Donor and Recipient Cell Surface Colony Stimulating Factor-1 Promote Neointimal Formation in Transplant-Associated Arteriosclerosis

Shungo Hiroyasu, Prameladevi Chinnasamy, Rong Hou, Kylie Hotchkiss, Isabel Casimiro, Xu-ming Dai, E. Richard Stanley, Nicholas E.S. Sibinga

Objective—Transplant-associated arteriosclerosis manifests as progressive vascular neointimal expansion throughout the arterial system of allografted solid organs, and eventually compromises graft perfusion and function. Allografts placed in colony stimulating factor (CSF)-1-deficient osteopetrotic (Csf1<sup>-/-</sup>/Csf1<sup>-/-</sup>) mice develop very little neointima, a finding attributed to impaired recipient macrophage function. We examined how CSF-1 affects neointima-derived vascular smooth muscle cells, tested the significance of CSF-1 expressed in donor tissue, and evaluated the contribution of secreted versus cell surface CSF-1 isoforms in transplant-associated arteriosclerosis.

Methods and Results—CSF-1 activated specific signaling pathways to promote migration, survival, and proliferation of cultured vascular smooth muscle cells. Tumor necrosis factor-α addition increased CSF-1 and CSF-1 receptor expression, and tumor necrosis factor-α-driven proliferation was blocked by anti-CSF-1 antibody. In a mouse vascular allograft model, lack of recipient or donor CSF-1 impaired neointima formation; the latter suggests local CSF-1 function within the allograft. Moreover, reconstitution of donor or recipient cell surface CSF-1, without secreted CSF-1, restored neointimal formation.

Conclusion—Vascular smooth muscle cells activation, including that mediated by tumor necrosis factor-α, can be driven in an autocrine/juxtacrine manner by CSF-1. These studies provide evidence for local function of CSF-1 in neointimal expansion, and identify CSF-1 signaling in vascular smooth muscle cells, particularly cell surface CSF-1 signaling, as a target for therapeutic strategies in transplant-associated arteriosclerosis. (Arterioscler Thromb Vasc Biol. 2012;33:00-00.)

Key Words: allograft ■ arteriosclerosis ■ chronic rejection ■ macrophage-colony stimulating factor ■ vasculopathy

Transplant-associated arteriosclerosis (TA) is an accelerated form of vascular obstructive disease in graft arteries that limits long-term success of solid organ transplantation. Advanced TA lesions contain a diffuse concentric neointima composed predominantly of vascular smooth muscle cells (VSMCs) and extracellular matrix proteins admixed with inflammatory cells. Compared with typical atherosclerosis, TA lesions contain less lipid, more infiltrating T cells and macrophages, and develop in months to years, rather than decades. Continuing neointimal expansion, restricted outward remodeling, and limited vasodilatory capacity impair arterial conduit function and lead ultimately to ischemia and graft failure.

Immunosuppressive regimens that successfully control acute rejection do not prevent TA. Whereas acute rejection is primarily destructive, TA is trophic—the result of progressive neointimal expansion. Early concepts of TA pathogenesis suggested that interferon (IFN)-γ-stimulated macrophages activated neointimal VSMCs by secreting cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-1β, and growth factors such as platelet-derived growth factor (PDGF)-BB and bFGF. There is considerable evidence that macrophages and VSMCs participate in an autonomous loop of mutual production and response to IFN-γ, even after initiating alloimmune responses have abated. Current models suggest 3 pathogenetically distinct stages in TA: early nonspecific vascular injury, leading to inflammatory cell recruitment; subsequent alloimmune response, with T lymphocytes and macrophages augmenting endothelial and medial injury through cytokine production; and late phase neointimal expansion of VSMCs (or similar cells) in response to cytokines and chemokines.

Colonial stimulating factor (CSF)-1 (also known as macrophage CSF) is the primary regulator of survival, proliferation,
and differentiation of macrophages. Osteopetrotic (op) mice possess an inactivating mutation in the \( \text{Csf1} \) gene, and homozygous \( \text{Csf1}^{-/-}/\text{Csf1}^{-/-} \) mice are CSF-1-deficient and have profound defects in macrophage formation. A macrophage role in TA pathogenesis was suggested by the finding that arterial allografts placed in \( \text{Csf1}^{-/-}/\text{Csf1}^{-/-} \) mice develop significantly less neointimal hyperplasia than those placed in wild-type (wt) mice. It remains unclear, however, whether protection from TA in \( \text{Csf1}^{-/-}/\text{Csf1}^{-/-} \) mice stems from loss of local CSF-1 function and impaired activity of macrophages (or other host-derived cells) within the transplanted organ, or from systemic effects of CSF-1-deficiency that might impinge more generally on immune function.

CSF-1 is expressed by multiple cell types, including endothelial and VSMCs in 3 distinct isoforms: one membrane-spanning, cell surface isoform (csCSF-1), and 2 secreted isoforms (secCSF-1), proteoglycan and glycoprotein, found in the circulation. CSF-1 signals are mediated by the CSF-1 receptor tyrosine kinase (CSF-1R) encoded by the \( \text{c}-\text{fms} \) gene. Among blood cells, the CSF-1R is expressed exclusively in cells of monocyte/macrophage lineage and their progenitors. It is, however, also expressed in oocytes, trophoblastic cells, osteoclasts, and other cell types. VSMCs in culture and in neointima can express CSF-1R and proliferate on stimulation by CSF-1, but how CSF-1 affects other salient VSMC activities is unknown. Similarly, whereas signaling intermediates activated by CSF-1 in myeloid cells are known to include Src family kinases (SFKs), extracellular signal-regulated kinases (ERKs), and phosphoinositide kinase-3 (PI3K), the signaling events downstream of the CSF-1R in VSMCs are unknown.

We recently developed and characterized several transgenic mouse lines useful for in vivo study of CSF-1 biology; these lines bear transgenes encoding selected isoforms of CSF-1 under control of the \( \text{Csf1} \) promoter and first intron. When this driver is coupled to a full length \( \text{Csf1} \) cDNA, the resultant transgene confers normal tissue-specific and developmental expression of CSF-1, including all 3 isoforms, and completely rescues the \( \text{Csf1}^{-/-}/\text{Csf1}^{-/-} \) phenotype. Analogous transgenes expressing exclusively cs or sec CSF-1 provide only partial, nonredundant rescue. By using these mice as donors or recipients in vascular allograft experiments, we can examine contributions made by different CSF-1 isoforms in either donor- or recipient-derived cells. These strategies allow us to expand findings based in cell culture with in vivo studies addressing the following questions: (1) is CSF-1 expression in donor tissues important for TA, (2) do systemic (sec) and exclusively local (cs) CSF-1 contribute differentially to TA, and (3) do the different CSF-1 isoforms have similar or distinct roles in donor and recipient?

We now report that, in addition to proliferation, CSF-1 stimulates neointimal VSMC migration and survival, activities that may both contribute to neointimal accumulation. These findings are complemented by our in vivo results, which indicate that both donor and recipient-derived csCSF-1 drive neointimal formation in TA. Together, these studies describe CSF-1–mediated auto/juxtacrine enhancement of VSMC growth, migration, and survival within the vessel wall.

### Methods

#### Mice

The CSF-1-deficient and 2 CSF-1 transgenic lines (\( \text{Csf1}^{-/-}/\text{Csf1}^{-/-} \), \( \text{TgN}(\text{FLCsf1})\text{Ers/+} \) (TgC/+), and \( \text{TgN}(\text{Csf1})\text{Ers/+} \) (TgCS/+), respectively), were crossed onto or developed in the FVB genetic background for ≥7 generations, as were \( \text{TgN}(\text{Csf1-Z})\text{Ers/+} \) (TgZ) mice, which express a \( \text{LacZ} \) reporter under control of \( \text{Csf1} \) regulatory elements. Transgene abbreviations are summarized in Table 1.

#### Arterial Transplantation

Our mouse orthotopic carotid arterial transplantation model (CAT; see Figure 1B in the online-only Data Supplement) has similarities to previously described aortic and carotid arterial transplant models. Transgenic donor–recipient combinations used are summarized in Table 2, and relevant effects on csCSF-1, secCSF-1, and systemic macrophage levels are shown in Table 3. Detailed descriptions of surgical methods, morphometry, cell culture, gene expression, and protein analyses can be found in the online-only Data Supplement.

#### Statistical Analysis

Quantitative results are expressed as means±SEM. Experiments shown were performed in triplicate or quadruplicate, and are representative of at least 3 repeated experiments, using tissues/cells pooled from at least 3 mice per sample, except as noted in Figure legends. Potential differences among values from multiple samples were assessed by unpaired t test or factorial ANOVA followed by Bonferroni multiple comparison test when appropriate.
CSF-1 Mediates VSMC Growth, Migration, and Survival via Distinct Signaling Pathways

We then assessed how CSF-1 affected cellular activities critical to vascular remodeling, including growth, survival under oxidative stress, and migration, and examined the contribution of various signaling pathways. In BrdU incorporation assays of proliferation, we stimulated allograft-derived VSMCs with CSF-1 (50 ng/mL) in the presence of inhibitors of ERK (MEK inhibitor PD98059), PI3K (wortmannin), p38 (SB203580), or SFKs (PP2). The ERK and SFK inhibitors suppressed CSF-1-induced BrdU incorporation to baseline levels, whereas the PI3K and p38 inhibitors had no effect (Figure 1B), despite clear reduction in pathway activity (Figure II in the online-only Data Supplement).

CSF-1 has important pro-survival effects, mediated by PI3K and Akt, in myeloid cells, and tissue macrophages in vivo and primary macrophages in culture are largely dependent on CSF-1 for both survival and proliferation. Whereas VSMC survival in routine culture is not CSF-1–dependent, the CSF-1–mediated Akt phosphorylation we found (Figure 1A) suggested that CSF-1 might enhance VSMC survival in stressed conditions. VSMCs encounter oxidative stress in vivo via H₂O₂ produced by macrophages, and H₂O₂ is commonly used as a pro-apoptotic stimulus for cultured VSMCs. Exposure to 5 μmol/L H₂O₂ caused readily detectable cell death in ≈20% of cells after 30 minutes (Figure 1C). CSF-1 treatment decreased this to ≈5%, indicating that CSF-1 has robust survival effects in allograft-derived VSMCs. Inhibition of PI3K totally reversed CSF-1–mediated survival, whereas inhibition of MEK, p38, and SFKs had little effect. Thus, CSF-1–mediated activation of PI3K promotes resistance of VSMCs to oxidative stress-induced cell death.

VSMC migration is also critical for neointimal formation. In Transwell assays, CSF-1 promoted VSMC chemotaxis; PDGF-BB, a very potent chemotactic stimulus, was tested for reference (Figure 1D, left). Inhibitor studies showed that this effect of CSF-1 required MEK and p38 activities, and was independent of SFK and PI3K (Figure 1D, right). Thus in these studies of allograft-derived VSMCs, CSF-1 stimulates multiple activities that contribute to VSMC accumulation in the arterial wall, and activates distinct combinations of signaling pathways to achieve each effect.

Table 1. Description of Transgenes and Mutations

<table>
<thead>
<tr>
<th>Transgene/Mutation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csf1-op&lt;sup&gt;9&lt;/sup&gt;</td>
<td>CSF-1 null</td>
</tr>
<tr>
<td>TgC&lt;sup&gt;13&lt;/sup&gt;</td>
<td>Full length CSF-1</td>
</tr>
<tr>
<td>TgCS&lt;sup&gt;9&lt;/sup&gt;</td>
<td>CSF-1, cell surface membrane spanning</td>
</tr>
<tr>
<td>Tgζ&lt;sup&gt;13&lt;/sup&gt;</td>
<td>Csf-1 promoter/first intron-lacZ</td>
</tr>
</tbody>
</table>

CSF indicates colony stimulating factor.

CSF-1 and Transplant-Associated Arteriosclerosis

Table 2. Mouse Donor and Recipient Transplant Combinations

<table>
<thead>
<tr>
<th>Group Designation</th>
<th>Donor</th>
<th>CSF-1 Genotype</th>
<th>Recipient</th>
<th>CSF-1 Genotype</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isograft</td>
<td>FVB</td>
<td>+/-</td>
<td>FVB</td>
<td>+/-</td>
<td>8</td>
</tr>
<tr>
<td>I</td>
<td>B10.A</td>
<td>+/-</td>
<td>FVB</td>
<td>+/-</td>
<td>9</td>
</tr>
<tr>
<td>II</td>
<td>B10.A</td>
<td>+/-</td>
<td>FVB</td>
<td>Csf1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>7</td>
</tr>
<tr>
<td>III</td>
<td>B10.A</td>
<td>+/-</td>
<td>FVB</td>
<td>Csf1&lt;sup&gt;+/+&lt;/sup&gt;; TgC/+</td>
<td>8</td>
</tr>
<tr>
<td>IV</td>
<td>B10.A</td>
<td>+/-</td>
<td>FVB</td>
<td>Csf1&lt;sup&gt;+/+&lt;/sup&gt;; TgCS/+</td>
<td>9</td>
</tr>
<tr>
<td>V</td>
<td>FVB</td>
<td>+/-</td>
<td>B10.A</td>
<td>+/-</td>
<td>9</td>
</tr>
<tr>
<td>VI</td>
<td>FVB</td>
<td>Csf1&lt;sup&gt;+/+&lt;/sup&gt;; Csf1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>B10.A</td>
<td>+/-</td>
<td>7</td>
</tr>
<tr>
<td>VII</td>
<td>FVB</td>
<td>Csf1&lt;sup&gt;+/+&lt;/sup&gt;; TgC/+</td>
<td>Balb/C</td>
<td>+/-</td>
<td>8</td>
</tr>
<tr>
<td>VIII</td>
<td>FVB</td>
<td>Csf1&lt;sup&gt;+/+&lt;/sup&gt;; TgCS/+</td>
<td>Balb/C</td>
<td>+/-</td>
<td>8</td>
</tr>
</tbody>
</table>

CSF indicates colony stimulating factor.

TNF-α Is a Major Inducer of Both CSF-1 and Its Receptor in Neointimal VSMCs

Medial and neointimal VSMCs harvested after vascular cannulation differ in their expression of CSF-1R. To test whether neointimal VSMCs in allografted arteries differ from donor medial VSMCs in their ability to express CSF-1, we prepared untransplanted aortic (medial) VSMCs (FVB wt) and cells derived from day 42 allografts (Table 2, group I). By immunocytochemistry, both medial- and allograft-derived cells were largely positive for expression of the VSMC markers calponin, α-smooth muscle actin, and smooth muscle 22α, whereas macrophage markers CD68 and Mac3 were seen only rarely (Figure III in the online-only Data Supplement and data not shown). Levels of Csf-1 and c-fms mRNA were measured by quantitative reverse transcription-polymerase chain reaction (Figure 2A). Compared with medial cells, allograft-derived cells in 2% fetal bovine serum expressed higher levels of c-fms and similar levels of Csf-1 transcripts. Interestingly, these levels increased robustly in allograft-derived cells stimulated with 20% fetal bovine serum. cCsf-1 mRNA levels were not serum-responsive in medial VSMCs, but more than doubled in allograft-derived VSMCs, whereas secCSF-1 transcripts increased more modestly. Similar patterns of overall expression and responsiveness were observed at the protein level (Figure IV in the online-only Data Supplement). These results indicate that allograft-derived, primarily neointimal VSMCs tend to express higher levels of both CSF-1R and cCSF-1 compared with medial VSMCs derived from normal arteries.

We then assessed how specific factors affected CSF-1 and CSF-1R expression in medial and allograft-derived cells.

Table 1.

<table>
<thead>
<tr>
<th>Transgene/Mutation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Csf1-op&lt;sup&gt;9&lt;/sup&gt;</td>
<td>CSF-1 null</td>
</tr>
<tr>
<td>TgC&lt;sup&gt;13&lt;/sup&gt;</td>
<td>Full length CSF-1</td>
</tr>
<tr>
<td>TgCS&lt;sup&gt;9&lt;/sup&gt;</td>
<td>CSF-1, cell surface membrane spanning</td>
</tr>
<tr>
<td>Tgζ&lt;sup&gt;13&lt;/sup&gt;</td>
<td>Csf-1 promoter/first intron-lacZ</td>
</tr>
</tbody>
</table>

CSF indicates colony stimulating factor.
VSMCs from wt FVB mouse aortae and from group I grafts were stimulated with growth factors and cytokines previously reported to increase Csf-1 and c-fms transcript levels, including PDGF-BB plus epidermal growth factor, TNF-α, and IFN-γ. Transcripts were measured by quantitative reverse transcription-polymerase chain reaction (Figure 2B). Of these factors, TNF-α and PDGF plus epidermal growth factor both induced Csf-1 and c-fms, with greater effects in neointimal than in medial VSMCs. Although the effect of IFN-γ effect was more modest, it showed selectivity for the csCsf-1 isoform, which is of particular interest given our in vivo findings (Figure 4, below). Although neointimal VSMCs are generally known to be more proliferative and synthetic than their medial counterparts, enhanced induction of both GS-1 and its receptor in such cells has not been previously reported.

We also studied CSF-1 protein expression in allograft-derived VSMCs stimulated with 2, 5, and 10 ng/mL of TNF-α for 48 hours, which allowed us to measure both secCSF-1 and csCSF-1 by ELISA (Figure 2C). Expression of both isoforms increased in a dose-dependent manner in response to TNF-α, consistent with the quantitative reverse transcription-polymerase chain reaction findings. These results point to TNF-α as a potentially important stimulus for CSF-1 signaling in VSMCs. Indeed, TNF-α is known to contribute importantly to TA: TNF-α blockade inhibits neointimal formation in a rabbit TA model, and interestingly, inactivation of TNF-α signaling specifically in donor tissue limits TA in allografted mouse hearts. Such observations prompted us to explore a potential link between TNF-α and CSF-1 signaling.

### Table 3. Transplantation Groups and Characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Combination</th>
<th>Cell Surface CSF-1</th>
<th>Circulating CSF-1</th>
<th>Circulating Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>B10.A to FVB</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>II</td>
<td>B10.A to op/op</td>
<td>Donor: normal; recipient: none</td>
<td>None</td>
<td>Decreased</td>
</tr>
<tr>
<td>III</td>
<td>B10.A to TgC&lt;sup&gt;13&lt;/sup&gt;</td>
<td>Donor: normal; recipient: ≈1/3</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>IV</td>
<td>B10.A to TgCS&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Normal</td>
<td>None</td>
<td>Decreased</td>
</tr>
<tr>
<td>V</td>
<td>FVB to Balb/C</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>VI</td>
<td>op/op&lt;sup&gt;6&lt;/sup&gt; to Balb/C</td>
<td>Donor: none; recipient: normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>VII</td>
<td>TgC&lt;sup&gt;13&lt;/sup&gt; to Balb/C</td>
<td>Donor: ≈1/3; recipient: normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>VIII</td>
<td>TgCS&lt;sup&gt;9&lt;/sup&gt; to Balb/C</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
</tbody>
</table>

CSF indicates colony stimulating factor.

Figure 1. Colony stimulating factor (CSF)-1 activates growth, survival, and migration of allograft-derived neointimal vascular smooth muscle cells (Table 2, group I) via multiple signaling pathways. A, Western analysis of phosphorylation events after CSF-1 addition. Effect of kinase inhibitors on CSF-1-stimulated B proliferation and C survival after H<sub>2</sub>O<sub>2</sub> stress, and D CSF-1-mediated chemotaxis. *P<0.001 vs all other groups; **P<0.05 vs indicated groups. ERK indicates extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide kinase-3; PDGF, platelet-derived growth factor.
BrdU, compared with wt VSMCs placed on fixed feeders from Csf1 op/Csf1op derived from α present. In overnight growth assays, nearly 40% of live cells by a porous membrane was lower, with no significant difference attributable to the presence or absence of CSF-1. These findings point to a relatively strong proliferative stimulus mediated through direct contact involving the csCSF-1 isoform.

To test this mechanism in living cells, we isolated neointimal VSMCs that express only the csCSF-1 isoform by harvesting cells from TgCS carotid arteries subjected to the flow-cessation model of vascular injury. These cells expressed the VSMC differentiation marker calponin and the CSF-1R, and increased csCSF-1 expression in response to TNF-α (Figure V in the online-only Data Supplement). We determined proliferation in BrdU incorporation assays after stimulation with TNF-α and exogenous CSF-1. To assess the role of CSF-1-mediated signaling, we added an anti-CSF-1 neutralizing antibody to some samples (Figure 3B). Exogenous CSF-1 increased proliferation 2-fold, an effect abolished by the antibody. TNF-α alone and TNF-α in combination with exogenous CSF-1 both increased proliferation; these effects were likewise reversed by the neutralizing antibody. CSF-1 acts exclusively through the CSF-1R, so these findings indicate that the growth-promoting effects of both CSF-1 and TNF-α are mediated through the CSF-1R. Because these TgCS-derived VSMCs do not express secCSF-1, the CSF-1-mediated increase in proliferation after TNF-α stimulation indicates that VSMC proliferation can be regulated by csCSF-1 in a local, autocrine/juxtacrine fashion.

**The csCSF-1 Isoform in Both Donor and Recipient Is Important in TA**

We then evaluated the relative expression of relevant factors and CSF-1 and CSF-1 isoforms over an extended time course after transplantation. Transcripts encoding TNF-α, PDGF-BB, epidermal growth factor, and IFN-γ all peaked at
day 29 after surgery; overall induction was most prominent for TNF-α (Figure 4A). CSF-1R (c-fms) transcripts were also elevated in allografted arteries, with highest levels also at day 29. Levels of sec and csCSF-1 transcripts showed some variation over time: secCSF-1 was relatively high at day 1, whereas both isoforms were increased at day 29. These data extend our findings in cultured VSMCs (Figure 2B) to the in vivo setting, showing expression of multiple factors in allografted arteries that may potentiate expression of CSF-1 and its receptor.

To identify the origin of cells in the grafted artery capable of expressing CSF-1, we used TgZ mice, in which the CSF-1 promoter/first intron drives expression of the LacZ gene in a pattern consistent with that of endogenous CSF-1. We tested these mice as either donor or recipient and detected the LacZ gene product by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining. Normal, untransplanted arterial sections showed no X-gal activity. With X-gal-positive, whereas with donor and recipient cells were found in the arterial media (Figure VI in the online-only Data Supplement). Thus both donor and recipient cells express CSF-1, and VSMCs, as well as macrophages, express the CSF-1R and can respond to CSF-1.

To test this idea in vivo, we performed a series of vascular allografts using Csfl+/Csfl+/ mice, reconstituted by transgenesis with specific CSF-1 isoforms, as recipients or donors in CAT. Representative immunohistochemical sections from wt allografts placed in wt transgenic mice are shown in Figure 4B (group I). Neointimal formation in wt grafts placed in mice lacking all CSF-1 (Csfl+/Csfl+; group II) was markedly attenuated, consistent with a previous study. Such a reduction was not seen in transgenic recipients expressing both sec and csCSF-1 (group III) or those expressing csCSF-1 only (group IV). This series of transplants shows that whereas recipient CSF-1 is essential for neointimal formation, the cs isoform alone is sufficient to drive neointimal formation, and the sec isoform is not required in the recipient.
Selective csCSF-1 Activity in the Recipient Uncouples Neointimal Formation and Inflammation

Although grafts placed in recipients expressing only csCSF-1 showed full restoration of neointimal formation, these arteries appeared to have fewer inflammatory cells than seen in other combinations with robust neointimal formation (Figure 4B, compare group IV versus groups I, III). Quantitation of the fraction of neointimal area occupied by nuclei in hematoxylin-eosin stained sections supported this observation (Figure 5A). To evaluate inflammatory activity, we performed immunohistochemical staining for the macrophage activation marker Mac3 (Figure 5B) and quantitated the fraction of cells positive for this marker (Figure 5C). In this analysis, groups II, III, and IV all scored lower than group I, indicating lower inflammation with any reduction in total Csf-1 expression in the recipient. Not surprisingly, the small neointimas from group II (in which recipients lack any Csf-1 and have globally reduced macrophage numbers) showed the lowest Mac3 activity; on other hand, group IV arteries showed a similar decrease in Mac3, despite their robust neointimas. Analysis of grafts with differing CSF-1 isoform expression placed in wt Balb/C recipients showed that neointimas from all groups stained diffusely for Mac3, regardless of differences in donor CSF-1 (groups V–VIII, not shown). These results indicate that neointimal formation does not necessarily correlate with infiltration of the graft by inflammatory cells, consistent with findings we and others have reported previously. Based on this analysis of day 42 grafts, we cannot exclude the possibility that the different CSF-1 isoforms affect pathogenetically significant inflammatory states at earlier time points. In the context of the present work, however, these observations suggest that factors driving VSMC accumulation in the neointima are still active when the recipient mouse lacks secCSF-1 and its associated inflammatory infiltrate.

Discussion

Our findings demonstrate that expression of the cs isoform of CSF-1 by both donor and recipient-derived cells is important for neointimal formation in a mouse model of TA. Although CSF-1 is best characterized as the primary factor promoting growth, survival, and differentiation of the monocyte/macrophage lineage, we find that CSF-1 can directly stimulate growth, migration, and survival of VSMCs, key activities that promote TA. Moreover, our results suggest an autocrine/juxtacrine regulatory mechanism in which VSMC accumulation is promoted by locally expressed csCSF-1 acting via the CSF-1R. These studies do not test the significance of CSF-1 isoforms in early inflammatory events that may contribute to eventual neointimal formation, though our cell culture and in vivo findings suggest the formation of a growth- and survival-promoting loop that does not depend on continued presence of inflammatory cells (ie, those recruited by secCSF-1, and lacking in csCSF-1–only recipients) at later time points within the established neointima.

We also tested the importance of CSF-1 in donor tissues by transplanting arteries from mice with varied expression of CSF-1 into wt recipients. As shown in Figure 4C, arteries from mice lacking CSF-1 (group VI) developed significantly smaller neointimas than those from wt controls with physiological CSF-1 expression (group V). Donor arteries from mice with essentially normal levels of circulating CSF-1 and substantially reduced csCSF-1 also showed impaired neointimal formation (group VII). On the other hand, transplanted arteries originating from mice with negligible circulating CSF-1 and near wt levels of csCSF-1 showed complete restoration of neointimal formation (group VIII, Figure 4C). These findings show that local (donor) expression of the csCSF-1 isoform is necessary and sufficient for neointimal formation. Taken together with the recipient studies shown in Figure 4B, these results define an important role in TA for csCSF-1 produces by both donor and recipient tissues.

Figure 5. Graft inflammatory activity and neointimal formation are dissociated in cell surface colony stimulating factor (csCSF)-1 recipient mice. Group I to IV transplanted arteries (day 42) were evaluated for inflammatory cell infiltration and macrophage activation. A, Fraction of neointimal area occupied by cell nuclei in hematoxylin-eosin-stained sections. For groups I, III, IV, n=8; for group II, n=5. *P<0.05 compared with group I. B, Representative arterial cross-sections stained for Mac3. NI and M mark the neo-intima and media, respectively. C, Cells staining positive for Mac3 were scored relative to the number of nuclei. *P<0.05 vs indicated samples. Sec indicates secreted; wt, wild type.
Multiple studies point to an important role for CSF-1 in vascular pathology, but the precise nature of this contribution is unclear, and the possibility that sec and csCSF-1 isoforms contribute differentially to vascular disease has not been assessed previously. Decreased TA in Csf-1−/−/Csf-1−/− transplant recipient mice, which lack all CSF-1, was attributed to systemic monocyte/macrophage depletion. Similarly, decreased lipid-driven (native vessel) atherosclerosis in LDLR−/−; Csf-1−/− mice was attributed to local CSF-1 acting to recruit and activate macrophages in the vessel wall. Nevertheless, the CSF-1R is expressed by cells other than monocyte/macrophages, including cultured VSMCs. Our findings both in vivo and in vitro provide evidence that CSF-1 acting locally in the vessel wall directly supports VSMC accumulation, as well as activating monocyte/macrophages, and suggest that this signaling axis is an important part of the contribution of CSF-1 to vascular pathogenesis.

Our results focus attention on the csCSF-1 isoform. Both sec and csCSF-1 isoforms were expressed in transplanted arteries (Figure 4), but restoration of neointimal formation in donor arteries otherwise lacking CSF-1 was only achieved with wt levels of the csCSF-1 isoform—the sec isoform was not sufficient. Neointimal VSMCs derived from day 42 allografts expressed more CSF-1 than normal aortic medial VSMCs, and csCSF-1 appeared more responsive to stimuli than secCSF-1, as seen in other cell types. Interestingly, the csCSF-1 isoform mediates comparatively more sustained proliferative effect on hematopoietic progenitors. Neointimal VSMCs also expressed more CSF-1R. Thus both ligand and receptor components of the CSF-1 regulatory loop are preferentially expressed more CSF-1R. Thus both ligand and receptor components of the CSF-1 regulatory loop are preferentially expressed by cells other than monocyte/macrophages, and suggest that this signaling axis is an important part of the contribution of CSF-1 to vascular pathogenesis.

In the clinical setting, TA has proven largely refractory to immunosuppressive therapies that prevent acute rejection. Clinical studies using agents such as sirolimus, which has both immunosuppressive and anti-proliferative activity, showed promising results 1 year after heart transplantation but in randomized but longer term protocols, consistent with the idea that control of immune cell proliferation may be key to prevention of neointimal formation and vascular obstruction. Our findings support this approach, while also pointing to control of VSMC proliferation as a desirable therapeutic goal in TA.

Understanding the role of CSF-1/CSF-1R signaling in neointimal formation has additional implications for the development of therapies to prevent TA. For one, suppression of immune cell function, whether directed at acquired lymphocyte-directed or innate macrophage-dependent immune responses, may do little to slow neointimal VSMC proliferation once the autocrine/juxtacrine CSF-1/CSF-1R loop is established. Factors that support this loop may prove to be better targets for intervention. In the mechanism we have outlined, these targets include TNF-α, as an important inducer of CSF-1 signaling in neointimal VSMCs, CSF-1 itself, and CSF-1R and its downstream mediators. Indeed, local TNF-α expression increases in neointimal VSMCs responding to mechanical arterial injury, and previous studies have shown that inhibition of TNF-α activity can limit neointimal formation. Like TNF-α, CSF-1 and CSF-1R targets are amenable to blockade by antibodies or with pharmacological inhibitors. Our findings suggest utility of even more selective therapies, perhaps via RNA interference or other approaches, which might be developed to specifically target csCSF-1.

Signaling pathways downstream of the CSF-1R in VSMCs may also provide targets for intervention. Structure–function analyses indicate that the outcome of CSF-1R activation can vary with cell type—thus signaling mechanisms defined in macrophages are not necessarily active in VSMCs. Consistent with studies in macrophages, we found that SFK and MEK/ERK pathways are necessary for CSF-1–stimulated VSMC proliferation, but not survival in the setting of oxidative stress, whereas the PI3K/Akt pathway is not essential for proliferative effects, but is required for survival. Conversely, we did not see a decrease in VSMC proliferation with PI3K inhibition, in distinction to a report that both PI3K/Akt and ERK signaling are required for optimal CSF-1–mediated mitogenesis in macrophages. To our knowledge, CSF-1–mediated chemotaxis of VSMCs has not been described previously; we find that it depends on the activities of both MEK and p38 mitogen-activated protein kinase, but not PI3K— unlike CSF-1–mediated migration in macrophages, which is PI3K-dependent.

Additional studies of VSMC-specific aspects of CSF-1–stimulated signaling may lead to identification of new targets more selective for this cell type, and in turn, to therapies that spare macrophage function and avoid further compromise of transplant recipient immunity.

Acknowledgments
We thank Xiao-Hua Zong and Ranu Basu for technical assistance.

Sources of Funding
Studies were supported by Atorvastatin Research Award (Pfizer) and National Institutes of Health (NIH) grants HL67944 to N.E.S.S., CA32551 and CA26504 to E.R.S, and 5P30-CA13330 to the Albert Einstein Cancer Center. S.H. received postdoctoral fellowship support from the American Heart Association, Heritage Affiliate. I.C. was supported in part by NIH T32 GM007491. X-M.D. was supported by an American Society of Hematology Fellow Scholar Award, and a Leukemia and Lymphoma Society Special Fellow Award.

Disclosures
None.

References


Donor and Recipient Cell Surface Colony Stimulating Factor-1 Promote Neointimal Formation in Transplant-Associated Arteriosclerosis
Shungo Hiroyasu, Prameladevi Chinnasamy, Rong Hou, Kylie Hotchkiss, Isabel Casimiro, Xu-ming Dai, E. Richard Stanley and Nicholas E.S. Sibinga

Arterioscler Thromb Vasc Biol. published online November 1, 2012;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636
The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2012/11/01/ATVBAHA.112.300264
Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/11/01/ATVBAHA.112.300264.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENT MATERIAL

Detailed Methods

Mice

Balb/C and B10.A-H2h2 mice were obtained from Jackson Laboratory. All mice were housed in pathogen-free conditions at the Albert Einstein College of Medicine. Male and female mice aged 8–12 weeks were used for transplantation experiments; lesion size in male and female mice was similar (not shown).

Carotid arterial transplantation

The standing committee of the Albert Einstein Institute for Animal Studies approved these studies. Our mouse orthotopic carotid arterial transplantation model (CAT; see supplement Figure IB) yielded reproducible neointimal formation with features characteristic of TA. The particular advantage of CAT is that it avoids abdominal surgery, which we found is poorly tolerated by Csf1op/Csf1op mice. We tested and adopted donor-recipient strain combinations using wt mice (supplement Figure IC). General anesthesia in mice was induced using an intraperitoneal injection of ketamine and xylazine (75 and 5 mg/kg, respectively). A midline incision was made in the donor mouse neck, and the entire left carotid artery was exposed and excised from its origin to bifurcation. The graft was washed and kept in ice-cold saline until use. In the recipient, the extrathoracic left carotid artery was isolated without touching the vagus nerve. Small vascular clamps (Roboz) were placed at both ends, and the vessel was excised leaving small cuffs at each end for anastomoses. The graft was positioned and trimmed to be
slightly shorter than the distance between the clamps. End-to-end anastomoses were performed with four to five interrupted 11-0 nylon sutures. The distal and proximal clamps were then removed. Hemostasis was usually achieved easily. The mice were kept warm until awake. Total surgical time was <1 h, and recipient mortality was <5%.

Allograft recipients were killed at different timepoints after CAT; based on preliminary studies, we chose day 42 as the principal timepoint for morphometric evaluation of TA. Grafts were fixed by perfusion with 4% paraformaldehyde/PBS (PFA/PBS) for 10 min at physiological pressure, postfixed with 4% PFA/PBS for 3 h, and washed with PBS. Before embedding, each graft was divided into 3 equal parts, and the ends were discarded to avoid possible effects related to the suture line. The central portion was embedded and sectioned (4 µm) for further analysis.

**Morphometric analysis**

Digital micrographs of H&E-stained sections were obtained, and the areas inside of the internal elastic lamina (IEL), and the luminal area (LA) were measured using the NIH ImageJ program on a Macintosh G4 computer running OSX. The percent neointimal area (% neointima) was calculated according to the formula, (IEL-LA)/IEL x 100.

**Immunocytochemistry**

Cells grown and fixed in coated chamber slides (BD Bioscience) were permeabilized with 0.4% Triton X-100 and blocked with 5% BSA/PBS. Primary antibodies used included anti-α-smooth muscle actin (IA4, Santa Cruz; 1:300), mouse anti-SM22α (Abcam, 1:300), rabbit anti-calponin (Abcam, 1:300), goat anti-CSF-1R1,2 (1:500), and rabbit anti-CD68 (Serotec, 1:500). Species-specific secondary antibodies were labeled
with Alexa Fluor 488, 555, or 594 fluorescent dyes (Molecular Probes), and staining was detected with an epifluorescent Olympus IX-81 or Axio Observer .Z1 microscope (Zeiss).

**Immunohistochemistry and X-gal staining**

Paraffin-embedded sections were stained with mouse anti-α-SMA IA4 (1:400), or rat monoclonal anti-Mac3 (Pharmingen; 1:200) using the indirect method. A rabbit anti-CSF-1R antiserum raised to a C-terminal peptide corresponding to amino acids 962–976 of the mouse CSF-1R was used at a final concentration of 1.25 µg/ml, and the specificity of staining was confirmed by preabsorbing the antibody with a 200-fold molar excess of the antigenic peptide. Signals were developed using an HRP-conjugated secondary antibody, DAB (3,3’-diaminobenzidine, Sigma) chromogen, and methylgreen counterstain. The fraction of cells expressing Mac3 was quantitated relative to total cell number (based on nuclear staining) by inspection of 4–5 neointimas per condition. For some studies, we determined the fraction of neointimal area occupied by cell nuclei using the LUT function of ImageJ to analyze hematoxylin-eosin-stained sections. Allografts derived from or placed in TgZ mice harvested 14 or 42 days after transplantation were fixed, serially dehydrated in 10, 15, and 20% sucrose/PBS, and embedded in OCT. Histochemical reactions with X-gal were performed overnight at 37°C on 8 µm cryostat sections.

**RNA extraction and QRT-PCR**

Total RNA was extracted using Trizol (Invitrogen). Sources included cultured medial VSMCs from normal aorta (FVB) or Group I neointimal VSMCs harvested 42 d after
transplantation. Cells were stimulated with medium containing 2% or 20% FBS, or with PDGF-BB (10 ng/ml) + EGF (3 ng/ml), TNF-α (10 ng/ml), or IFN-γ (50 U/ml) for 8 h prior to RNA extraction. Values were normalized relative to those from medial VSMCs cultured with 2% FBS or vehicle.

For in vivo studies, we used Group I allografts at days 1, 6, 29 and 42 after transplant, with 3 grafts pooled at each timepoint. Native heart and carotid artery isografts (FVB=>FVB) harvested at day 42 served as controls. For cell culture studies, total RNA was extracted from 1 x 10⁶ cells, and reverse-transcribed into cDNA using SuperScriptIII (Invitrogen). Real-time PCR was performed using a SYBR Green QRT-PCR Kit (Invitrogen) and the MX3000P Real-Time PCR System (Stratagene). Relative values were obtained using the standard curve method, normalized by gapdh (Figure 2A) or rpl13A (Figures 2B, 4), and expressed as relative values in comparison with the specific control group (detailed methods found in User Bulletin #2, ABI Prism 7700, Applied Biosystems).

Primer sequences designed for QRT-PCR were:

- **secCsf-1**
  - CACTGAGGACTCACAACCTCATCCTT;
  - ACCCAGTTAGTGCCCCAGTGAAGAT

- **csCsf-1**
  - AACAGCTTTTGCTAAGTGCTCTAGCCG;
  - AGGTGGAAGACAGACTCAGGGATCT

- **c-fms**
  - TCCACCGGGACGTAGCA;
  - CCAGTCCAAAGTCCCCAATCT
Each primer pair crosses intron-exon junctions to suppress genomic DNA amplification.

**Cell Culture**

Primary culture VSMCs were established using collagenase-elastase digestion\(^3\) from \(wt\) FVB aortae, carotid allografts 42 days after CAT (Table 2, Group I), and \(wt\) FVB carotid arteries subjected to flow-cessation injury\(^4\). For the last technique, mice were killed 28 days after arterial ligation, and cells prepared from the readily apparent neointima. Cells were maintained in DMEM (Invitrogen) supplemented with penicillin/streptomycin, L-
glutamine, non-essential amino acids and 20% FBS (HyClone), subcultured weekly, and used between passages 3 and 6. VSMCs showed a characteristic hill-and-valley pattern; staining for multiple lineage markers is shown in supplement Figure III.

In stimulation experiments for RNA extraction, primary VSMCs (1x10^5) were grown in 100 mm dishes for 48 h, and rendered relatively quiescent by culture in 2% FBS for 48 h. Total RNA was harvested 8 h after stimulation with 20% FBS, mouse CSF-1 (50 ng/ml, Sigma), mouse PDGF-BB (10 ng/ml, Cell Signaling) + mouse EGF (3 ng/ml, Chemicon), mouse TNF-α (10 ng/ml, Sigma), or mouse IFN-γ (50 U/ml, US Biological).

**Cell proliferation, survival, and migration**

Quiescent VSMCs were stimulated for 24 h with TNF-α (10 ng/ml, Sigma) and/or CSF-1 (50 ng/ml, Sigma). For some studies, chemical inhibitors of PI3K (wortmannin, 100 nM, Calbiochem), MEK (PD98059, 50 μM, Calbiochem), p38 (SB203580, 0.5 μM, Cell Signaling), and c-Src (PP2, 10 μM, Calbiochem, or SU6656, 5 μM, Sigma) were added. In other studies, anti-CSF-1 neutralizing antiserum was added to some wells (final dilution, 1:1000). BrdU (10 μM) was added 6 h before fixation. Cellular DNA was denatured with hydrochloric acid and stained with anti-BrdU antibody (1:200, Abcam) and DAPI counterstain. Photomicrographs of random 10X epifluorescent fields (6 per chamber) were obtained, and cells were counted using the ImageJ program. Proliferation was evaluated according to the formula, 100 x (BrdU positive/DAPI positive) cells per field; data presented reflect the mean of 6 fields per chamber.

To assess growth stimulation by fixed cellular monolayers, CSF-1-expressing (Group I) or CSF-1-deficient (Csf1<sup>op</sup>/Csf1<sup>op</sup>) VSMCs were grown to confluence, stimulated with
TNF-α for 6 h, washed with PBS, fixed with 4% PFA/PBS for 20 min, and washed extensively with PBS. Responder CSF-1-deficient VSMCs were rendered quiescent in DMEM/0.4%HS for 48 h, trypsinized, and added to the fixed cells. BrdU incorporation was assessed overnight.

For survival studies, Group I VSMCs at 80% confluence were cultured in 0.4% HS for 24 h before exposure to H₂O₂. Some cells were pretreated with specific kinase inhibitors for 5 min, and all cells were stimulated with CSF-1 (50 ng/ml) or vehicle for an additional 5 min before H₂O₂ (5 µM) addition. Viability was assessed after 30 min using the LIVE/DEAD system (Invitrogen). For each sample, we scored 6 randomly-selected epifluorescent photomicrographic fields for dead (red)/(live (green) + dead (red) cells using ImageJ software.

Migration studies were performed in Transwell culture. Quiescent VSMCs (30,000 per well) were added to the upper chambers and after 4 h at 37°C, migration was scored by DAPI nuclear staining of cells on the underside of the membrane. PDGF-BB (10 ng/ml) or CSF-1 (50 ng/ml) was added to upper, lower, or both Transwell chambers. In some studies, cells were preincubated with kinase inhibitors for 15 min prior to addition to upper chamber.

**CSF-1 ELISA**

Group I VSMCs (1x10⁵) were cultured in 10 ml dishes for 48 h in DMEM supplemented with 0.4% horse serum prior to stimulation with 0, 2, 5, or 10 ng/ml of TNF-α for 48 h. CSF-1 protein levels were detected using an ELISA (RnD Systems). We measured
secCSF-1 in 10 ml of culture medium without concentration, and csCSF-1 released from washed monolayers by treatment with 2 ml 0.05% trypsin-EDTA.

**Western Analysis**

Total protein from Group I VSMCs was extracted 0, 15, 30, 60, and 120 min after stimulation with 20 ng/ml CSF-1, separated by SDS-PAGE (20 µg/lane), and transferred to Immobilon-P membrane (Millipore). After blocking in Tris pH 8.0, NaCl 150 mmol/L, and 0.1% Tween-20 (TBST) with 5% (w/v) non-fat milk, blots were incubated overnight at 4°C with 1:1000 dilutions of either rabbit polyclonal antibodies in TBST with 5% non-fat milk (Phospho-Src Tyr<sup>416</sup>, Phospho-Src Tyr<sup>527</sup>, non-Phospho-Src Tyr<sup>527</sup>, and Phospho-Akt Thr<sup>308</sup>, mouse monoclonal antibody in TBST with 5% non-fat milk (non-Phospho-Src Tyr<sup>416</sup>), or rabbit monoclonal antibodies in TBST with 5% BSA (Phospho-ERK1/2, Phospho-JNK, Phospho-p38 MAPK) (Cell Signaling). Blots were incubated with HRP-conjugated secondary antibodies and visualized by ECL (Amersham). Protein loading was assessed with anti-fibronectin or anti-tubulin antibodies (Labvision; 1:500 and 1:250, respectively).

**References (Supplement Material)**

3. Oakes BW, Batty AC, Handley CJ, Sandberg LB. The synthesis of elastin, collagen, and glycosaminoglycans by high density primary cultures of neonatal


Figure Legends

Supplement Figures

Figure I. Characterization of TA response in orthotopic arterial allografts. (A) Immunohistochemical analysis 42 days after orthotopic transplantation of an aortic segment from an FVB donor mouse into a BALB/c recipient. The specimen was perfusion fixed and stained with hematoxylin-eosin (H&E) or antibodies against the indicated antigens to identify proliferation (PCNA), smooth muscle (SM, α-actin), and macrophages (MP, Mac-2). An arrow in the H&E panel indicates the internal elastic lamina, which bounds the neointima; an asterisk marks the vessel lumen. (B) Left, Schematic depiction of the orthotopic transplant model developed for these studies, with gray segment representing the transferred carotid arterial graft. Right, critical steps during the procedure: 1) harvest of donor arterial segment, 2) excision of recipient segment, 3) graft sutured in place, and 4) clamp release, with restoration of perfusion and no significant bleeding at anastomoses. (C) Summary of morphometric analysis of neointimal formation from pilot studies using isografts and 2 different donor-recipient combinations.

Figure II. Analysis of pharmacologic inhibitor efficacy in Group I neointimal VSMCs. Cultured cells were treated with inhibitors at the indicated concentrations for 30 minutes, stimulated with CSF-1 for 15 minutes, and cellular lysates analyzed by immunoblotting using phosho-specific antibodies that reflect pathway activity. Actin or tubulin antibodies were also used to assess protein loading. Asterisks indicate the inhibitor concentrations used in functional studies (Figure 1).
**Figure III.** Allograft-derived neointimal cells express smooth muscle marker genes. Cultured cells derived from Group I allografts were fixed and stained with DAPI to identify cell nuclei (blue) and specific antibodies for macrophage (CD68, red) and smooth muscle markers (green): (A) calponin, (B) α-smooth muscle actin (αSMA), and (C) SM22α. Cell staining was quantitated by counting cells positive for the indicated markers out of total nuclei in at least 5 fields per slide. Cells staining for CD68 were uncommon.

**Figure IV.** Evaluation of CSF-1 and CSF-1R expression by VSMCs. Cells were cultured in low (2%) or high (20%) concentrations of FBS, as indicated. (A) Sec and cs CSF-1 levels were determined in medial and neointimal VSMCs (Group I) by ELISA, as described in methods. (B) CSF-1R expression was evaluated by immunocytochemistry, using a primary goat anti-CSF-1R antibody (see supplement Figure V). Positive-staining cells were counted in at least 6 fields per condition. Total cell number was evaluated by nuclear staining with DAPI. *, $P < .05$ vs. corresponding medial sample (sec, 20% FBS). **, $P < .01$ vs. corresponding medial sample (cs, 20% FBS), and vs. low serum neointimal sample (cs, 2% FBS). ***, $P < .02$ vs 2% FBS neointimal cells.

**Figure V.** Neointimal SMCs from the $TgCS$ mouse express the CSF-1R and csCSF-1. Cells were stained with DAPI to identify nuclei, and with (A) rabbit anti-calponin (stained green) and (B) goat anti-CSF-1R (stained red) antibodies. Panel C shows merged
images, with arrows indicating some cells expressing both signals. Bar graph shows ELISA-based quantitation of sec and csCSF-1 production by these cells after stimulation with TNF-α (0–10 pg/ml).

**Figure VI.** Both donor and recipient cells produce CSF-1 in allografted arteries. X-gal staining reflects activation of the CSF-1 promoter driving a lacZ reporter gene in frozen sections from either B10A to TgZ (wt => TgZ) or TgZ to Balb/C (TgZ => wt) grafts 42 d after transplantation. Arrowheads indicate X-gal-positive cells, and NI and M indicate the neointima and media, respectively. Scale bar, 100 µm. The bar graph depicts the fraction of cells that stained X-gal-positive in the two reciprocal allograft combinations.

**Figure VII.** Diffuse expression of the CSF-1R in the neointima. Carotid arterial allografts 42 d after transplantation, stained with rabbit anti-CSF-1R antibody (left), or anti-CSF-1R antibody preabsorbed with excess CSF-1R C-terminal peptide antigen (right). Arrowheads indicate CSF-1R-positive cells. The bar graph shows evaluation of CSF-1R expression in Group I allograft arteries. Neointimal cells stained positive for CSF-1R were scored relative to the total number of cell nuclei in 6 or more fields per sample. Untransplanted artery sections were scored as reference.
Figure I, Hiroyasu et al

donor recipient % neointimal area
Isograft  FVB/NJ  FVB/NJ  1.2±0.5  (n=7)
Allograft-1  FVB/NJ  Balb/CJ  50.4±14.1 (n=3)
Allograft-2  B10.A(2R)  FVB/NJ  48.7±14.5 (n=5)
Figure II, Hiroyasu et al

PD98059 (µm)

<table>
<thead>
<tr>
<th>0</th>
<th>10</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-ERK1/2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Wortmannin (nm)

<table>
<thead>
<tr>
<th>0</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>150</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Akt (T308)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SB203580 (µm)

<table>
<thead>
<tr>
<th>0</th>
<th>.1</th>
<th>.25</th>
<th>.5</th>
<th>.75</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-p38</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PP2 (µm)

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>25</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Src (Y416)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>actin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure III, Hiroyasu et al.
Figure IV, Hiroyasu et al.

A

B

Csf-1 (pg/ml)

20%FBS + + + +

sec cs sec cs

**

*

medial neointimal

20%FBS + +

sec cs

***

medial neointimal

CSF-1R-positive cells /total nuclei

20%FBS + +
Figure V, Hiroyasu et al.
Figure VI, Hiroyasu et al.
anti CSF-1R  negative control

Figure VII, Hiroyasu et al.