Inhibition of Thrombin Receptor Signaling on α-Smooth Muscle Actin+ CD34+ Progenitors Leads to Repair After Murine Immune Vascular Injury

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Objective—The goal of this study was to use mice expressing human tissue factor pathway inhibitor (TFPI) on α-smooth muscle actin (α-SMA)+ cells as recipients of allogeneic aortas to gain insights into the cellular mechanisms of intimal hyperplasia (IH).

Methods and Results—BALB/c aortas (H-2d) transplanted into α-TFPI–transgenic (Tg) mice (H-2b) regenerated a quiescent endothelium in contrast to progressive IH seen in C57BL/6 wild-type (WT) mice even though both developed aggressive anti-H-2d alloresponses, indicating similar vascular injuries. Adoptively transferred Tg CD34+ (but not CD34-) cells inhibited IH in WT recipients, indicating the phenotype of α-TFPI–Tg mice was due to these cells. Compared with syngeneic controls, endogenous CD34+ cells were mobilized in significant numbers after allogeneic transplantation, the majority showing sustained expression of tissue factor and protease-activated receptor-1 (PAR-1). In WT, most were CD45+ myeloid progenitors coexpressing CD31, vascular endothelial growth factor receptor-2 and E-selectin; 10% of these cells coexpressed α-SMA and were recruited to the neointima. In contrast, the α-SMA+ human TFPI+ CD34+ cells recruited in Tg recipients were from a CD45- lineage. WT CD34+ cells incubated with a PAR-1 antagonist or taken from PAR-1-deficient mice inhibited IH as Tg cells did.

Conclusion—Specific inhibition of thrombin generation or PAR-1 signaling on α-SMA+ CD34+ cells inhibits IH and promotes regenerative repair despite ongoing immune-mediated damage. (Arterioscler Thromb Vasc Biol. 2012;32:42-49.)

Key Words: coagulation ■ immune system ■ thrombin ■ transplantation ■ vascular biology

Intimal hyperplasia (IH) developing after immune-mediated injury is associated with vascular remodeling, progressive loss of lumen, and chronic ischemia of downstream tissues. This process underpins chronic rejection after transplantation, in which the injury is mediated by the alloimmune response.

See accompanying article on page 3

The precise cellular mechanisms by which IH develops are not clearly understood. A commonly accepted model proposes that T cells and macrophages infiltrating the tunica intima and adventitia1,2 make cytokines that induce changes in medial vascular smooth muscle cells (SMCs), causing them to migrate, proliferate, and become modulated SMCs characteristically seen in IH.3 Interferon γ (IFN-γ) emerges as an important cytokine influencing SMC phenotype in this model.4

Data from rodent models of transplant arteriosclerosis (TA)5–7 indicate that a significant proportion of α-smooth muscle actin (α-SMA)-expressing neointimal cells are recipient derived. Although labeled SMCs by some authors, recent data indicate that these cells are inflammatory leukocytes, lacking expression of markers of a true SMC lineage, such as myosin heavy chain and calponin.8 The role and importance of these recipient α-SMA+ leukocytes in the pathogenesis of IH is unknown.

Our previous work partly addressed this. We examined bone marrow (BM)-derived α-SMA+ cells following mechanical endoluminal injury.9–11 Tissue factor (TF)–initiated thrombin generation and protease-activated receptor-1 (PAR-1) stimulation on these cells was necessary for IH to develop. Transgenic mice expressing an anticoagulant fusion protein based on human tissue factor pathway inhibitor (hTFPI) under the control of a modified α-SMA promoter (α-TFPI–Tg mice) failed to develop the IH seen in wild-type (WT) mice. Instead, the neointima contained α-SMA+ hTFPI-expressing cells covered by a quiescent endothelium; ie, the arteries were repaired back to a preinjured state. BM chimera and adoptive transfer experiments showed that repair was dependent on transgene expression by CD34+ cells.9 Additionally, inhibition of PAR-1 on transferred CD34+ cells

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42
induced the same phenotype. In an attempt to understand neointima formation in WT mice, we showed that thrombin induced proliferation, inhibited apoptosis, and promoted the dominant outgrowth of a minority population of circulating α-SMA+/CD34+ cells (accounting for approximately 3% of all circulating CD34+ cells), which had a phenotype similar to those found in the neointima of WT animals. We concluded that progressive IH was due to the effects of locally generated thrombin/PAR-1 interactions on these CD34+ cells once recruited to the neointima.

Because this “single-hit” mechanical injury model has few clinical counterparts, we have continued this work using mouse aortas across a full major histocompatibility complex mismatch (H-2d to H-2b) without immunosuppression, a model that involves rapid destruction of the endothelium of transplanted allografts and continuous immune-mediated damage to the donor vessel resulting in IH within 4 to 6 weeks.

Materials and Methods
An expanded Methods section is available in the supplemental materials, available online at http://atvb.ahajournals.org.

Animals and Experimental Models
C56BL/6 (WT) and BALB/c mice (Harlan Olac Ltd, Bicester, United Kingdom) ROSA-enhanced yellow fluorescent protein (EYFP), heterozygous α-TFPI-Tg, and homozygous PAR-1- and PAR-4-deficient mice were bred and maintained at Imperial College London. All had been maintained for more than 10 generations on a BL/6 background. All procedures were approved by United Kingdom Home Office.

Aortic Transplantation
Mice were transplanted using a sleeve anastomosis technique. Briefly, a 5-mm segment of infrarenal donor aorta, flushed with heparin, was transplanted into recipient aorta. Blood flow was confirmed by direct inspection after the clamps were removed.

Skin Transplantation
Full-thickness tail skin was grafted on the lateral flanks of recipients 14 days after aortic transplantation and protected under sterile gauze covered by plaster for 9 days. Rejection was counted when no viable skin remained.

Wire-Induced Endoluminal Carotid Artery Injury
This injury was performed as previously described. Briefly, a 100-μm-diameter wire was introduced and withdrawn 3 times into the common carotid via the external carotid artery before the external carotid artery was tied off. After confirming restoration of normal blood flow through the common carotid, the skin was closed, and animals were allowed to recover.

Morphometric Analysis and Immunohistology
Sections were prepared and examined as previously described. For analysis of intimal and medial areas, at least 3 random sections were examined from each of ≥5 arteries. For immunofluorescence analysis, images were examined using the MetaMorph system (Universal Imaging, Downingtown, PA). For confocal, a Leica DM-IRBE microscope was used, and images were acquired using Leica TCS-NT software. Infiltration by immune cells was analyzed by manual counting at ×400 magnification. Average counts were derived from examination of at least 6 random sections from each artery. For the antibodies (Abs) used, see the supplemental materials.

CD34+ Cell Separation and Injection
CD34+ cells were isolated as previously described and had an average purity of 95%. For comparative immunophenotyping using immunocytofluorescence, at least 300 cells were counted at ×200 magnification from at least 3 random fields from 3 different wells. For functional experiments, CD34+ cells were purified from mice 2 to 4 days after wire-induced injury, incubated in vitro for 1 hour, before 7.5×107 to 1×108 cells were injected intravenously into aortic recipients 1 week posttransplantation.

Assessment of Antidonor Immune Responses
Antidonor Ab titers were assessed on donor splenocytes by flow cytometry. Circulating IFNγ was analyzed by ELISA (R&D Systems). BM-derived dendritic cells (DCs) were isolated and cultured as described elsewhere. T Cells were prepared from splenocytes and lymph node cells. Proliferation was assessed by [3H]thymidine incorporation.

Statistical Analysis
Data are presented as means±SEM. Significance of the difference between 2 groups was determined by unpaired Student t or log rank test. Values of P<0.05 were considered statistically significant.

Results
Phenotype of Aortic Transplants
Six weeks after transplantation into C57BL/6 (H-2b, WT) recipients, BALB/c (H-2b) aortas showed florid IH with neointimal areas and neointimal/media ratios ≈10-fold higher than those of syngeneic controls (Figure 1A–1C). Immunofluorescence of frozen sections showed widespread staining for TF colocalized with α-SMA in neointima (Figure 1G). To confirm that recipient α-SMA+ cells were TF+ we transplanted BALB/c aortas into ROSA-EYFP mice expressing EYFP on all cells (Figure 1H).

Aortas transplanted into α-TFPI-Tg mice (H-2b) did not develop IH (Figure 1D–1F). Immunofluorescence revealed no intimal TF staining, but there was a rim of recipient hTFPI on the luminal aspect (Figure 1I). IH did not develop when syngeneic grafts were transplanted into either strain (Figure 1C and 1F).

These data confirm that recipient α-SMA+TF+ cells infiltrate the intima of transplanted vessels and that expression of hTFPI by these cells is associated with absence of IH.

Immune Activation and Effector Mechanisms in WT Versus α-TFPI-Tg
Titers of donor-specific, anti-BALB/c IgM and IgG Abs were similar in WT and α-TFPI-Tg recipients (Supplemental Figure IA and IB), as was the density of T cells, CD68+ macrophages and CD11c+ DCs infiltrating transplanted vessels (Supplemental Figure IC and ID).

There were no differences in the proportions of IFNγ-secreting CD4+ or CD8+ T cells from spleen (Supplemental Figure IE and IF) or lymph nodes (Supplemental Figure IIA and IIB) in WT or α-TFPI-Tg recipients. Levels of circulating IFNγ at 2 weeks were also similar (Supplemental Figure IG).

T cells and DCs from each strain had a similar phenotype. Importantly, in α-TFPI-Tg mice, there was no fusion protein expressed by either cell (Supplemental Figure IIC and IID). In vitro proliferative alloresponses to BALB/c DCs by T cells from WT and α-TFPI-Tg mice were similar (Supplemental Figure IIE), and DCs from each strain promoted similar proliferative T cell responses by allogeneic T cells (Supplemental Figure IIIF). Finally, WT and α-TFPI-Tg recipients
rejected BALB/c skin grafts at a similar tempo, even after a previous BALB/c aortic graft (Supplemental Figure IH), indicating similar alloimmune response to BALB/c aortas were comparable in WT and -TFPI-Tg mice. Therefore differences in the nature or intensity of the alloimmune injury were unlikely to account for the aortic phenotypes observed.

Adoptive Transfer of CD34<sup>+</sup> Cells After Aortic Transplantation

Peripheral blood leukocytes were purified from WT or -TFPI-Tg mice after endoluminal injury, separated into CD34<sup>+</sup> and CD34<sup>-</sup> fractions, and injected into either WT or -TFPI-Tg recipients 1 week posttransplantation. Endoluminal injury was used as a mechanism to enrich vascular progenitors within the CD34<sup>+</sup> population.10

The CD34<sup>+</sup>, but not CD34<sup>-</sup> fractions significantly affected the phenotype of IH. CD34<sup>+</sup> cells from -TFPI-Tg mice inhibited the development of IH in WT recipients, whereas CD34<sup>-</sup> cells from WT mice promoted IH in -TFPI-Tg recipients (Figure 2A and 2B).

-TFPI<sup>+</sup> staining was detectable on the luminal aspect of the graft 2 weeks after injection of cells from -TFPI-Tg mice (Figure 2C). With cells from ROSA-EYFP mice, YFP<sup>+</sup> cells were seen throughout the neointima 6 weeks after injection (Figure 2D). Both these results illustrate that injected CD34<sup>+</sup> cells are recruited into the alogeneic aorta.

Following our previous demonstration that hTFPI on these cells was acting to prevent thrombin generation,9,10 CD34<sup>+</sup> cells from WT or ROSA-EYFP mice were incubated with a selective PAR-1 antagonist before injection. Compared with controls, these cells prevented IH (Figure 2A and 2B). In addition, CD34<sup>+</sup> cells from PAR-1-deficient animals prevented IH (Figure 2A and 2B). Controls from a second strain, deficient in the low-affinity thrombin receptor PAR-4, also inhibited IH, but to a significantly lesser degree than cells from PAR-1 knockout mice.

All these data indicate that CD34<sup>+</sup> cells have an important influence on the hyperplastic response following alloimmune injury and that inhibiting thrombin generation or PAR-1 signaling on these cells is sufficient to prevent IH.

Phenotype of Endogenous Circulating CD34<sup>+</sup> Cells

There was a significant increase in the number of circulating CD34<sup>+</sup> cells after transplantation (Figure 3A). Beyond day 3, numbers fell rapidly back to baseline after syngeneic transplantation but remained high after allogeneic transplantation. Cell numbers were similar in -TFPI-Tg mice (data not shown).

TF<sup>+</sup> cells accounted for ≈75% of the CD34<sup>+</sup> cells in the circulation after allogeneic transplantation but only 20% after syngeneic transplantation. Cells expressing PAR-1 made up to 50% of the CD34<sup>+</sup> cells after allogeneic transplantation but were only a minority population after syngeneic transplantation, accounting for <10% of the CD34<sup>+</sup> cells. Finally, -SMA<sup>+</sup> cells were found, making up ≈10% of CD34<sup>+</sup> cells.
in both strains after allogeneic transplantation but <1% cells on day 7 after syngeneic transplantation.

Comparing the phenotype of CD34⁺ cells in WT with those of α-TFPL-Tg mice 7 days after allogeneic transplantation, the same proportion expressed TF, PAR-1 (Figure 3B), vascular endothelial growth factor receptor-2 (VEGFR-2), α-SMA, tyrosine kinase with immunoglobulin and EGF homology domains (TIE)-2, and CD31, but there were significant differences in the expression of these molecules by α-SMA⁺CD34⁺ cells (Table). The majority in WT expressed PAR-1 alongside CD45, CD11b, CD68, F4/80, Ly-6C, CD11b, CD68, F4/80, Ly-6C, CD115, CX3CR1, and CCR2, indicating that they were myeloid progenitors. In addition, almost all the α-SMA⁺ cells from WT coexpressed VEGFR-2, CD31, TIE-2, and E-selectin, suggesting an angiogenic phenotype. In contrast, the majority of α-SMA⁺CD34⁺ cells from α-TFPL-Tg mice were negative for CD45, CD11b, CD115, F4/80, Ly-6C, CX3CR1, CCR2, and PAR-1 and expressed no VEGFR-2, CD68, CD31, or E-selectin. In these mice, expression of the hTFPI fusion protein was found on both the CD45⁺ and CD45⁻ subpopulations, confirming that the promoter driving transgene expression was active in both
lineages (Figure 3C). In contrast, despite immunocytofluorescence examination of >8000 individual CD34⁺ cells from the peripheral blood of 4 different groups of transplanted α-TFPI-Tg recipients, we detected no α-SMA or hTFPI expression that could not be accounted for by contaminating CD34⁺ cells, indicating that the fusion protein was only expressed by circulating CD34⁺ cells.

The differences between WT and α-TFPI-Tg mice were due entirely to the expression of the TFPI fusion protein, as an anti-hTFPI monoclonal Ab, injected at the time of transplantation, was associated with the appearance of significant numbers of CD31⁺CD45⁺ cells in these mice (Supplemental Table II). Moreover, these changes could be reversed if the Ab was administered with a PAR-1 (but not a PAR-2) antagonist (Supplemental Table II), suggesting that the effect of TFPI was to inhibit signaling through PAR-1 but not PAR-2.

These data indicate profound TF and PAR-1-dependent differences between strains in the mobilization and phenotype of CD34⁺ cells posttransplantation, with the α-TFPI-Tg mice having reduced proportions of circulating CD45⁺ myeloid progenitors (particularly CD45⁺α-SMA⁻ cells), increased proportions of CD45⁺α-SMA⁺ cells, reduced expression of PAR-1 by α-SMA⁺ cells, and an absence of α-SMA⁻ cells coexpressing VEGFR-2, CD31, and E-selectin.

### Recruitment of Cells to Aorta After Injury

To link these differences in endogenous CD34⁺ cells to IH or repair, we studied recruitment of cells to the transplanted allografts using confocal microscopy. As previously reported, donor endothelial cells were rapidly lost within 24 to 72 hours in both WT and α-TFPI-Tg (Figure 4A). In WT, new α-SMA-expressing cells were visible on the luminal aspect by day 7; these cells coexpressed CD31, CD45, and E-selectin, consistent with recruitment of the majority population of circulating CD34⁺α-SMA⁺ cells. By day 14, IH was becoming established, and there was widespread expression of CD31 and CD45 throughout the neointima, with no evidence of a new endothelium being formed (Figure 4).

In α-TFPI-Tg recipients, cells expressing hTFPI were detectable on the luminal aspect of the transplanted aorta by day 3; these were CD34⁺ but negative for CD31, CD45 (Figure 5), and E-selectin (not shown), consistent with recruitment of the majority population of endogenous CD34⁺α-SMA⁺ cells in these mice. Recruitment of these cells was associated with the appearance of CD31⁺ cells on the luminal aspect; these were also CD34⁺ but CD45⁻ and E-selectin-negative and appeared in areas distinct from the

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**Table.** Proportion of CD34⁺α-SMA⁺ Cells Expressing the Indicated Molecules, Expressed as Percentage (±SEM) of Total CD34⁺α-SMA⁺ Cells

<table>
<thead>
<tr>
<th>Phenotype of Circulating CD34⁺α-SMA⁺ Cells, d 7 (%)</th>
<th>WT</th>
<th>α-TFPI-Tg</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TF</td>
<td>100 (±0)</td>
<td>88 (±7.2)</td>
<td>NS</td>
</tr>
<tr>
<td>PAR-1</td>
<td>91.7 (±4.8)</td>
<td>9.2 (±5.8)</td>
<td>0.0004</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>81.3 (±3.2)</td>
<td>0</td>
<td>0.000001</td>
</tr>
<tr>
<td>CD31</td>
<td>98 (±2)</td>
<td>0</td>
<td>0.0000001</td>
</tr>
<tr>
<td>TIE-2</td>
<td>83.4 (±1.8)</td>
<td>59.1 (±8.2)</td>
<td>0.04</td>
</tr>
<tr>
<td>E-selectin</td>
<td>100 (±31)</td>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>CD45</td>
<td>81.7 (±5.4)</td>
<td>18.7 (±7.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>CD68</td>
<td>69.6 (±16.1)</td>
<td>0</td>
<td>0.01</td>
</tr>
<tr>
<td>F4/80</td>
<td>80.2 (±6.4)</td>
<td>8.4 (±5.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Ly6-C</td>
<td>65.7 (±13.6)</td>
<td>5.4 (±2.9)</td>
<td>0.01</td>
</tr>
<tr>
<td>CD11b</td>
<td>73.9 (±7.8)</td>
<td>11.1 (±7.3)</td>
<td>0.004</td>
</tr>
<tr>
<td>CD115</td>
<td>71.5 (±14.7)</td>
<td>13.4 (±5.0)</td>
<td>0.02</td>
</tr>
<tr>
<td>CCR2</td>
<td>84.4 (±1.3)</td>
<td>16.9 (±3)</td>
<td>0.00003</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>83.4 (±1.8)</td>
<td>20.8 (±4.2)</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

Data are derived from 6 mice. α-SMA indicates α-smooth muscle actin; WT, wild-type; α-TFPI-Tg, α-tissue factor pathway inhibitor–transgenic; TF, tissue factor; TIE, tyrosine kinase with immunoglobulin and EGF homology domains; PAR-1, protease-activated receptor; VEGFR-2, vascular endothelial growth factor receptor-2; NS, not significant.

*Percentage (±SEM) of CD34⁺α-SMA⁺ cells expressing these molecules.*

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**Figure 4.** Recruitment of cells to aortas in wild-type (WT) recipients. A, Three-color immunohistochemical analysis of frozen sections from BALB/c aortas stained with 4',6-diamidino-2-phenylindole (DAPI) (blue), anti-α-smooth muscle actin (anti-α-SMA) (red), and anti-CD31 (green). B to E, Three-color immunohistochemical analysis of frozen sections from allogeneic aortas harvested from WT mice. Day 0—20 hours posttransplantation. All sections were stained with DAPI (blue) and anti-α-SMA or E-selectin as indicated (red) (B) or anti-CD31 or CD45 as indicated (green) (C). D, Overlay images. E, Enlarged images from the boxed areas in D. Yellow indicates colocalization. Rows from same day are consecutive sections. Results are representative of 3 independent experiments.
hTFPI-expressing cells. By day 28, a new quiescent endothelium had regenerated in the allografts transplanted into α-TFPI-Tg recipients (Figure 5).

These data confirm that the α-SMA+ cells recruited to the luminal aspect of transplanted allografts in both strains had a phenotype consistent with that of the CD34+α-SMA+ cells found in the circulation.

Phenotype of Mouse Aortas After Adoptive Transfer of CD34+ Cells
Aortas in WT mice injected with CD34+ cells from α-TFPI-Tg were examined by immunofluorescence; these developed a quiescent endothelium lacking expression of α-SMA or E-selectin (Supplemental Figure IIIA), even though the cells were injected 1 week after transplantation, at a time when recruitment of endogenous CD45+α-SMA+ cells was under way. The same appearance was also seen when WT (not shown) or ROSA-EYFP CD34+ cells incubated with PAR-1 antagonist (Supplemental Figure IIIC) were injected. Results from the latter indicated that injected CD34+ cells contributed to the new quiescent endothelial layer, as well as the α-SMA+ cells beneath. Finally, WT recipients injected with CD34+ PAR-1-deficient cells also developed a quiescent endothelium (Supplemental Figure IVA), in contrast to when PAR-4-deficient cells were used (Supplemental Figure IVB); although IH was reduced by these cells (see Figure 2), the neointima that developed had a WT appearance, with widespread staining for TF, CD31, and E-selectin.

These data indicate that thrombin or PAR-1 inhibition on CD34+ cells is sufficient to promote endothelial regeneration, despite an ongoing immune response against the donor vessel and the presence of significant numbers of recipient CD45+α-SMA+ progenitors circulating in these mice.

Discussion
This study offers 3 novel insights into TA by demonstrating, first, that IH is associated with a distinct subset of CD34+CD45+α-SMA+ cells circulating after allogeneic transplantation, whereas regenerative repair seen in α-TFPI-Tg mice is associated with a distinct subset of CD45+α-SMA+ cells; second, that IH can be prevented in the face of an aggressive antidonor immune response by specific manipulation of thrombin generation or PAR-1 receptors on CD34+ cells; and third, that this manipulation promotes regenerative repair. An important point about the regenerative repair was that it occurred in the face of an aggressive immune response to the donor tissue, without specific modulation by immunosuppressive drugs.

Coagulation proteins have previously been implicated in the pathophysiology of TA, though with little understanding of the mechanisms involved. For example, chronic rejection is associated with extensive fibrin deposition within arteriosclerotic vessels in human renal allografts18 and with widespread TF expression in rat and human cardiac allografts.19–21 Moreover, a potent thrombin inhibitor, hirudin, has been shown to attenuate experimental TA in rat cardiac allografts.22 Our data now provide a mechanistic explanation for the link between TF and IH, through thrombin/PAR-1 signaling on circulating CD34+ cells.

PAR-1 is the archetypal member of a family of G-protein-coupled, 7 transmembrane-domain cell surface receptors, designated PAR-1 to PAR-4,23–26 through which coagulation proteases mediate many of their proinflammatory effects. Thrombin, which cleaves PAR-1, -3, and -4,27 influences the phenotype of numerous cell types in this way.26 Having previously linked IH with PAR signaling on newly recruited CD34+ cells in a model of wire-induced endovascular injury,10 we now show that the same mechanism is relevant in an allogeneic transplantation model, in which the continuous immune-mediated injury is more representative of the chronic lesions seen in inflammatory vascular disease.

The immune response to transplanted aortas in α-TFPI-Tg mouse recipients was the same as in WT. Both exhibited comparable in vitro antidonor direct T cell allosresponses, stimulation of T cells by DCs, production of antidonor Ab (strongly implying comparable indirect T-cell activation);
infiltration of the graft by macrophages, T cells, and DCs; and finally, rejection of H-2d skin grafts by naïve and donor-primed animals. Others have shown that IFNγ, produced by infiltrating immune cells, is the critical cytokine driving IH, as illustrated by the severely attenuated lesions developing in recipient mice lacking IFNγ35 and, importantly, the IH that develops in immunodeficient mice lacking immune cells after administration of IFNγ.29,30 We found that the proportions of CD4+ and CD8+ T cells staining for IFNγ in lymph nodes and spleen of both WT and α-TFPI-Tg recipients were similar, as were the amounts of IFNγ detectable in the circulation posttransplantation. All these data strongly suggest that BALB/c grafts in both strains were subject to the same immune-mediated injury and were equally exposed to the cytokine stimulus driving IH. These findings are not surprising, considering that the α-TFPI-Tg mice do not have a systemically anticoagulated phenotype9 and that, within the leukocyte population, the hTFPI fusion protein is expressed by only a small proportion of CD34+ cells.

In their 2001 article using the same transplantation model, using β-galactosidase-Tg mice, Shimizu et al reported that neonontal SMCs were derived from the recipient BM cells rather than those of the donor,5 a conclusion that has since been reached by others working in different models of IH.31 These findings have remained controversial, in part because of the disputed ability of BM-derived cells to give rise to SMCs and because recent publications have directly contradicted the earlier data.32,33 Recently, Iwata et al, working in 3 separate models of IH including TA, reported significant numbers of recipient α-SMA+SM22α+ cells in the neointima, but these also expressed CD115, CD11b, F4/80, and Ly-6C, markers of the monocyte/macrophage lineage rather than established markers of differentiated SMCs such as smooth muscle myosin heavy chain or calponin.8 The authors concluded that although BM-derived α-SMA+ cells were involved in vascular remodeling, they were myeloid cells and not true SMC progenitors. Our data are compatible with this work.

Moreover, we have shown that myeloid progenitors expressing markers of the classic or inflammatory lineage make up the majority (60%–80%) of the CD34+ cells mobilized by WT mice into the circulation posttransplantation and demonstrated that some of these express α-SMA, making up nearly 10% of all CD34+ cells by day 7. Many of these α-SMA+ cells also expressed TIE-2, VEGFR-2, CD31, and E-selectin, indicating a hemangiocyte or vascular leukocyte lineage.34 Although at their peak these cells numbered no more than 30,000/mL in the circulation, these were the cells that were recruited to the luminal aspect of the allograft and that accumulated in the expanding neointima. The coexpression of α-SMA with TF and endothelial proteins, such as CD31, P-selectin, and E-selectin, in the neointima has been previously documented by us10 and by other groups working in IH56 with the luminal cells described as pseudoendothelial by some.36

These distinct CD45+α-SMA+ cells were only found circulating after allogeneic transplantation and not syngeneic transplantation, suggesting that the inflammatory environment generated by the ongoing alloresponse was responsible. Although we have not addressed which mediators are involved, it is probable that IFNγ plays a role, given its established link with TA. Other features specific to allogeneic transplantation were the sustained expression of TF and PAR-1 by the majority of circulating CD34+ cells.

That TF expression was relevant was demonstrated by the vessels transplanted into α-TFPI-Tg mice, which had a radically different phenotype compared with those of WT. Not only was IH inhibited, but also transplanted allogeneic vessels appeared to have undergone regenerative repair, characterized by replacement of donor endothelium with quiescent cells sitting on a layer of new α-SMA+ cells.

Although small numbers of circulating CD34+α-SMA+hTFPI+ cells in these mice were CD45+, the majority were predominantly negative for CD45, CD11b, CD115, F4/80, Ly-6C, CX3CR1, CCR2 and PAR-1 and lacked expression of VEGFR-2, CD68, CD31, and E-selectin. This phenotype was directly dependent on the TFPI fusion protein. To our knowledge, this is the first description of the 2 subsets of α-SMA+CD34+ cells posttransplantation and of a link between them through coagulation proteases.

Recruitment of these cells to the allogeneic vessel wall was followed by CD34+CD45− endothelial cells, which eventually formed a new endothelium. The same appearance was seen after adoptive transfer of purified CD34+ cells from PAR-1 deficient mice and WT cells that had first been incubated with a selective PAR-1 antagonist. All these results indicate that TF was most likely the trigger to thrombin generation, allowing signaling through PAR-1, and also suggested that inhibition of PAR-1 signaling on circulating CD34+ cells was the most important determinant of the repair phenotype. However, the precise molecular signals resulting in the sequential recruitment of α-SMA+ and CD31+ cells to the allogeneic vessel are not clear.

For adoptive transfer experiments, CD34+ cells were isolated from syngeneic or congenic mice that had undergone wire-induced carotid artery injury 2 days previously, so that they were enriched for CD34+ cells mobilized in the context of vascular injury. We have assumed that recruitment of injected cells to the neointima is important for subsequent inhibition of IH, but we cannot exclude the possibility that they also act at remote sites to influence the mobilization or phenotype of endogenous CD34+ cells (or other types of cells).

In summary, this work indicates that the phenotype of alloimmune-mediated vascular injury (the phenotype of rejection) in this model is determined by the phenotype of mobilized repair cells, not by the nature of the immune response. These data should open new avenues of research into the quality and character of the endogenous repair response under conditions of ongoing inflammation. They should lead to a reevaluation of previous data obtained from mouse models of TA and prompt a reevaluation of the mechanisms underpinning human chronic rejection. They also have significance for the investigation and treatment of inflammatory vascular diseases, in which more work needs to be done to explore why endogenous repair mechanisms fail to operate.

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Disclosures
None.

References
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Supplemental Material

Inhibition of thrombin receptor signalling on α-smooth muscle actin* CD34* progenitors leads to repair after murine immune vascular injury.

Chen – Repair by PAR-1 inhibition on myeloid progenitors

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Detailed Materials and Methods

Animals and experimental models. WT and BALB/c mice were purchased from Harlan Olac Ltd (Bicester, United Kingdom). ROSA-EYFP\(^1\), heterozygous \(\alpha\)-TFPI-Tg\(^2\) and CD31-TFPI-Tg\(^3\), homozygous PAR-1-\(^4\) and PAR-4-deficient mice\(^4\) were bred and maintained in the Biological Services Unit of Imperial College London. ROSA-EYFP mice express the yellow fluorescence protein in all cells and were used to track recipient cells in the donor vessel. Mice were used at 8 – 12 weeks of age. All animal experimental procedures were approved by UK Home Office. Recovery surgery was performed using appropriate analgesia consisting either fentanyl /fluanisone ('Hypnorm', 0.4 ml/kg, Janssen, Oxford, UK) in combination with midazolam ('Hypnovel', 5 mg/ml, Roche, Basel, Switzerland). The mixed compounds (1 part Hypnorm, 2 parts water, 1 part Hypnovel) were administered intraperitoneally at 0.1 ml/10 g. For other animals, Buprenorphine (Alstoe Veterinary, York, UK) was administered subcutaneously at a dose of 0.01 mg/Kg post-surgery (before recovery of consciousness). Animals were anesthetised by inhalation of isoflurane supply. Isoflurane vaporization for induction of anaesthesia(20-30 seconds), surgical intervention (30-40 minutes) and maintenance of anaesthesia (3.0 – 5.0 hr) were 4.0%, 1.5% and 1.0%, respectively.

Aortic transplantation: Mice were transplanted using a sleeve anastomosis technique as we have previously used\(^5\). Briefly, a 5mm segment of infrarenal donor aorta was removed after flushing with 300\(\mu\)l of N saline containing 50\(\mu\)l of heparin before transplantation into the same position of the recipient aorta. Blood flow was confirmed by direct inspection after the clamps removed. \(N=5-6\) per group

Skin transplantation: Full thickness tail skin from BALB/c mice was grafted on beds prepared on the lateral flanks of recipients 14 days post aortic transplantation. Graft sites were protected under sterile gauze covered by plaster removed on day 9. Grafts
were observed daily afterward and were considered rejected when no viable skin remained. Graft survival in the groups was compared using the log-rank test.

Wire-induced carotid artery injury: This was performed as we previously described. Briefly, a 100μm diameter wire was introduced and withdrawn three times into the common carotid via the external carotid artery before the external carotid artery was tied off. After confirming restoration of normal blood flow through the common carotid, the skin was closed and animals were allowed to recover.

Morphometric analysis and immunohistology. Sections were prepared and examined as previously described. Briefly, aortas were isolated and embedded in OCT (VWR International, Dorset, UK) by freezing with dry ice, sectioned at 5μm thickness and fixed in methanol at –20°C fixed in methanol. For analysis of intimal and medial areas, sections were stained using the Accustain™ Elastin Stain kit (Sigma) and examined under an Olympus U-ULH Optical microscope (Olympus Optical Co. Ltd, Tokyo, Japan) using Image-Pro Plus™ version 3.0 software (Media Cybernetics, Silver Spring, MD, USA). At least three random sections were examined from each of five grafts / wire-injured arteries. For IF analysis, sections were examined with Plan-NEOFLUAR objectives using a KTL/CCD-1300/Y/HS camera from Princeton Instruments (Trenton, NJ). Images were analyzed using the MetaMorph imaging system (Universal Imaging, Downingtown, PA). Some images are presented as overlays on the brightfield image to highlight the position of the elastic laminae. Infiltration of aortas by recipient immune cells was analysed by manual counting at x400 magnification. At least six random sections from each graft were measured, and average counts calculated.

Progenitor cell separation and injection. Whole mouse blood was collected 2-3 days post-injury. CD34+ cells were isolated using a stem cell separation kit according to the manufacturer's instructions (Stemcell Technology, London, UK). The average purity of the CD34+ cell populations was approximately 95% as assessed by flow cytometry. For comparative immunophenotyping using immunocytofluorescence,
CD34+ cells were plated into a 24 well plate at 5x10^3 cells/well, centrifuged at 1000 rpm, fixed with 4% paraformaldehyde (PFA), washed with PBS for 3 times, centrifuged at 1800 rpm for 2 min and then labelled with one or more of the Ab as listed below. For some analyses, fixation occurred after first layer staining. All wells were stained with DAPI. Labelled cells were analyzed using an immunofluorescence microscopy (Axiovert S100 TV; Zeiss, Welwyn Garden City, UK). To determine subpopulation densities, at least 300 cells were counted at X200 magnification from at least 3 random fields from 3 different wells.

For functional experiments, CD34+ cells were purified from mice 2-4 days after injury, seeded in a 24-well plate at 2x10^4 cells /ml and cultured in Iscove’s MDM (Sigma) supplemented with 2% FBS (Stemcell Technology) for 1-5 days. Cells were incubated with either thrombin (Enzyme Research Laboratories Ltd, Swansea, UK) or inactive thrombin (Cambridge Bioscience, Cambridge, UK), both at 50nM, Mercaptopropionyl-Phe-Cha-Arg-Lys-Pro-Asn-Asp-Lys-NH2 (PAR-1 antagonist) or Trans-cinnamoyl YPGKF-NH2 (PAR-4 antagonist) (both from Peptides International Inc. Louisville, KY 40224, USA) at 10µM.

7.5 x10^5 -1x10^6 freshly prepared CD34+ cells were injected into aortic recipients through a tail vein 1 week post-transplantation. In some experiments, cells were cultured in either medium, 500nM thrombin or 10µM PAR-1 antagonist for 1 hour, before injection.

**Ab for IF or immunocytofluorescence.** The Ab used were monoclonal rat anti-mouse CD34, CD68 (Serotec, Oxford, United Kingdom), and CD31, CD3, CD4, CD8 (BD Bioscience Pharmingen, Oxford, United Kingdom); polyclonal rabbit anti–TF, VEGF-R2 (ABR-Affinity BioReagents,Golden, CO), SM22α, P-selectin (BD Bioscience Pharmingen), anti-PAR 1 and anti-PAR 4 (Autogenbioclear, Wiltshire, UK) and human TFPI (American Diagnostica, Stamford, CA, USA); mouse
monoclonal anti-PAR 2 \(^1\) (Zymed, California, USA) and anti–human α-SMA (Sigma-Aldrich, St Louis, MO, USA); chicken anti–mouse E-selectin (R&D Systems, Minneapolis, MN, USA); hamster anti-mouse CD11c (Serotec). Second layer staining was with either goat anti-rat IgG-FITC, goat anti-rabbit IgG-FITC, sheep anti-mouse IgG-FITC or rabbit anti–chicken IgG-FITC (all from Sigma-Aldrich), rabbit anti-hamster IgG-FITC (BD Biosciences), or horse anti-mouse IgG–Texas red (Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with DAPI for three colour IF. For human sections the following were used; FITC-labelled sheep anti-human VWF (Serotec), CD31 (Antibodies GmbH, Aachen, Germany) and TF (Affinity Biologicals, Ancaster, Canada), mouse anti-human E-selectin \(^8\) or Cy3-labelled mouse anti-α-SMA (Sigma).

**Assessment of anti-donor Ab and circulating cytokines.** Blood was obtained from euthanized recipient mice 2 weeks post-transplantation by cardiac puncture, diluted with one-tenth volume of 3.2% sodium citrate and spun at 250g for 5 minutes at 4°C to isolate plasma, which was stored at –80°C until used. For assessment of anti-donor Ab, spleens from BALB/c mice aged 6 to 10 weeks were minced and passed through a 70µm nylon cell strainer (BD Bioscience, Bedford, MA, USA) before digestion in 0.2% collagenase D (Boehringer Mannheim, Germany) in DMEM medium at 37°C for 30 minutes. Red blood cells were lysed using ACK buffer. The remaining cells were suspended in PBS/BSA at 1x10\(^6\) cells/ml, incubated with mouse Seroblock FcR (AbD Serotec, Oxford, UK) for 10 min at RT and then with serial dilutions of the recipient mouse plasma for 1 hour at RT. After three brief washes in PBS, cells were incubated with either goat anti-mouse IgG or IgM (Sigma, UK) for 1 h at 4°C in the dark. As a negative control, cells were stained with plasma from a non-transplanted mouse. Flow cytometric analysis was performed as described below.

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\(^1\) Anti-PAR reagents recognize both cleaved and non-cleaved forms of the receptor.
Circulating IFN\(\gamma\) was analysed by enzyme-linked immunosorbent assay (ELISA) (from R&D Systems) according to the manufacturer’s instructions.

**Immune activation assays.** BM-derived DC were cultured as described elsewhere \(^9\). Briefly, BM cells were flushed out from the femurs and tibiae of mice and passed through a nylon cell strainer. Red blood cells were lysed using ACK buffer. After washing, cells were seeded in tissue culture flasks or plates at concentration of 1x10\(^6\)/ml in RPMI growth medium plus 2.5% supernatant from granulocyte-macrophage colony-stimulating factor (GM-CSF) producing hybridoma cell line. On day 3, non-adherent cells were discarded and fresh medium was added. On day 6, half the medium (plus non-adherent cells) was removed from culture and discarded. Fresh RPMI growth medium with 1.25% GM-CSF hybridoma supernatant was added back to the rest of the culture. On day 6, 1\(\mu\)g/ml LPS (Escherichia coli serotype 0128:B12, Sigma) or 1\(\mu\)M dexamethasone (Sigma) was added to the culture medium for 24 hours to produce strongly or poorly immunogenic DC respectively. Cells were harvested on day 8.

For co-culture assays, splenocytes and lymph node cells (mesenteric and axillary) were prepared as above. To enrich for T cells, cell suspensions underwent 2 rounds of adherence for 45 minutes onto plastic culture flasks at 37°C. Non-adherent cells were incubated with the following monoclonal Ab; anti-MHC II (M5114), anti-CD16/32 and anti-CD45R (B220; all from eBioscience, Insight, Wembley, UK) with or without anti-CD8 or anti-CD4 (BD Pharmingen). T cells were negatively selected using sheep-anti-rat magnetic beads (Dynal). To assess proliferation, stimulator cell proliferation was inhibited with \(\gamma\)-irradiation. Plates were pulsed with [\(^3\)H]TdR for 18 hours (overnight) on day 5. Proliferation was measured as [\(^3\)H]TdR incorporation by liquid scintillation spectroscopy.

**Flow cytometric analysis** was performed on a FACSCalibur flow cytometer and analysed using Cellquest (BD BioSciences, UK) or Flojo (Treestar, USA) software.
2×10⁵ cells were washed in PBS containing 5% heat inactivated FCS then stained with the appropriate concentration of Ab (see above) in buffer in 96 well V-bottom plates and incubated for thirty minutes in the dark at 4°C. After three washes, they were stained with a relevant secondary Ab before further washing. Cells were analysed immediately or fixed in 2% paraformaldehyde for analysis within three days. For intracellular cytokine staining, splenocytes or lymph node cells were isolated as above and stimulated with PMA (Sigma) at 50ng/ml plus ionomycin (Calbiochem) at 500ng/ml for 4 hours. Brefeldin A (Sigma) was added at 10ng/ml for the last 2 hours of incubation. Cells were washed and incubated with rat anti-mouse CD4-APC (BD Pharmingen). Following further washing, cells were fixed in 2% paraformaldehyde for 20 minutes at room temperature then washed. Cells were permeabilised by incubating in PBS 5% FCS with 0.5% saponin (Sigma) for 10 minutes, and then stained with rat anti-mouse IFN-γ-FITC (BD Pharmingen) and washed thoroughly prior to analysis by flow cytometry.

**Statistical analysis.** Data are presented as means ±SEM. Significance of the difference between 2 groups was determined by unpaired Student t or log rank test. Values of P<0.05 were considered statistically significant.
Supplemental References


7. Chen D, Abrahams JM, Smith LM, McVey JH, Lechler RI, Dorling A. Regenerative repair after endoluminal injury in mice with specific antagonism


Supplementary Figure Legends

Supplementary Figure I. Adaptive immune response in WT (white symbols/columns) vs. α-TFPI-Tg (black) recipients.

A&B: Flow cytometric analysis of circulating anti-BALB/c Ab in serum from day 14 assessed by binding to BALB/c splenocytes (A). Data points represent means (± SEM) from 5 individual mice. Comparing WT vs. α-TFPI-Tg, p=NS all dilutions.
Profiles from single mouse (B) showing binding of specific serum diluted 1 in 4.

Control serum from non-transplanted mouse.

C: Analysis of CD4\(^+\), CD8\(^+\), CD68\(^+\) and CD11c\(^+\) cells infiltrating adventitia and neointima at 6 weeks. Graphs show mean density (± SEM) of 18 counts, obtained from 3 random sections from each of 6 recipients. At least 100 cells per section were counted. Comparing WT vs. α-TFPI-Tg, p=NS all comparisons.

D: Two colour IF (overlayed on brightfield) of representative sections. CD4\(^+\), CD8\(^+\), CD68\(^+\) and CD11c\(^+\) cells (all green). Red = α-SMA.

E&F: Flow cytometric analysis of (E) CD4\(^+\)IFN\(_\gamma\)\(^+\) (F) CD8\(^+\)IFN\(_\gamma\)\(^+\) cells purified from spleens of recipient mice 2 weeks post-transplantation. Data points represent individual mice. Inverted triangles, syngeneic grafts; squares, BALB/c grafts. In both, *p<0.05; **p=NS.

Similar data for LN cells are presented in supplementary figure 2.

G: Mean ± SEM IFN\(_\gamma\) levels in peripheral blood (n=5). Each sample tested in duplicate by ELISA. Squares –syngeneic grafts. Circles –BALB/c grafts. Comparing allograft recipients, p=NS at all time points.

H: Survival of BALB/c skin grafts transplanted 2 weeks post-aortic transplantation.
Inverted triangles –syngeneic aortas. Squares, BALB/c aortas. Comparing allograft recipients, p=NS at all time points.
Supplementary Figure II. Additional data on the adaptive immune responses in WT (white symbols/columns) vs. α-TFPI-Tg (black) mice.

A & B: Flow cytometric analysis of (A) CD4⁺IFNγ⁺ (B) CD8⁺IFNγ⁺ cells purified from lymph nodes of mice 2 weeks post-transplantation. Data points represent individual mice, with mean shown by horizontal line. Inverted triangles = recipients of syngeneic grafts. Squares, recipients of BALB/c grafts. In both, *p<0.03; **p=NS. [NB some data from WT mice in B have been presented before in HM Clarke et al, Transplantation 88, 653 (Sep 15, 2009)]

C & D: Flow cytometric analysis of resting and activated CD4+ T cells (C) and DC (D) from WT and α-TFPI-Tg mice. Filled-in profiles = isotype control.

E&F: Functional analysis of CD4⁺ T cells (E) and DC stimulatory capacity (F).

Results are expressed as mean cpm +/- SEM after ³H-thymidine uptake by 2 x 10⁵ recipient (E) or BALB/c (F) CD4⁺ T cells in triplicate wells after 5 day culture with BALB/c (E) or recipient (F) BM-derived LPS-activated DC.

Supplementary Figure III. Immunohistology after adoptive transfer CD34⁺ cells

Phenotypic analysis of BALB/c aortas transplanted in WT mice with adoptive transfer of 1 x 10⁶ CD34⁺ cells. N=5 mice per group. All aortas harvested at 6 weeks. Some IF images have been overlayed on the brightfield image to illustrate elastic laminae.

'Co-localisation' = two (left column) and three (right column) colour overlay images, where yellow = co-localisation. Blue = DAPI.

A: CD34⁺ cells from α-TFPI-Tg mice. Red = α-SMA; Green = TF, P-selectin (P-sel), CD31 or E-selectin (E-sel) as indicated.

B: CD34⁺ cells from EYFP mice, incubated in vitro with PAR-1 antagonist. Red = TF, CD31 or E-selectin (E-sel) as indicated; Green = YFP.

Supplementary Figure IV. Immunohistology after adoptive transfer CD34⁺ cells
Phenotypic analysis of BALB/c aortas transplanted in WT mice with adoptive transfer of 1 x 10⁶ CD34⁺ cells. N=5 mice per group. All aortas harvested at 6 weeks. Some IF images have been overlayed on the brightfield image to illustrate elastic laminae.

‘Co-localisation’ = two (left column) and three (right column) colour overlay images, where yellow = co-localisation. Blue = DAPI.

A: CD34⁺ cells from PAR-1-deficient mice. Red = α-SMA; Green = TF, P-selectin (P-sel), CD31 or E-selectin (E-sel) as indicated.

A: CD34⁺ cells from PAR-4-deficient mice. Red = α-SMA; Green = TF, P-selectin (P-sel), CD31 or E-selectin (E-sel) as indicated.
Supplementary Table I: Proportion of CD34+ cells expressing the indicated molecules, expressed as % (±SEM) of total CD34+ cells. Data derived from 6 mice

<table>
<thead>
<tr>
<th>Phenotype of circulating CD34+ cells – Day 7</th>
<th>% (±SEM) of CD34+ cells expressing these molecules</th>
<th>p value</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>α-TFPI-Tg</td>
<td></td>
</tr>
<tr>
<td>TF</td>
<td>71 (±9)</td>
<td>74 (±6)</td>
</tr>
<tr>
<td>PAR-1</td>
<td>52 (±5)</td>
<td>43 (±8)</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>84 (±7)</td>
<td>73 (±9)</td>
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<tr>
<td>α-SMA</td>
<td>9.4 (±0.7)</td>
<td>9.6 (±0.7)</td>
</tr>
<tr>
<td>CD31</td>
<td>26 (±6)</td>
<td>27 (±5)</td>
</tr>
<tr>
<td>TIE-2</td>
<td>70 (±4)</td>
<td>62 (±6)</td>
</tr>
<tr>
<td>E-selectin</td>
<td>25 (7.8)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>CD45</td>
<td>68 (±5)</td>
<td>36 (±4)</td>
</tr>
<tr>
<td>CD68</td>
<td>44 (±9)</td>
<td>2 (±1)</td>
</tr>
<tr>
<td>F4/80</td>
<td>46 (±6)</td>
<td>25 (±4)</td>
</tr>
<tr>
<td>Ly6-C</td>
<td>58 (±6)</td>
<td>29 (±5)</td>
</tr>
<tr>
<td>CD11b</td>
<td>63 (±7)</td>
<td>33 (±6)</td>
</tr>
<tr>
<td>CD115</td>
<td>63 (±10)</td>
<td>35 (±7)</td>
</tr>
<tr>
<td>CCR2</td>
<td>64 (±4)</td>
<td>53 (±5)</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>70 (±4)</td>
<td>58 (±6)</td>
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</table>
Supplementary Table II: Proportion of CD34⁺ α-SMA⁺ cells in α-TFPI-Tg mice expressing the indicated molecules, expressed as % (±SEM) of total CD34⁺ α-SMA⁺ cells. Data from 6 mice.

<table>
<thead>
<tr>
<th>Phenotype of circulating CD34⁺ cells – Day 7</th>
<th>% (±SEM) of CD34⁺ α-SMA⁺ cells expressing these molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>With control Ig</td>
<td>With anti-TFPI</td>
</tr>
<tr>
<td>CD45⁺</td>
<td>14.3 (±3)</td>
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<tr>
<td>CD31⁺</td>
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