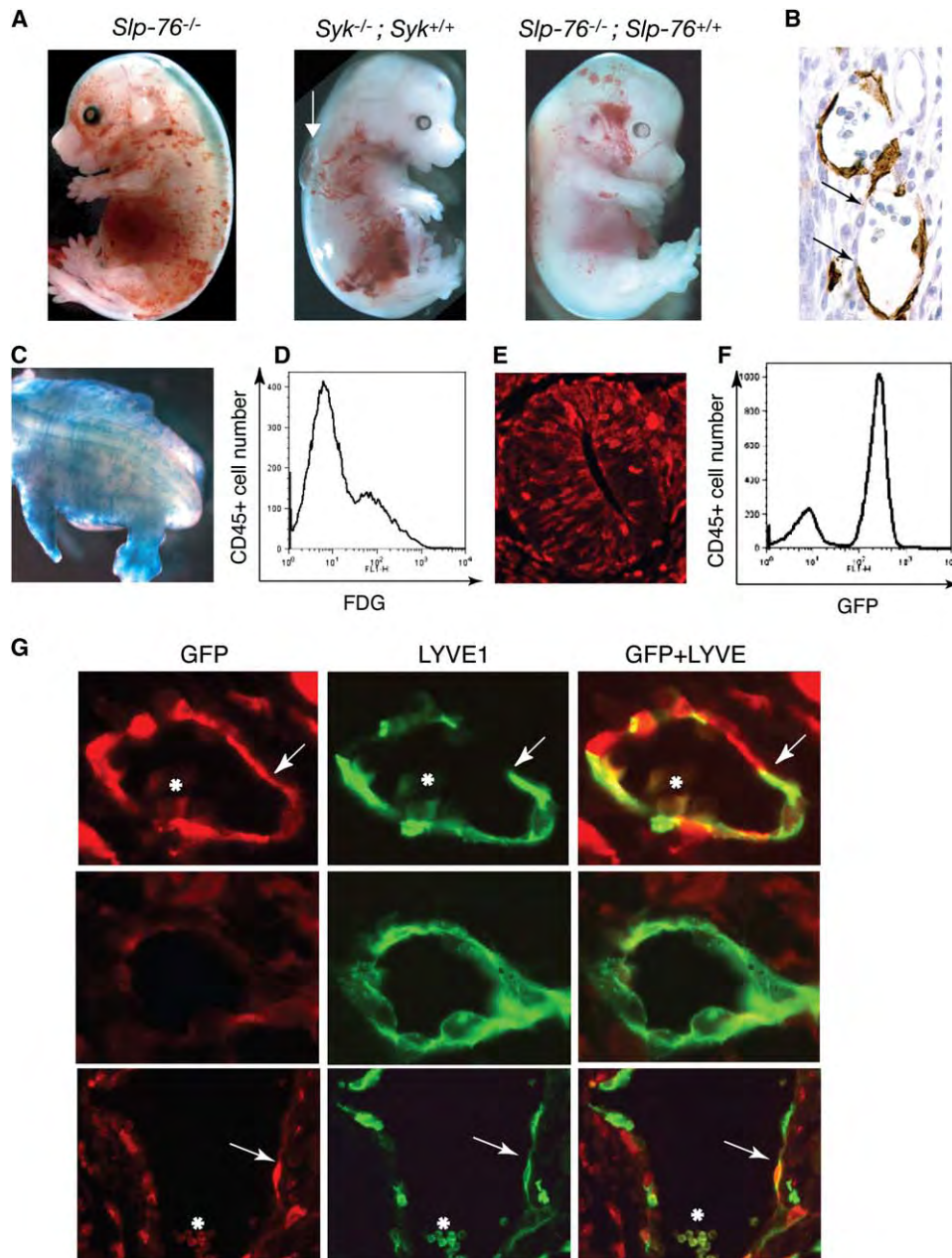


Figure 4. Cell-Autonomous Conferral of Vascular Mixing by Deficient Cells in Chimeric Embryos

(A) Chimeric E14.5 embryos generated by injection of either *Syk*-deficient or *Slp-76*-deficient ES cells into wild-type blastocysts derived from ROSA26 mice are shown. The white arrow indicates a region of edema overlying a focal region of blood-filled lymphatics in a *Syk*^{-/-}; *Syk*^{+/+} chimeric embryo.

(B) Identification of blood-lymphatic heterotypic vessels in chimeric embryos with vascular mixing. LYVE1 immunostaining of chimeric embryos with visible vascular phenotypes revealed vessels composed of LYVE1⁺ and LYVE1⁻ endothelial cells identical to those seen in deficient embryos (Abtahian et al., 2003).

(C) Contribution of wild-type cells in a chimeric embryo demonstrated by X-gal staining. X-gal staining of the whole embryo of the *Syk*^{-/-}; *Syk*^{+/+} chimeric embryo in (A) is shown.

(D) Contribution of wild-type cells to fetal liver hematopoietic tissue demonstrated by detection of β -galactosidase with the fluorescent substrate FDG in CD45⁺ hematopoietic cells. The result shown is representative of studies performed on six embryos with gross and histologic evidence of blood-filled lymphatics.

(E) Contribution of GFP⁺ *Syk*-deficient cells in chimeric embryos generated by injection of GFP⁺, *Syk*^{-/-} ES cells into wild-type blastocysts. GFP is detected as red immunofluorescence in cells of the developing trachea.

(F) Identification of GFP⁺, *Syk*^{-/-} cells in fetal liver hematopoietic tissue of a chimeric embryo using flow cytometry.

(G) *Syk*-deficient endothelial cells are found in blood-lymphatic heterotypic vessels. The contribution of *Syk*-deficient cells to vessels containing LYVE1⁺; LYVE1⁻ heterotypic (top and bottom panels) and LYVE1⁺ homotypic endothelium (middle panel) was determined using dual immunofluorescent staining for GFP (red) and LYVE1 (green). Note the presence of blood cells (asterisk) in heterotypic vessels (found in regions of the embryo with vascular mixing) but not in homotypic vessels (found in regions of the embryo without vascular mixing). The vessels shown are representative of 27 heterotypic and 13 homotypic vessels examined in three embryos.

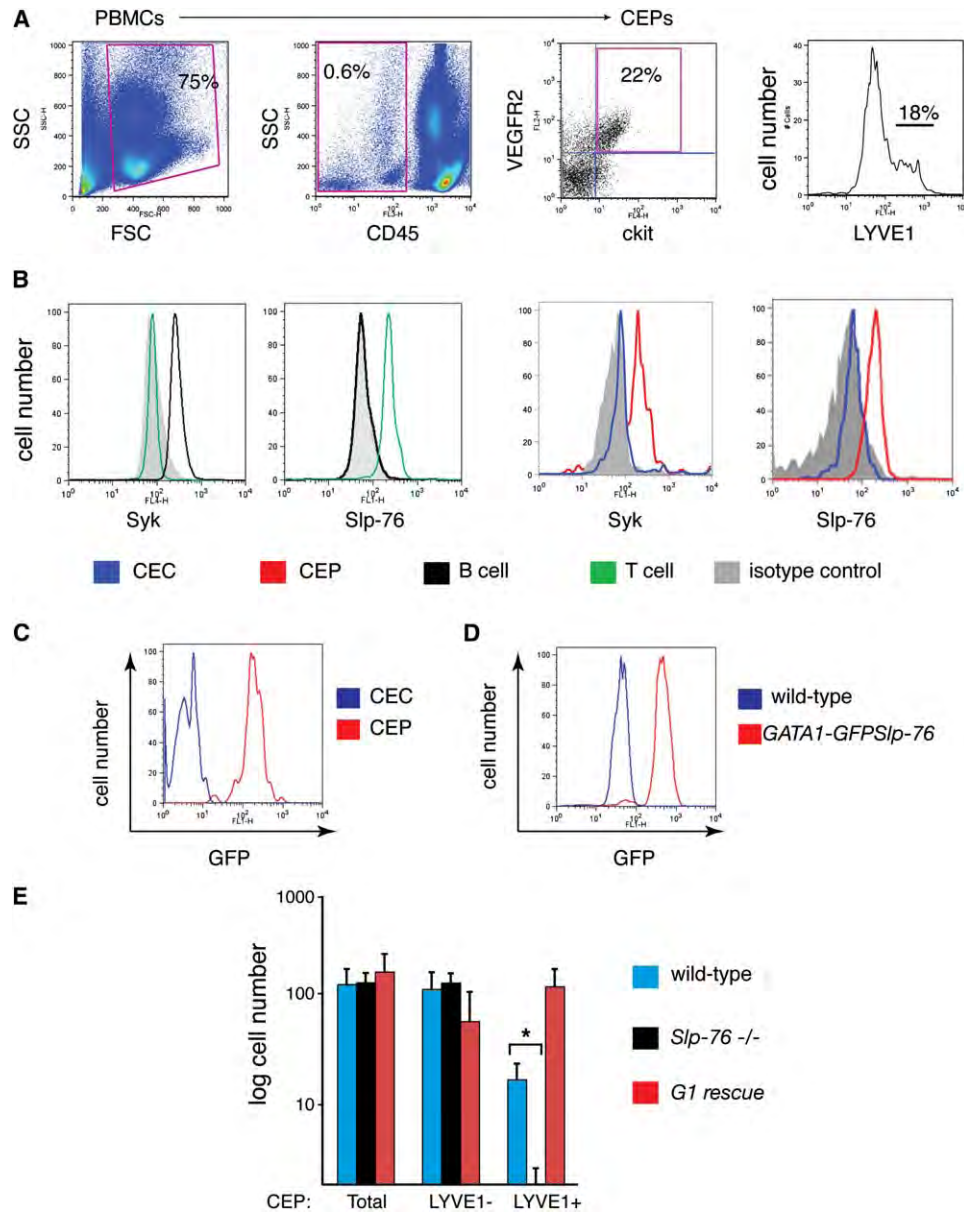


Figure 5. Expression and Function of Syk and Slp-76 in Circulating Endothelial Precursors

(A) Flow cytometric identification of circulating endothelial precursors (CEPs). The strategy to identify CEPs from total peripheral blood mononuclear cells (PBMCs) using cell surface markers is shown. The boxed cell population identifies the population of cells selected by each gate. The far right panel shows LYVE1 expression in a population of CD45⁻, ckit⁺, VEGFR2⁺ CEPs.

(B) Syk and Slp-76 are expressed in CEPs but not circulating endothelial cells (CECs). In lymphocytes, Syk is detected in B (black lines) but not T (green lines) cells, while Slp-76 is detected in T but not B cells (top panels). Background staining of lymphocytes obtained using an isotype-matched control antibody is shown in gray. Both Syk and Slp-76 are expressed in CD45⁻; VEGFR2⁺; ckit⁺ CEPs, but not in CD45⁻; VEGFR2⁻; ckit⁻ CECs (bottom panels).

(C) CEPs but not CECs are hematopoietic. CEPs and CECs were isolated from animals that had been lethally irradiated and reconstituted with GFP-expressing marrow to test for hematopoietic origin.

(D) The GATA1-GFP/Slp-76 transgene drives GFP/Slp-76 expression in CEPs.

(E) Slp-76 is required to generate LYVE1⁺ CEPs. Total, LYVE1⁻, and LYVE1⁺ CEPs were measured in the blood of wild-type (blue bars, n = 7), Slp-76-deficient (black bars, n = 6), and *G1 rescue* (red bars, n = 7) mice. Mean and standard deviation are shown. *p = 0.001 by Student's t test.

(Papayannopoulou and Nakamoto, 1993; Papayannopoulou et al., 1998) and precursor cell homing to the vasculature (Jin et al., 2006). Progenitor cell release from the bone marrow is a complex event that requires transmigration from the osteal zone to the vascular zone and across the bone marrow sinusoidal endothelium (Heissig et al., 2002). While little is known regarding

cellular egress from the fetal liver, because that organ is less structured and complex than bone marrow it is possible that a migration defect that confers a competitive advantage to wild-type cells in the adult bone marrow would not do so in the fetal liver. Such a migration defect might also explain the connections made between blood and lymphatic vessels, as blood-borne

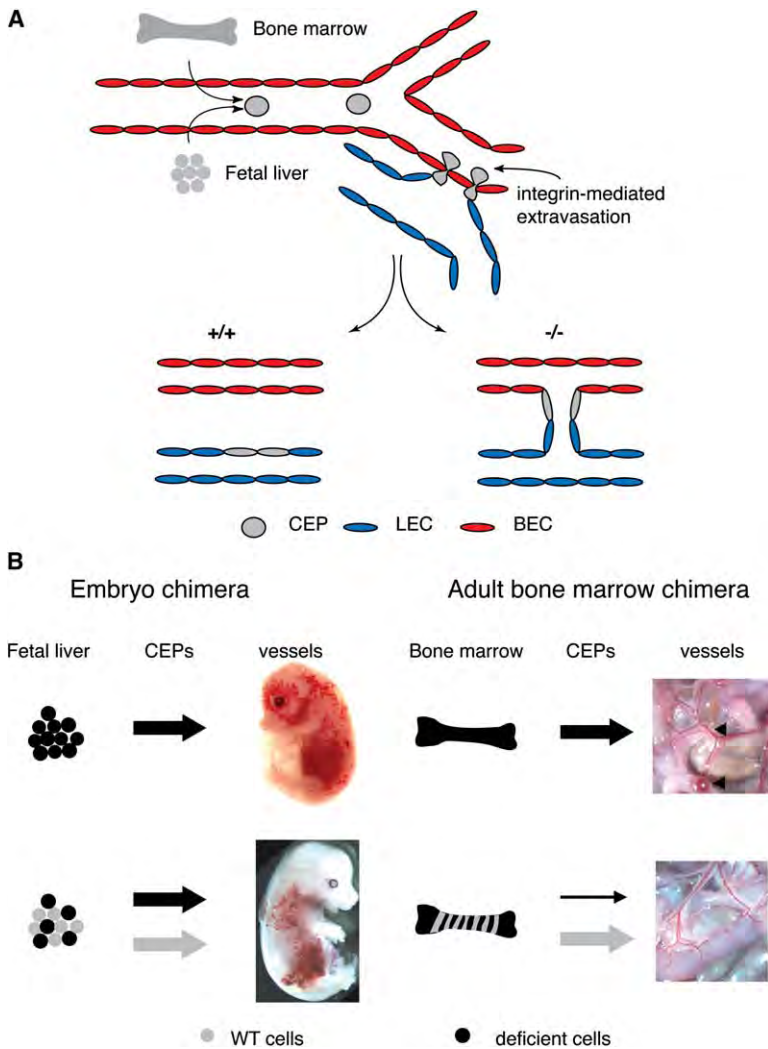


Figure 6. A Model of Blood-Lymphatic Mixing Conferred by Hematopoietic Endothelial Precursors Lacking Syk or Slp-76

(A) A model by which hematopoietic endothelial progenitor cells lacking Syk or Slp-76 create blood-lymphatic connections is shown. Endothelial progenitors from the fetal liver or bone marrow must enter and then exit the blood vascular system to contribute to lymphatic vessel growth or repair. Loss of integrin signaling through Syk and Slp-76 is postulated to hinder migration both into and out of the blood. Incomplete exit from blood vessels results in the formation of blood-lymphatic connections and heterotypic vessels containing LYVE1⁺ and LYVE1⁻ endothelial cells.

(B) A competitive model to explain blood-lymphatic vascular mixing in embryo chimeras but not adult bone marrow chimeras. More stringent cell migration required to enter the blood from the bone marrow than the fetal liver is postulated to confer a competitive advantage to wild-type endothelial progenitors that prevents the development of vascular mixing in the adult bone marrow chimera. The hematopoietic tissue, circulating endothelial precursor cell population, and vascular phenotype for each type of experiment are shown. Arrow thickness indicates relative contribution of wild-type (gray) and deficient (black) cells to the circulating endothelial precursor pool. Arrowheads indicate blood-filled mesenteric lymphatics and Peyer's patches in the gut of an affected adult bone marrow chimera.

endothelial precursors destined for the lymphatic system must first completely exit the blood vascular system and failure to do so could result in blood-lymphatic connections (see model, Figure 6). Whether the vascular phenotype in mice lacking Syk and Slp-76 arises due to loss of signaling downstream of integrin receptors in hematopoietic endothelial precursors is an important area of future investigation that will require the development of new techniques to isolate and/or generate enough of these rare cells to directly study integrin-dependent function such as migration.

Regardless of the nature of the defect, the vascular phenotype of mice lacking Syk or Slp-76 links our understanding of the hematopoietic-endothelial cell relationship in developing and adult animals. Recently, a significant field has emerged studying the role of hematopoietic precursor cells in adult angiogenesis associated with tumor growth and vascular ischemia. Clinical trials suggest an important role for these cells in human vascular disease (Losordo and Dimmeler, 2004; Werner et al., 2005), and both blocking and accelerating the release of hematopoietic endothelial precursor cells have been proposed as novel therapies. However, the fact that endothelial precursor cell function has been studied

primarily using induced angiogenic responses in animals that have undergone hematopoietic reconstitution has sparked debate over the very nature of these cells, as well as whether they serve an equally important role in natural angiogenic responses. Studies of mice lacking Syk and Slp-76 provide essential evidence that hematopoietic endothelial precursors are genuine contributors to vascular growth in vivo, and suggest the presence of progenitor cell diversity that may expand both the biological role and therapeutic potential of these cells.

Experimental Procedures

Mice

Syk-deficient and Slp-76-deficient mice have been previously described (Clements et al., 1998; Turner et al., 1995). ROSA26 and Tie2-GFP transgenic mice were obtained from Jackson Labs. GATA1-GFP/Slp-76 transgenic mice were generated by cloning an eGFP-Slp-76 fusion protein into the Not1 site of a GATA1 minigene generously provided by Dr. Stuart Orkin (McDevitt et al., 1997). Transgenic mice were genotyped by PCR using primer sequences specific for GFP. All mice were housed under pathogen-free conditions at the University of Pennsylvania in accordance with National Institute of Health guidelines and approved animal protocols.

Antibodies

Surface antibodies used include anti-murine CD3 ϵ (145-2C11), CD4 (GK1.5), CD8 α (53-6.7), CD11b (M1/70), CD11c (HL3), CD25 (7D4), CD44 (1M7), CD45 (clone 30-F11), CD45R (RA3-6B2), CD90.2 (50-2.1), CD117 (2B8), CD144 (11D4.1), flk1 (Avas 12 α 1), Ly-6A/E (Sca-1, clone E13-161.7), Ly-6G (Gr-1, clone RB6-8C5), NK-1.1 (PK136), Pan-NK cells (DX5), and TER-119 (all obtained from BD PharMingen). Polyclonal rabbit anti-mouse LYVE1 was generated as previously described (Prevo et al., 2001). Intracellular antibodies included mouse IgG_{2a} anti-murine SLP-76 (clone MS76; eBioscience) and a mouse monoclonal anti-Syk antibody generously provided by Dr. Arthur Weiss, University of California at San Francisco (clone 5F5.2; Chu et al., 1999). Secondary antibodies included: goat anti-mouse IgG + IgM-fluorescein isothiocyanate (FITC) (polyclonal), mouse anti-rat IgG_{1/2a}-biotin (clone G28-5), streptavidin-allophycocyanin (APC), streptavidin-FITC, streptavidin-peridinin chlorophyll-a (Sav-PerCP) (all BD PharMingen), APC-conjugated goat anti-mouse IgG (H+L) (Molecular Probes), and goat anti-rabbit IgG (PharMingen).

Flow Cytometric Analysis

Blood collected from the inferior vena cava of anesthetized adult mice was washed in collecting media (Iscove's modified Dulbecco's media, 5% heat-inactivated fetal calf serum [FCS], 5 mM β -mercaptoethanol, and 20 mM EDTA), spun down, and incubated with 1:200 dilution of purified CD16/CD32 antibody (Fc Block; BD PharMingen) for 10 min at 4°C prior to subsequent antibody stains. After the final antibody staining, the cells were washed once more in collecting media, spun down, and resuspended in red blood cell lysis buffer (Becton Dickinson). For intracellular staining, cells other than CEPs were fixed with 1% paraformaldehyde for 10 min, washed, and resuspended in permeability buffer (2% saponin in staining media) for 10 min at room temperature. CEPs were permeabilized and fixed by addition of 1 ml ice-cold methanol. After permeabilization, cells were stained with a 1:50 concentration of anti-murine Syk monoclonal antibody or anti-murine Slp-76 antibody. Goat anti-mouse FITC was used as a secondary antibody. Cells were stained according to standard protocols and examined on a Becton Dickinson FACS Caliber or Becton Dickinson LSRII cytometer. FlowJo software (Tree Star) was used to analyze the data. When analyzing CEPs, a minimum of 1,500,000 cells was collected per sample; a minimum of 60 CD45⁻ flk1⁺ CD117⁺ cells had to be acquired to be included in this study.

Platelet Signaling Studies

Platelets were isolated from anesthetized adult mice as previously described (Chen and Kahn, 2003). Washed platelets (1×10^6) were stimulated with convulxin (CVX; Alexis Biochemicals), AYPGKF (University of Pennsylvania Protein Chemistry Laboratory), or ADP (Chronolog) in the presence of PE-conjugated anti-murine CD62P antibody (BD PharMingen) for 20 min at 37°C and P-selectin surface expression was quantified by flow cytometry.

Measurement of Neutrophil Respiratory Burst

Bone marrow-derived neutrophils were isolated as previously described (Newbrough et al., 2003). Ninety-six-well Immulon 4 HBX plates (Thermo Labsystems) were coated with 150 μ g/ml fibrinogen (Sigma) in PBS, 15 μ g/ml poly-RGD (Sigma) in PBS, or 20 μ g/ml anti-CD18 (C71/16) in carbonate buffer. Antibody-coated wells were blocked with 10% FCS for 30 min. One hundred microliters of prewarmed neutrophils suspended at 4×10^6 cells/ml in Hank's balanced salt solution, 10 mM HEPES, 100 mM ferricytochrome C (Sigma Aldrich), and 1 mM Ca²⁺ was added to the wells. Adherent respiratory burst was measured through oxidation-induced changes in ferricytochrome C absorbance as previously described (Newbrough et al., 2003). When indicated, 50 ng/ml murine TNF α , 10 μ M fMLP, or 100 nM PMA was added.

Neonatal Skin Cell Preparation

Neonatal skin was minced, and resuspended in a 10 ml volume of Dispase (BD Biosciences) and collagenase P (Sigma). The samples were incubated for 1 hr at 37°C with shaking, followed by a passage through two 70 μ m nylon cell strainers (BD Falcon). The cell suspen-

sions were washed twice with staining media and stained as described.

Histology and Immunohistochemistry

Tissues were fixed in 2% paraformaldehyde, and paraffin-embedded sections were studied as described at <http://www.uphs.upenn.edu/mcrrc/histology/histologyhome.html>. Dual staining for GFP was accomplished using goat anti-LYVE1 (R&D Systems) and rabbit anti-GFP (Research Diagnostics).

Generation of Chimeric Embryos

Chimeric embryos were generated by injecting Syk-deficient and Slp-76-deficient embryonic stem cells into blastocysts harvested from ROSA26 mice using standard methods. *Slp-76*^{-/-} and *Syk*^{-/-} ES cells were generated as previously described (Mortensen et al., 1992). Individual Syk-deficient ES cell clones expressing GFP were isolated following exposure to GFP-expressing lentivirus as previously described (Chen et al., 2006).

β -Galactosidase Staining of Chimeric Embryos

Embryos were fixed at 4°C for several hours in 2% paraformaldehyde and then washed for 2 hr with room temperature PBS. After the last rinse, the embryos were placed in foil-covered scintillation vials containing staining solution (2 mM MgCl₂, 5 mM K⁺ ferricyanide, 5 mM K⁺ ferrocyanide, 1% NP-40, 1 \times PBS, and 0.1% X-gal dissolved in dimethylformamide) and gently rocked for 24 hr at 37°C. The embryos were then rinsed four to five times in PBS over the course of several hours, until the yellow tint was no longer present. The embryos were subsequently stored in 2% paraformaldehyde.

Detection of β -Galactosidase⁺ Hematopoietic Cells in Chimeric Embryos

Individual embryonic fetal livers were minced and passed through two 70 μ m nylon filters to create single-cell suspensions. The cells were then prepared according to standard protocols using fluorescein di- β -D-galactopyranoside (FDG) from Molecular Probes to detect cells expressing β -galactosidase. Viable CD45⁺ cells were analyzed for fluorescein expression.

Statistics

Means and standard deviations are shown with the number of samples for each group indicated as the N. p values shown were calculated using the two-tailed Student's t test.

Supplemental Data

Supplemental Data include one table and five figures showing additional information regarding GATA1-GFP⁺ Slp-76 transgenic mice, *Syk*^{+/+}; *Syk*^{-/-} embryo chimeras, identification and characterization of circulating endothelial precursor cells, and comparison of phenotypes in *Syk*^{+/+}; *Syk*^{-/-} chimeric embryos and adult radiation chimeras and are available at <http://www.developmentalcell.com/cgi/content/full/11/3/349/DC1/>.

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