Abstract—Transgenic mice with endothelium-specific endothelin-1 (ET-1) overexpression exhibit endothelial dysfunction and vascular remodeling, oxidative stress, and inflammation. We previously observed that monocytes/macrophages play a role in angiotensin II, aldosterone, and deoxycorticosterone acetate/salt-induced vascular remodeling, oxidative stress, and inflammation using a model with reduced monocytes/macrophages, the osteopetrotic (Op) mouse, which has a mutation in the macrophage colony stimulating factor (CSF1) gene. However, it is unknown whether monocytes/macrophages are implicated in adverse vascular effects of ET-1. We hypothesized that reduction in monocytes/macrophages would blunt ET-1–induced vascular injury. We performed a study on 4- to 6-month-old male mice with endothelium-specific ET-1 overexpression (eET-1), reduction in CSF1 (Csf1op/op), or both (eET-1/Csf1op/op), and their wild-type littermate control mice. There was no difference in systolic blood pressure between groups. Endothelial function and vascular structure were determined on a pressurized myograph. Endothelium-dependent relaxation in response to acethylcholine was similar in eET-1 and eET-1/Csf1op/op mice. Media:lumen ratio and media cross-sectional area were ≈1.5-fold greater in eET-1 than in wild-type mice (P<0.05), which was not observed in mice deficient in CSF1. ET-1–induced oxidative stress measured by dihydroethidium staining (P<0.05) and NADPH oxidase activity assessed with lucigenin chemiluminescence (P<0.05) were blunted by CSF1 deficiency. ET-1 caused a 2.5-fold increase in monocyte/macrophage infiltration compared with wild-type mice (P<0.001), which was blunted in the mice deficient in CSF1. Reduction of monocyte/macrophage-dependent inflammation in mice overexpressing ET-1 in endothelium results in reduced vascular remodeling and oxidative stress, providing evidence for a role of monocytes/macrophages and innate immunity in ET-1–induced vascular injury. (Hypertension. 2013;62:112-117.) • Online Data Supplement

Key Words: CSF1 ▪ innate immunity ▪ macrophage colony stimulating factor ▪ monocytes ▪ osteopetrosis mutation ▪ oxidative stress ▪ vascular remodeling

Endothelin-1 (ET)-1, a 21-aa peptide produced by endothelial and other cells, is one of the most potent vasoconstrictors.1,2 Plasma ET-1 is increased in patients with essential hypertension and in hypertension associated with systemic disorders, in atherosclerosis and heart failure, as well as in other cardiovascular and metabolic disorders, and in chronic kidney disease.3 ET-1 plays a role in the development of hypertension by causing vascular damage and inflammation.4,5 Transgenic mice overexpressing ET-1 specifically in endothelial cells (eET-1) present endothelial dysfunction and vascular remodeling, oxidative stress, and inflammation.6,7

The immune system plays a role in hypertension and vascular injury.8-10 The innate immune system is involved in angiotensin (Ang) II, deoxycorticosterone acetate (DOCA)/salt, and aldosterone-induced hypertension and vascular damage.11-13 Ang II, DOCA/salt, and aldosterone infusion caused an increase in vascular monocyte/macrophage infiltration. Ang II and DOCA/salt-induced hypertension and vascular remodeling, oxidative stress, and inflammation were blunted in a model with reduced monocytes/macrophages, the osteopetrotic (Op) mouse, which is deficient in macrophage colony stimulating factor (CSF)11,12 because of a thymidine (T) insertion in the coding region of the colony stimulating factor (Csf1) gene that generates a stop codon 21 bp downstream.14 Similarly, aldosterone-induced endothelial dysfunction and vascular oxidative stress were decreased in Csf1op/op mice.13 The innate immune system could play a role in ET-1–induced vascular injury, because the inflammation observed in eET-1 is characterized by an increase in vascular monocyte/macrophage infiltration.7 However, this remains to be demonstrated.

To test the hypothesis that innate immunity participates in ET-1–induced vascular injury, we studied whether vascular injury induced by increased expression of ET-1 is prevented in eET-1 mice crossed with Csf1op/op mice that have reduced monocyte/macrophage-dependent inflammation.

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Reduced Macrophage-Dependent Inflammation Improves Endothelin-1–Induced Vascular Injury

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Methods
Additional Materials and Methods are mentioned in the online-only Data Supplement.

Animals
The study was approved by the Animal Care Committee of the Lady Davis Institute for Medical Research and McGill University, followed recommendations of the Canadian Council for Animal Care, and was in agreement with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. We used 4- to 6-month-old male mice with endothelium-specific ET-1 overexpression (eET-1), reduction in CSF1 (Csf1<sup>Op</sup>/<sup>+</sup>), or both (eET-1/Csf1<sup>Op</sup>/<sup>+</sup>), and their wild-type (WT) littermate control mice. Mice were generated by crossing heterozygous Csf1<sup>Op</sup>/<sup>+</sup> mice (C57BL/6)<sup>11–13</sup> with eET-1 transgenic mice overexpressing the human ET-1 driven by the Tie-2 promoter/enhancer–conferring endothelial-specific expression (C57BL/6).<sup>6</sup> Mice were genotyped for the presence or absence of eET-1 by polymerase chain reaction and for Op mutation within Csf1 gene by polymerase chain reaction followed by BstXI restriction digestion (Figure S1 in the online-only Data Supplement). Systolic blood pressure was measured by tail-cuff method using MC4000 blood pressure analysis system (Hatteras Instruments, Cary, NC). Mice were anesthetized with 3% isoflurane mixed with O<sub>2</sub> at 1 L/mL, depth of anesthesia was confirmed by rear foot squeezing, blood was collected by cardiac puncture for plasma oxygen species production and monocyte/macrophage infiltration.

Statistical Analysis
Data are presented as means±SEM. Comparisons were made by 1-way ANOVA, followed by a Student–Newman–Keuls post hoc test. A 2-way ANOVA was used to compare the contractile response to ET-1 before and after BQ123. A value of P<0.05 was considered significant.

Results
Physiological Parameters
Increased expression of ET-1 and reduction in CSF1 did not affect body weight, systolic blood pressure, tibia length, and weight of the heart or kidneys (Table). Increased expression of ET-1 caused hypertrophic remodeling of mesenteric arteries. Media:lumen ratio and media cross-sectional area of mesenteric resistance arteries were ≈1.5-fold greater in eET-1 mice compared with WT mice. This was prevented by CSF1 reduction (Figure 1D and 1E). The stress–strain curve was unaffected by increased ET-1 expression (Figure 1F). As previously observed,<sup>13</sup> CSF1 deficiency reduced vascular stiffness of mesenteric resistance arteries as demonstrated by a rightward shift in the stress–strain curve compared with WT mice.

Endothelium-Dependent and Endothelium-Independent Responses
Endothelium-dependent relaxation responses to acetylcholine were not altered by overexpression of ET-1 and reduction in CSF1 (Figure 1A). In presence of the NO synthase inhibitor L-NAME, acetylcholine-induced relaxation was reduced to ≈41% in WT and eET-1 mice, whereas it was reduced to ≈75% in Csf1<sup>Op</sup>/<sup>+</sup> and eET-1/Csf1<sup>Op</sup>/<sup>+</sup> mice (Figure 1B). These results suggest that reduction in CSF1 altered the mechanisms mediating acetylcholine-induced relaxation. Endothelium-independent relaxation to the NO donor sodium nitroprusside was unaltered by overexpression of ET-1 and CSF1 deficiency (Figure 1C).

ET-1 Overexpression–Induced Vascular Remodeling Was Prevented by CSF1 Deficiency
Increased expression of ET-1 caused hypertrophic remodeling of mesenteric arteries. Media:lumen ratio and media cross-sectional area of mesenteric resistance arteries were ≈1.5-fold greater in eET-1 mice compared with WT mice. This was prevented by CSF1 reduction (Figure 1D and 1E). The stress–strain curve was unaffected by increased ET-1 expression (Figure 1F). As previously observed,<sup>13</sup> CSF1 deficiency reduced vascular stiffness of mesenteric resistance arteries as demonstrated by a rightward shift in the stress–strain curve compared with WT mice.

ET-1 Overexpression–Induced Reduction in Contractile Response to ET-1 Was Blunted by CSF1 Reduction
eET1 mice exhibited a reduced contractile response to ET-1 compared with WT mice, change that was blunted by reduced CSF1 (Figure 2A). The ETA receptor blocker BQ123 reduced maximal contractile responses to ET-1 to a similar extent in all groups (Figure 2B).

CSF1 Deficiency Prevented ET-1 Overexpression–Induced Oxidative Stress
Increased expression of ET-1 induced a 5-fold increase in reactive oxygen species generation in the aorta of eET-1 mice compared with WT mice, which was prevented by CSF1 deficiency (Figure 3A and Figure S2). ET-1 caused a 7-fold elevation in NADPH oxidase activity in mesenteric arteries of eET1 mice compared with WT mice, which was prevented by reduced CSF1 (Figure 3B).

CSF1 Reduction Prevented ET-1 Overexpression–Induced Inflammation
Monocyte/macrophage infiltration increased 2.5-fold in aorta of eET1 mice compared with WT mice (Figure 4 and Figure

Table. Physiological Parameters of Animals

<table>
<thead>
<tr>
<th>Parameters</th>
<th>WT</th>
<th>eET-1</th>
<th>Csf1&lt;sup&gt;Op&lt;/sup&gt;/&lt;sup&gt;+&lt;/sup&gt;</th>
<th>eET1/Csf1&lt;sup&gt;Op&lt;/sup&gt;/&lt;sup&gt;+&lt;/sup&gt;</th>
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<tr>
<td>Body weight, g</td>
<td>40.8±2.6</td>
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<td>SBP, mmHg</td>
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<td>106±4</td>
<td>117±3</td>
<td>111±3</td>
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<tr>
<td>Heart weight, mg</td>
<td>175±15</td>
<td>185±8</td>
<td>175±24</td>
<td>166±5</td>
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<tr>
<td>Kidneys weight, mg</td>
<td>680±56</td>
<td>819±123</td>
<td>523±48</td>
<td>601±24</td>
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<tr>
<td>Tibia length, mm</td>
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<td>25.0±0.2</td>
<td>24.0±0.3</td>
<td>24.3±0.2</td>
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<tr>
<td>Body weight/tibia length</td>
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<td>1.8±0.1</td>
<td>1.6±0.1</td>
<td>1.7±0.1</td>
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<tr>
<td>Heart weight/tibia length</td>
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<td>7.4±0.3</td>
<td>7.3±1.1</td>
<td>6.8±0.2</td>
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<tr>
<td>Kidneys weight/tibia length</td>
<td>28.4±2.1</td>
<td>32.8±5.0</td>
<td>21.8±2.0</td>
<td>24.7±0.9</td>
</tr>
</tbody>
</table>

Body and tissue weights and tibia length were determined in WT, eET-1, Csf1<sup>Op</sup>/<sup>+</sup>, and eET-1/Csf1<sup>Op</sup>/<sup>+</sup> mice. Values are means±SEM, n = 8 to 10.

Csf indicates colony stimulating factor; eET1, endothelium-specific endothelin-1 overexpression; SBP, systolic blood pressure; and WT, wild-type.
S3). Most of the increase in monocyte/macrophage infiltration was found in the adventitia and perivascular fat. As previously observed,\textsuperscript{13} \(\text{Csf1}^{\text{Op/+}}\) mice presented a 60% reduction in monocyte/macrophage infiltration compared with WT mice. Monocyte/macrophage infiltration was not different in eET-1/\(\text{Csf1}^{\text{Op/+}}\) mice compared with \(\text{Csf1}^{\text{Op/+}}\) mice.

**Discussion**

During the past few years, it has become increasingly apparent that not only is inflammation a mechanism participating in vascular injury associated with high blood pressure but also that the immune system is a major contributor to the process of inflammation in many cardiovascular conditions.\textsuperscript{8,9,15} Both the innate and the adaptive immune responses have been implicated in cardiovascular disease, particularly in hypertensive renal pathology\textsuperscript{16} and, more recently, in vascular and heart disease.\textsuperscript{12,17–19} Indeed, it was previously showed that the innate immune system is involved in Ang II and DOCA/salt-induced hypertension and vascular injury and in aldosterone-induced vascular damage in a blood pressure–independent fashion.\textsuperscript{11–13} Other investigators have as well more recently confirmed a

**CSF1 Reduction Does Not Alter ET-1 Overexpression**

As we previously described,\textsuperscript{6} plasma ET-1 level was increased 9-fold in eET-1 mice compared with WT mice (Figure 5). Basal or increased plasma ET-1 levels were not affected by \(\text{Csf1}^{\text{haploinsufficiency}}\). Plasma ET-1 level in \(\text{Csf1}^{\text{Haplo}}\) was unchanged relative to WT mice and was increased 16-fold in eET-1/\(\text{Csf1}^{\text{Haplo}}\) mice compared with \(\text{Csf1}^{\text{Haplo}}\) mice. Thus, the reduced vascular injury observed in eET-1/\(\text{Csf1}^{\text{Haplo}}\) mice is not attributable to changes or a decrease in ET-1 overexpression.

Figure 2. Endothelin-1 (ET-1) overexpression–induced reduction in contractile response to ET-1 was blunted by colony stimulating factor (CSF) reduction. Contractile response curve to ET-1 (A) and contractile response to 10\(^{-6}\) mol/L ET-1 in the absence (control) and presence (+BQ123) of ET\(_{\text{a}}\) receptor antagonist BQ123 (B) were determined in mesenteric arteries of wild-type (WT), endothelium-specific endothelin-1 overexpression (eET-1), colony stimulating factor-deficient (\(\text{Csf1}^{\text{Op/+}}\)), and eET-1/\(\text{Csf1}^{\text{Op/+}}\) mice. Values are means±SEM. *\(<0.05\) vs wild-type (WT) and ‡\(<0.01\) and ‡‡\(<0.001\) vs respective control. n=6 to 10 for A and 7 to 9 for B.

Figure 3. Colony stimulating factor (CSF1) reduction prevented endothelin-1 (ET-1) overexpression–induced oxidative stress. Production of reactive oxygen species (ROS) using dihydroethidium (DHE) staining of aorta (A) and NADPH oxidase activity in mesenteric arteries using lucigenin chemiluminescence (B) were determined in wild-type, endothelium-specific endothelin-1 overexpression (eET-1), colony stimulating factor-deficient (\(\text{Csf1}^{\text{Op/+}}\)), and eET-1/\(\text{Csf1}^{\text{Op/+}}\) mice. Quantification of ROS levels expressed as relative fluorescence units (RFU)/\(\mu\)m\(^2\) is presented in A. NADPH oxidase activity is expressed as relative light units (RLU)/mg of dry tissue weight. Values are means±SEM, n=3 to 4 for A and 4 to 7 for B. *\(<0.01\) and **\(<0.001\) vs wild-type.
role for macrophages/monocytes in Ang II–induced actions on blood pressure and the vasculature.6,7 Thus, although different forms of hypertension are associated with a role of immune mechanisms, whether all experimental models of hypertension have an immune component is unknown. The present study demonstrates for the first time that ET-1–induced vascular hypertrophic remodeling and oxidative stress are prevented by CSF1 deficiency, which leads to an impaired monocyte and macrophage production and maturation,20 thus demonstrating a role of the latter in this particular model. CSF1 deficiency leads also to deficiency in osteoclast production and differentiation in the bone. However, there is no reason to believe that osteoclasts play a role in ET-1–induced vascular damage in the present model.

Enhancement of ET-1 expression in eET-1 mice was associated with a decreased contractile response to ET-1, hypertrophic remodeling and vascular oxidative stress, and inflammation.6,7 However, increased ET-1 expression did not result in endothelial dysfunction. The latter phenotype could be attributable to additional backcrossing of the mice in the C57BL/6 background or to an accidental selection for a NO synthase–independent relaxation mechanisms. The decrease in monocyte/macrophage infiltration in the adventitia could play a role in the change in the relaxation mechanisms in Csf1<sup>op/op</sup> mice. This was not found in previous studies using the Csf1<sup>op/op</sup> mice<sup>11–13</sup> and could also result from backcrossing of the mice in the C57BL/6 background.

WT littermate mice were used to control for the gain in ET-1 expression or deficiency in CSF1. WT littermate mice also controlled for the background and environment. This was possible because they are littermates of eET-1 and Csf1<sup>op/op</sup> mice. All the mice, including the eET-1/Csf1<sup>op/op</sup> mice, were produced by mating eET-1 mice with Csf1<sup>op/op</sup> mice. Both eET-1 and Csf1<sup>op/op</sup> mice have the same C57BL/6 background. Csf1<sup>op/op</sup> mice were used to control for gain in ET-1 expression in the presence of CSF1 deficiency. Csf1<sup>op/op</sup> mice are littermates of eET-1/Csf1<sup>op/op</sup> mice and control also for the background and environment.

Using Csf1<sup>op/op</sup> and Csf1<sup>op/op</sup> mice, we had concluded that the innate immune system plays a role in Ang II, DOCA/salt, and aldosterone-induced vascular injury.11–13 Deficiency of CSF1 prevented Ang II and DOCA/salt-induced endothelial dysfunction, hypertrophic remodeling, oxidative stress and inflammation, and aldosterone-induced endothelial dysfunction and oxidative stress. In this study, reduction in CSF1 blunted ET-1–induced hypertrophic remodeling and oxidative stress. The common finding between all these studies is that deficiency or reduction in CSF1 decreased vascular monocyte/macrophage infiltration and blunted the oxidative stress response to Ang II, DOCA/salt, aldosterone, and ET-1. These data support a role for monocytes/macrophages and the innate immune system in the development of vascular oxidative stress, and its contribution to vascular injury. However, what triggers the innate immune system response remains to be clarified.

The adventitia is a complex structure of blood vessels, constituted by an extracellular matrix scaffold containing fibroblasts, blood and lymphatic vessels (and, thus, endothelial
cells), nerves, progenitor cells, and immune cells, including macrophages. \(^2^\) Perivascular fat is closely associated to the adventitia. Overexpression of ET-1 in endothelial cells caused an increase in monocyte/macrophage infiltration in the adventitia and associated perivascular fat, which is similar to findings in mice infused with Ang II or aldosterone. \(^2^,^3^,^4^\) However, the mechanisms by which Ang II, aldosterone, or ET-1 cause an increase in immune cell infiltration remain unknown. Vascular inflammation could be initiated and perpetuated in the adventitia, according to the outside-in hypothesis. \(^2^\) Increased expression of ET-1 in endothelium or within the adventitia could act in paracrine fashion directly on monocytes or macrophages or via other cells, such as the smooth muscle cells. In this study and previously, we found that a deficiency in CSF1 impaired monocyte/macrophage infiltration induced by ET-1, Ang II, and DOCA/salt in rodents, the latter a hypertensive model with an important ET-1–dependent component. CSF1 is a critical factor for monocyte production, monocyte to macrophage differentiation, and macrophage maturation. \(^2^\) Op mice have a deficiency in peripheral blood monocytes and in macrophages in tissues, \(^2^,^2^,^5^\) and in addition, residual macrophages in tissues of Op mice present an immune phenotype. \(^2^\) Furthermore, CSF1 is expressed locally in endothelial cells, smooth muscle cells, monocytes, and macrophages. \(^2^\) Therefore, it can be expected that a deficiency in CSF1 will have a profound effect on the bone marrow monocyte production and, at the vascular level, on monocyte to macrophage differentiation and macrophage maturation, resulting in blunting of vascular inflammation.

The adaptive immune system has also been implicated in Ang II, DOCA/salt, and aldosterone-induced vascular damage. \(^1^,^2^,^3^,^4^\) Whether it is involved in ET-1–induced vascular injury is unknown. In addition, it has not been determined whether the activation of the innate immune system precedes or accompanies activation of the adaptive immune response. However, it is reasonable to think that activation of innate immunity stimulates the adaptive immunity because macrophages could act as antigen-presenting cells to T lymphocytes.

ET-1 and the innate immune system could play a role in the development of vascular injury in patients with hypertension. Parissis et al. \(^2^\) have observed a significant correlation between elevated plasma ET-1 and peripheral monocyte–related inflammatory markers in patients with hypertension with or without hypercholesterolemia. Further work is required to demonstrate this mechanism in humans.

In conclusion, by crossing mice that overexpress ET-1 in the endothelium with mice with deficient macrophages as a consequence of a mutation in the Csf1 gene, we have extended the findings of the role of the innate immune system to a rodent model of vascular injury associated with ET-1 overexpression. These results unambiguously implicate innate immunity in inflammatory responses to ET-1.

**Perspectives**

Monocytes/macrophages and, therefore, the innate immune system play a role in vascular injury in different conditions, leading to the development or maintenance of hypertension by increasing oxidative stress. These include the actions of Ang II and mineralocorticoids, to which ET-1 is now added. Additional work is required to reveal the mechanism of activation of the innate immune system in this paradigm. Discovery of mechanisms involved in the activation of the innate immunity could allow discovery of novel therapeutic targets for the treatment of hypertension.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is New?**

- Reduced colony stimulating factor (CSF1) prevented endothelin-1 (ET-1)–induced hypertrophic remodeling and oxidative stress.

**What Is Relevant?**

- The results of this study and of preceding studies support a role for the innate immunity, monocytes/macrophages, and oxidative stress in the development of vascular injury associated with hypertension.

- Determination of the mechanism causing activation of the innate immune system could lead to the discovery of novel therapeutic targets for hypertension.

**Summary**

Reduction in monocyte/macrophage-dependent inflammation blunted ET-1–induced mesenteric resistance artery hypertrophic remodeling and vascular oxidative stress, which was demonstrated by crossing eET-1 with Csf1<sup>OP/OP</sup> mice. Discovery of the mechanism of activation of the innate immune system could reveal new therapeutic targets for hypertension.