Bone Marrow Angiotensin AT\textsubscript{1} Receptor Regulates Differentiation of Monocyte Lineage Progenitors From Hematopoietic Stem Cells


**Background**—The angiotensin II (Ang II) type 1 (AT\textsubscript{1}) receptor is expressed in bone marrow (BM) cells, whereas it remains poorly defined how Ang II regulates differentiation/proliferation of monocyte-lineage cells to exert proatherogenic actions.

**Methods and Results**—We generated BM chimeric apoE\textsuperscript{-/-} mice repopulated with AT\textsubscript{1}-deficient (Agtr1\textsuperscript{-/-}) or wild-type (Agtr1\textsuperscript{+/+}) BM cells. The atherosclerotic development was significantly reduced in apoE\textsuperscript{-/-}/BM-Agtr1\textsuperscript{-/-} mice compared with apoE\textsuperscript{-/-}/BM-Agtr1\textsuperscript{+/+} mice, accompanied by decreased numbers of BM granulocyte/macrophage progenitors (GMPe-Kit\textsuperscript{+} Sca-1\textsuperscript{-} Lin\textsuperscript{-} CD34\textsuperscript{-} CD16/32\textsuperscript{+}) and peripheral blood monocytes. Macrophage-colony-stimulating factor (M-CSF)–induced differentiation from hematopoietic stem cells (HSCs; c-Kit\textsuperscript{+} Sca-1\textsuperscript{-} Lin\textsuperscript{-}) to promonocytes (CD11b\textsuperscript{hi} Ly-6Ch\textsuperscript{lo}) was markedly reduced in HSCs from Agtr1\textsuperscript{-/-} mice. The expression of M-CSF receptor c-Fms was decreased in HSCs/promonocytes from Agtr1\textsuperscript{-/-} mice, accompanied by a marked inhibition in M-CSF–induced phosphorylation of PKC-\(\delta\) and JAK2. c-Fms expression in HSCs/promonocytes was mainly regulated by TNF-\(\alpha\) derived from BM CD45\textsuperscript{-} CD34\textsuperscript{-} stromal cells, and Ang II specifically regulated the TNF-\(\alpha\) synthesis and release from BM stromal cells.

**Conclusions**—Ang II regulates the expression of c-Fms in HSCs and monocyte-lineage cells through BM stromal cell–derived TNF-\(\alpha\) to promote M-CSF–induced differentiation/proliferation of monocyte-lineage cells and contributes to the proatherogenic action. (**Arterioscler Thromb Vase Biol. 2009;29:1529-1536.**)

**Key Words:** bone marrow progenitors ■ angiotensin ■ monocyte ■ atherosclerosis ■ M-CSF

The angiotensin II (Ang II) type 1 (AT\textsubscript{1}) receptor exerts proatherogenic actions.\textsuperscript{1} AT\textsubscript{1} receptor–deficient (Agtr1\textsuperscript{-/-}) mice showed a significant reduction of atherosclerotic development,\textsuperscript{2,3} and treatment with AT\textsubscript{1} receptor blocker (ARB) reduced the size of atherosclerotic lesions both in experimental animals and humans.\textsuperscript{4} AT\textsubscript{1} receptors are present in a variety of cells, including endothelial cells, vascular smooth muscle cells, and bone marrow (BM) stem cells and progenitors.\textsuperscript{5,6} Recently, Cassis et al demonstrated that Ang II–induced atherosclerosis was significantly attenuated in LDL receptor–deficient (LDLr\textsuperscript{-/-}) mice whose BM cells were repopulated with Agtr1\textsuperscript{-/-} cells.\textsuperscript{7} Fukuda et al also reported that atherosclerotic lesion development was significantly reduced in apoE-deficient (apoE\textsuperscript{-/-}) mice with Agtr1\textsuperscript{-/-} marrow.\textsuperscript{8} However, no information regarding the role of the BM-AT\textsubscript{1} receptor on the differentiation/proliferation and properties of BM stem cells and progenitors has been reported in these previous studies.

Monocytes and macrophages play a crucial role in the pathogenesis of atherosclerosis, which is characterized by plaque progression, destabilization, and subsequent plaque rupture, through foam cell formation, migration/proliferation of resident vascular smooth muscle cells, and degradation of extracellular matrix.\textsuperscript{9} Along with the previous studies showing the effect of diet-induced hypercholesterolemia on BM and leukocyte,\textsuperscript{10} Swirska et al reported that hypercholesterolemia induced a surprisingly profound expansion of blood monocytes as well as BM monocyte-lineage cells.\textsuperscript{11} However, the relative contribution of the BM renin–angiotensin system to hypercholesterol-emia-associated monocytosis has not been fully investigated.\textsuperscript{12}

In the present study, we focused on the action of the AT\textsubscript{1} receptor expressed in BM cells and studied whether (1) Ang II affects the differentiation/proliferation from BM stem cells into monocyte-lineage cells, and (2) hypercholesterolemia-associated monocytosis contributes to the development of AT\textsubscript{1}-mediated atherosclerosis. Our results demonstrated for the first time that (1) Ang II promotes M-CSF–induced differentiation from hematopoietic stem cells (HSCs; c-Kit\textsuperscript{+} Sca-1\textsuperscript{-} Lin\textsuperscript{-}) into monocyte-lineage cells through up-regulation of the M-CSF receptor c-Fms, and that (2) TNF-\(\alpha\)...
Kit atherosclerotic lesions in apoE cells of 2-month-old female apoE (NY) and Tanabe Seiyaku Co Ltd (Osaka, Japan), respectively. BM mice (C57BL/6) were obtained from Taconic Co Ltd (Germantown, NY). Monocytes were isolated from BM mono-nuclear cells (CD11b^high^Ly-6G^low^), thus leading to controlled by Ang II specifically regulates the c-Fms expression in promonocytes (CD11b^high^Ly-6G^low^), thus leading to the increased numbers of circulating monocytes that modulate AT1-mediated proatherogenic activities.

Methods

A full description of all methods can be found in the Data Supplement (available online at http://atvb.ahajournals.org).

Animal Preparation

ApoE^−/−^ mice (C57BL/6) and AT1a receptor-deficient (Agtr1^−/−^) mice (C57BL/6) were obtained from Taconic Co Ltd (Germantown, NY) and Tanabe Seiyaku Co Ltd (Osaka, Japan), respectively. BM cells of 2-month-old female apoE^−/−^ recipient mice were repopulated with male Agtr1^−/−^ or Agtr1^+/+^ cells. The percentage chimerism determined by transplanting GFP-overexpressing BM cells was 96±2% of peripheral blood mononuclear cells. Furthermore, BM CD45^−^CD34^+^stromal cells, HSCs, and myeloid progenitors (MP: c-Kit^+^Sca-1^−^Lin^−^) were almost completely (more than 99%) replaced by GFP-positive cells (supplemental Figure I). All animal experiments were conducted according to the Guidelines for Animal Experiments at Kyoto Prefectural University School of Medicine.

Statistical Analysis

All data are expressed as the mean±SE. Mean values were compared using ANOVA. If a statistical significant effect was found, Fisher test was performed to detect the difference between the groups. P<0.05 was considered statistically significant.

Results

BM-AT1 Deficiency Attenuates Atherosclerosis Concomitant With the Reduction of BM Monocyte-Lineage Cells

Consistent with the previous reports, apoE^−/−^/BM-Agtr1^−/−^ mice showed a significant reduction of atherosclerotic lesions compared with apoE^−/−^/BM-Agtr1^+/+^ mice (31%, P<0.05; Figure 1A). At 3 months after BMT, the numbers of white blood cells and monocytes were similar between the 2 groups (supplemental Table I). However, after 2 months of a Western diet feeding, white blood cells and monocytes were significantly less abundant in apoE^−/−^/BM-Agtr1^−/−^ mice than apoE^−/−^/BM-Agtr1^+/+^ mice by 40% and 39%, respectively (P<0.05; supplemental Table II).

HSCs (c-Kit^+^Sca-1^−^Lin^-^) have been shown to differentiate into common myeloid progenitors (CMP:c-Kit^+^Sca-1^−^Lin^-^CD34^+^CD16/32^-^) and then granulocyte/macrophage progenitors (GMP:c-Kit^+^Sca-1^−^Lin^-^CD34^+^CD16/32^-^), followed by the terminal differentiation into BM monocytes (CD11b^high^Ly-6G^low^). We examined BM-AT1−me-
diated effects on the differentiation/proliferation of HSCs and monocyte-lineage cells by flow cytometric analysis. The number of GMP was much lower in apoE−/−/BM-Agrt1−/− mice (34%, P<0.05), whereas HSCs and CMP did not differ between the 2 groups (Figure 1B). The expression level of CCR2 on monocyte-lineage cells was not impaired in Agrt1−/− mice (supplemental Figure II).

M-CSF–Induced Macrophage-Colony–Forming Activity Is Attenuated in BM Cells From AT1-Deficient Mice

We first compared the numbers of HSCs, CMP, and GMP between Agrt1+/+ and Agrt1−/− mice under steady-state condition without hypercholesterolemia. There was no difference between the 2 genotypes of mice, suggesting that the steady-state development of monocyte-lineage cells is relatively well preserved in Agrt1−/− mice (supplemental Figure IIIA, supplemental Table III). We next performed a macrophage-colony–forming assay to investigate whether the response to M-CSF is attenuated in BM cells from Agrt1−/− mice. Stimulation by M-CSF markedly increased the number of macrophage-colony units in BM cells from Agrt1+/+ mice, which was remarkably diminished in BM cells from Agrt1−/− mice (P<0.01; supplemental Figure IIIB), suggesting that BM-AT1 is crucially implicated in M-CSF–induced differentiation/proliferation of HSCs into monocyte-lineage cells.

M-CSF–Induced Differentiation From HSCs to Monocyte-Lineage Cells Is Suppressed in HSCs From AT1-Deficient Mice

We examined the time course of differentiation of HSCs from Agrt1+/+ mice into monocyte-lineage cells with or without M-CSF. Stimulation by M-CSF preferentially increased the number of promonocytes (CD11bhighLy-6Glow) terminally differentiated from myeloid progenitor (MP; c-Kit+Sca-1+Lin−; supplemental Figure IV). We next compared the differentiation potential of HSCs between Agrt1+/+ and Agrt1−/− mice (Figure 2). In the absence of M-CSF, the numbers of myeloid progenitors and promonocytes (CD11bhighLy-6Glow) did not differ between the 2 genotypes. In contrast, stimulation by M-CSF markedly increased the number of promonocytes in both groups, whereas the extent was significantly attenuated in HSCs from Agrt1−/− mice (38%, P<0.01).

c-Fms Expression Is Inhibited in AT1-Deficient Mice

The expression of M-CSF receptor c-Fms was examined by flow cytometry. Consistent with the previous finding,16 the expression level of c-Fms (CD115) was gradually upregulated during the developmental stage from HSCs to promonocytes in Agrt1+/+ mice, whereas the expression was severely decreased in all developmental stages in Agrt1−/− mice (Figure 3). The mRNA expression of c-Fms was also suppressed by 71% in Agrt1−/− mice (P<0.05; supplemental Figure V).

We also examined the effect of hypercholesterolemia on the c-Fms expression. Four-week Western diet feeding significantly increased the expression level of c-Fms (CD115) in all populations of HSCs, myeloid progenitors, and promono-
cytes compared with chow diet feeding (supplemental Figure VI). In contrast, BM-AT1 expression was not affected by the Western diet feeding (supplemental Figure VII). These findings suggest that in the hypercholesterolemic setting, M-CSF–mediated growth of monocyte-lineage cells is enhanced by an increase in its receptor c-Fms expression.

Phosphorylation of PKC-δ and JAK2 Is Inhibited in Monocyte-Lineage Cells From AT1-Deficient Mice

To investigate the effect of reduced c-Fms expression on its downstream signals, we examined the phosphorylation of PKC-δ and JAK2, which are known to be essential in M-CSF–induced differentiation/proliferation of monocyte-lineage cells.17,18 In c-Kit+Lin− population including HSCs (c-Kit+Sca-1+Lin−) and myeloid progenitors (c-Kit+Sca-1−Lin−) from Agrt1+/+ mice, the peak phosphorylation levels of PKC-δ and JAK2 were observed at 5 and 30 minutes after M-CSF stimulation, respectively (supplemental Figure VIII A). The M-CSF–induced phosphorylation levels of PKC-δ and JAK2 at each time point were dramatically diminished in HSCs and myeloid progenitors from Agrt1−/− mice (80% and 75%, respectively, P<0.01; supplemental Figure VIIIIB and VIIC). These findings were also confirmed by Western blot analysis (supplemental Figure IX). We further examined the effect of PKC-δ inhibitor (rottlerin) or JAK2 inhibitor (AG490) on M-CSF–induced differentiation/proliferation of BM monocyte-lineage cells. Administration of rottrlerin (10μmol/L) or AG490 (50μmol/L) into the culture medium completely diminished the M-CSF–induced increase in the number of promonocytes (supplemental Figure X).

The Expression of c-Fms on Promonocytes Is Not Affected by Ang II or ARB

We next studied how AT1 signals regulate the c-Fms expression on HSCs/promonocytes. The result from the in vitro culture assay showed that 4-day treatment with Ang II (1 μmol/L) or ARB (10 μmol/L) did not affect the expression levels of c-Fms on HSCs, myeloid progenitors, and promonocytes (only data in promonocytes shown in Figure 4), and also did not affect M-CSF–mediated growth of HSCs, myeloid progenitors, and promonocytes (supplemental Figure XIA), suggesting that AT1 receptor–mediated signals are not directly involved in the expression of c-Fms on HSCs and BM monocyte-lineage cells nor the differentiation from HSCs to promonocytes.

TNF-α Restores the Impaired Expression of c-Fms on Promonocytes From Agrt1−/− Mice

To further elucidate the mechanism by which Ang II regulates the expression of c-Fms, we next focused on the BM stromal cells (CD45−CD34−) other than hematopoietic-lineage cells, and examined the expression of TNF-α, because TNF-α has been reported to regulate the c-Fms expression in various cell types.19,20 Interestingly, the expression level of TNF-α was extremely higher in the purified CD45−CD34− BM stromal cells compared with that in promonocytes (supplemental Figure XII A). Immunohistochemical analysis also showed that TNF-α–positive staining was mostly colocalized with BM stromal cells (supplemental Figure XII B). Furthermore, we found that the number of...
CD45⁺CD34⁻ BM stromal cells was markedly diminished in Agtr1⁻/⁻ mice compared with the Agtr1⁺/⁺ mice (Figure 5A).

We further examined the effect of TNF-α on c-Fms expression in promonocytes from Agtr1⁻/⁻ mice. Consistent with the previously reported data,¹⁹,²⁰ 4-day treatment with TNF-α (50 ng/mL) upregulated (65% versus control, \( P < 0.01 \)) the expression level of c-Fms in promonocytes from Agtr1⁺/⁺ mice (Figure 4). Interestingly, the similar extent of
TNF-α–mediated induction of c-Fms was also observed in promonocytes from Agtr1−/− mice (Figure 4), indicating that TNF-α–mediated expression of c-Fms in HSCs and promonocytes is not impaired by AT1 deficiency.

**AT1 Signals Regulate Growth of BM Stromal Cells and TNF-α Expression**

The effects of AT1 deficiency and ARB on the number of BM stromal cells and their expression of TNF-α were studied. Real-time PCR analysis showed that AT1 mRNA expression is detectable in myeloid progenitors, promonocytes, and BM CD45−CD34− stromal cells, whereas no significant expression is observed in HSCs (supplemental Figure XIII). One-week administration of ARB (Olmesartan: 3 mg/kg/d) into the wild-type mice profoundly reduced the percentage fraction of BM stromal cells, the extent of which was similar to that in Agtr1−/− mice (Figure 5A). Furthermore, ARB treatment significantly decreased the expression level of TNF-α mRNA in BM stromal cells (Figure 5C). Considering that Ang II did not affect the c-Fms expression (Figure 4) or the proliferation (supplemental Figure XIA) of HSCs, myeloid progenitors, and promonocytes, it is likely that the target of ARB is BM stromal cells, and Ang II directly regulates their growth and TNF-α synthesis/release, leading to the modulation of c-Fms expression on BM monocyte-lineage cells in a paracrine fashion.

**ARB Reduces Atherosclerosis Accompanied by a Reduction of Monocyte-Lineage Cells Without Affecting Serum M-CSF Levels**

We studied the effect of ARB on monocyte-lineage development in apoE−/− mice fed a Western diet. The atherosclerotic

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**Figure 3.** Inhibition of M-CSF receptor expression on ablation of marrow AT1. A, Flow cytometry for c-Fms expression in HSCs, myeloid progenitors (MP: c-Kit+ Sca-1− Lin−), and promonocytes (CD11bhigh Ly-6Glow) from Agtr1+/+ and Agtr1−/− mice under steady-state condition without hypercholesterolemia. B, Quantitative analysis showing a significant inhibition of c-Fms expression in each fraction. Values are the mean±SE for at least 4 mice in each group. *P<0.05 vs Agtr1+/+.
lesion area showed a significant reduction in ARB-treated mice compared with hydralazine- and saline-treated mice (supplementary Figure XIVA). Whereas the number of myeloid progenitors was significantly increased by 4-week Western diet feeding, it was completely reduced in ARB-treated mice (supplementary Figure XIVB), consistent with the results from BM chimeric mice. Furthermore, the frequency of circulating monocytes (CD11b<sup>hi</sup>Ly-6G<sup>hi</sup>Ly-6Ch<sup>hi</sup>) in saline- and hydralazine-treated mice was completely diminished in ARB-treated mice (supplementary Figure XIVB). The serum M-CSF concentration was significantly elevated by 4-week Western diet feeding but was not suppressed by ARB treatment (supplementary Figure XIVC). These findings suggest that the decreased number of monocyte-lineage cells in ARB-treated hypercholesterolemia mice is not attributable to a decrease in serum M-CSF levels but to the direct actions of ARB on BM cells.

Discussion

The present study demonstrated that Ang II affects the expression profile of the M-CSF receptor c-Fms on HSCs and monocyte-lineage cells through BM stromal cell-derived TNF-α, and thereby regulates M-CSF-mediated differentiation/proliferation of BM monocyte-lineage cells followed by the mobilization of monocytes, which contributes to the AT<sub>1</sub>-mediated proatherogenic actions. These findings provide novel information on the BM tumour-nangiotensin system and a unique opportunity to develop therapeutic strategies targeting BM stem cells for the prevention of atherosclerotic cardiovascular disease.

In contrast with Ang II–induced atherosclerosis, the role of BM-AT<sub>1</sub> on hypercholesterolemia-induced atherosclerosis is controversial. Fukuda et al demonstrated that apoE<sup>−/−</sup> mice repopulated with Agtr1<sup>−/−</sup> marrow showed a modest but significant reduction of atherosclerotic lesion development, whereas ablation of BM-AT<sub>1</sub> receptor in LDLr<sup>−/−</sup> mice had no effect on atherosclerosis, suggesting that the different models used may differ in their consequence of BM-AT<sub>1</sub> on atherosclerosis. Indeed, Strawn et al demonstrated that native LDL significantly upregulated AT<sub>1</sub> receptor expression on CD34<sup>+</sup> cells, which was completely diminished by treatment with a neutralizing LDL receptor antibody, suggesting that hypercholesterolemia–induced expression of AT<sub>1</sub> receptor is comparatively higher in apoE<sup>−/−</sup> mice than LDLr<sup>−/−</sup> mice. In addition, Daugherty et al demonstrated that hypercholesterolemia extensively increased the plasma Ang II concentration in LDLr<sup>−/−</sup> mice, which was completely abolished in Agtr1<sup>−/−</sup> mice. Taken together, it is quite likely that BM-AT<sub>1</sub> receptor activation is more implicated in the hypercholesterolemia–induced atherosclerosis in apoE<sup>−/−</sup> mice rather than LDLr<sup>−/−</sup> mice.

BM stem cells are primed for multilineage gene expression and can differentiate into all types of blood cells. M-CSF is the principal regulator of proliferation and terminal differentiation of monocyte-lineage cells. We found that M-CSF–induced colony forming activity was dramatically attenuated in BM cells from Agtr1<sup>−/−</sup> mice, and that in vitro differentiation of HSCs from Agtr1<sup>−/−</sup> mice was significantly reduced in the presence of M-CSF. We further demonstrated that M-CSF receptor c-Fms expression and its downstream signaling were impaired. In hypercholesterolemia, activated endothelial cells, vascular smooth muscle cells, and inflammatory leukocytes have been shown to secrete a variety of cytokines, chemokines, and growth factors, including M-CSF. In this study, we showed that serum M-CSF levels were significantly elevated in apoE<sup>−/−</sup> mice fed a Western diet (supplementary Figure XIVc). Accordingly, Ang II–mediated action in the differentiation/proliferation of monocyte-lineage cells is considered to be more augmented in various pathological conditions as well as atherosclerosis, in which serum M-CSF levels were elevated.

The M-CSF receptor c-Fms is encoded by the <i>c-fms</i> protooncogene, whose expression is predominantly regulated by the transcription factor Pu.1. Agtr1<sup>−/−</sup> mice do not show any phenotype of low growth rate, tooth deficiency, severe osteopenosis, reduced bone marrow cellularity, or...
depletion of circulating monocytes, all of which were observed in Csf1r−/− mice, in which the c-Fms gene is genetically disrupted. Likewise, Agtr1−/− mice do not show any of the phenotypes observed in PU.1−/− mice. TNF-α directly stimulates BM blood osteoclast precursor genesis by enhancing c-Fms expression. TNF-α increased the expression level of c-Fms on promonocytes from Agtr1−/− mice to the same extent as Agtr1+/+ mice (Figure 4), suggesting that TNF-α–mediated expression of c-Fms is not impaired by AT1 deficiency. Given the reduced expression of TNF-α in BM cells from Agtr1−/− mice (Figure 5B), it is conceivable that decreased expression of c-Fms in monocyte-lineage cells from Agtr1−/− mice is primarily attributable to the impaired TNF-α–mediated actions.

Bone marrow niche plays an important role in the differentiation and proliferation of HSCs, in which BM stromal cells and mesenchymal stem cells (MSCs) regulate localization, self-renewal, and differentiation of HSCs through the secretion of cytokines and growth factor, cell-to-cell interactions, and the influence of extracellular matrix proteins. Matsushita et al reported that BM-MSCs expressed AT1 receptor and secreted Ang II. BM stromal cells and BM-MSCs have been reported to secrete TNF-α as well as M-CSF. Our present study demonstrates that TNF-α derived from BM stromal cells upregulates the c-Fms expression on HSCs and BM monocyte-lineage cells, and that AT1 deficiency is indirectly involved in the regulation of c-Fms expression by inhibiting the proliferation of BM stromal cells. Considering that ARB treatment of the wild-type mice inhibits the proliferation of BM stromal cells (Figure 5), and that AT1 signals activate ERK1/2 and Akt pathways in mesenchymal stem cells, it is likely that Ang II plays an important role in the proliferation of BM stromal stem cells rather than HSCs. In fact, the mRNA expression level of AT1 is much higher in BM stromal cells, whereas no expression was detected in HSCs (supplemental Figure XIII). Further studies will be needed to elucidate how Ang II differentially regulates the proliferation and differentiation of BM stem cells. ARB treatment significantly attenuated macrophage-colony-forming activity in a dose-dependent manner (supplemental Figure XIB). Ang II has been shown to augment the number of macrophage-colony forming units. In contrast with these findings, neither Ang II nor ARB treatment affected the M-CSF–induced differentiation of monocyte-
lineage cells in vitro culture assay. Colony forming unit assays were performed using total BM cells that include nonhematopoietic lineage cells such as BM stromal cells and BM-MSCs. The discrepant result from in vitro culture assay seems to be attributable to the effects of Ang II or ARB on BM stromal cells and BM-MSCs. Thus, the target of ARB is BM monocyte-lineage cells through TNF-α mediated up-regulation of c-Fms expression on BM monocyte-lineage cells.

In conclusion, our findings demonstrate that Ang II promotes the M-CSF-mediated differentiation/proliferation of BM monocyte-lineage cells through TNF-α-mediated up-regulation of c-Fms expression, and that the TNF-α is mainly derived from BM stromal cells growth-controlled by Ang II and specifically regulates the c-Fms expression on monocyte-lineage cells, thus leading to the increased numbers of circulating monocytes that modulate AT₁-mediated proatherogenic activities.

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Disclosures
None.

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Supplement Figure I

% of maximum

GFP

HSC

MP

BM CD45-CD34- stromal cells

apoE+/apoE-/- BM-GFP-tg
Supplement Figure II

A

B

Agtr1+/+ Agtr1−/−

Promonocytes

CCR-2 expression (MFI)

Supplement Figure II
Supplement Figure IV

A

Day 2  Day 3  Day 4  Day 5  

M-CSF(-)  

M-CSF(+)  

CD11b  
c-Kit  Sca-1  

promonocytes  

B

Cell count  

M-CSF  

Day 3  Day 4  Day 5  

HSC  MP  Promonocytes (CD11b<sup>high</sup>Ly-6G<sup>low</sup>)

(Cells/dish)

(50000  40000  30000  20000  10000  0)

#  *
Supplement Figure V

C-Fms mRNA level (fold)

Agtr1^{+/+}  Agtr1^{-/-}

Promonocytes

*
Supplement Figure VI

A

B

% CD115 positive cells

Chow
Western
Chow
Western
Chow
Western

HSC
MP
Promonocytes

*
Supplement Figure VIII

A

0 min 1 min 5 min 15 min 30 min

B

Agtr1+/+ Agtr1-/-

C

p-PKCδ

p-JAK2

% of phosphorylated cells

*  #
Supplement Figure IX

A

Agtr1+/+  Agtr1−/

- phospho-PKCδ
- PKCδ
- phospho-JAK2
- JAK2

B

Relative pPKCδ ratio

Relative pJAK2 ratio

0
0.2
0.4
0.6
0.8
1.0
1.2

Agtr1+/+  Agtr1−/

*
Supplement Figure X

M-CSF (50 ng/ml) - + + + +
AG490 (50 μM) - - + -
Rottlerin (10 μM) - - - +

Agtr1+/+
Supplement Figure XI

A

<table>
<thead>
<tr>
<th>Condition</th>
<th>Total cell</th>
<th>HSC</th>
<th>MP</th>
<th>promonocyte</th>
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<tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Ang II (1µM)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>ARB (10µM)</td>
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</tr>
<tr>
<td>ARB (10µM)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B

![Bar graph showing CFU-M colonies per dish](image)

- Olmesartan (µM): (-), 1, 10, 100
- CFU-M colonies per dish: 0, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 8500, 9000, 9500, 10000, 10500, 11000, 11500, 12000

- N.D.: Not detectable
- *: Significant difference
- **: Highly significant difference

Graph A shows the effect of Olmesartan on cell counts for different conditions.

Graph B presents the CFU-M colonies per dish for various concentrations of Olmesartan.
Supplement Figure XIII

AT1a receptor mRNA level (fold)

N.D.  

* 

** 

Total BM cells  

HSC  

MP  

Promonocytes  

CD45^-CD34^-cell
Supplement Figure XIV

A

Control Saline Hydralazine Olmesartan

Atherosclerotic lesion area (mm²)

B

Control Saline Hydralazine Olmesartan

C

Control Saline Hydralazine Olmesartan

Serum M-CSF (pg/mL)

%LY6Chigh circulating monocytes (CD11bhighLY6Glo)

# * †

Supplement Figure XIV
## Supplement Table 1
Circulating Blood Cells in Chimeric Mice 3 Months after Bone Marrow Transplantation

<table>
<thead>
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<th>RBC x 10⁴/µl</th>
<th>WBC/µl</th>
<th>Neutrophil/µl</th>
<th>Lymphocyte/µl</th>
<th>Monocyte/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BM-Agtr1⁺/⁺</strong></td>
<td>8</td>
<td>773 ± 24</td>
<td>5500 ± 686</td>
<td>2012 ± 229</td>
<td>3317 ± 520</td>
<td>179 ± 53</td>
</tr>
<tr>
<td><strong>BM-Agtr1⁻/⁻</strong></td>
<td>8</td>
<td>763 ± 26</td>
<td>4894 ± 790</td>
<td>2138 ± 330</td>
<td>2554 ± 526</td>
<td>191 ± 32</td>
</tr>
</tbody>
</table>

All data are shown as mean ± SE. RBC, red blood cells; WBC, white blood cells. Values are the mean ± SE.
Supplement Table 2
Circulating Blood Cells in Chimeric Mice Fed a Western Diet for 2 Months

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>RBCx10⁴/µl</th>
<th>WBC/µl</th>
<th>Neutrophil/µl</th>
<th>Lymphocyte/µl</th>
<th>Monocyte/µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM-Agtr1⁺⁺</td>
<td>8</td>
<td>766 ± 14</td>
<td>5555 ± 840</td>
<td>2269 ± 407</td>
<td>2851 ± 450</td>
<td>496 ± 130</td>
</tr>
<tr>
<td>BM-Agtr1⁻⁻</td>
<td>7</td>
<td>690 ± 19###</td>
<td>3317 ± 534#</td>
<td>1902 ± 334</td>
<td>1055 ± 193##</td>
<td>305 ± 42#</td>
</tr>
</tbody>
</table>

All data are shown as mean ± SE. RBC, red blood cells; WBC, white blood cells. Values are the mean ± SE. #P<0.05, ##P<0.01 vs. Agtr1⁺⁺.
# Supplement Table 3
Circulating Blood Cells in Donor Agtr1\(^{+/+}\) and Agtr1\(^{-/-}\) Mice

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>RBC(\times 10^4/\mu l)</th>
<th>WBC/(\mu l)</th>
<th>Neutrophil/(\mu l)</th>
<th>Lymphocyte/(\mu l)</th>
<th>Monocyte/(\mu l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agtr1(^{+/+})</td>
<td>9</td>
<td>802 ± 13</td>
<td>2944 ± 427</td>
<td>1038 ± 251</td>
<td>1777 ± 356</td>
<td>125 ± 40</td>
</tr>
<tr>
<td>Agtr1(^{-/-})</td>
<td>10</td>
<td>821 ± 12</td>
<td>2500 ± 183</td>
<td>857 ± 100</td>
<td>1433 ± 143</td>
<td>121 ± 22</td>
</tr>
</tbody>
</table>

All data are shown as mean ± SE. RBC, red blood cells; WBC, white blood cells. Values are the mean ± SE.


**Supplement Table 4**

Hemodynamic Data and Lipid Profile in Chimeric Mice Fed a Western Diet for 2 Months

<table>
<thead>
<tr>
<th>Donor</th>
<th>n</th>
<th>Body weight</th>
<th>Mean blood pressure</th>
<th>Heart rate</th>
<th>Total cholesterol</th>
<th>Triglycerides</th>
<th>LDL cholesterol</th>
<th>HDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(g)</td>
<td>(mmHg)</td>
<td>(bpm)</td>
<td>(mg/dl)</td>
<td>(mg/dl)</td>
<td>(mg/dl)</td>
<td>(mg/dl)</td>
</tr>
<tr>
<td>Agtr 1&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>8</td>
<td>26.2 ± 2.2</td>
<td>85.3 ± 4.8</td>
<td>618 ± 39</td>
<td>469 ± 39</td>
<td>44.0 ± 19.0</td>
<td>109.8 ± 10.3</td>
<td>11.8 ± 3.1</td>
</tr>
<tr>
<td>Agtr 1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>7</td>
<td>26.8 ± 2.4</td>
<td>80.7 ± 4.0</td>
<td>559 ± 35</td>
<td>432 ± 26</td>
<td>18.0 ± 4.2</td>
<td>99.8 ± 8.0</td>
<td>8.5 ± 1.2</td>
</tr>
</tbody>
</table>

Values are the mean ± SE. No significant differences between the genotypes with Student’s t test.