Lysozyme M–Positive Monocytes Mediate Angiotensin II–Induced Arterial Hypertension and Vascular Dysfunction

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Background—Angiotensin II (ATII), a potent vasoconstrictor, causes hypertension, promotes infiltration of myelomonocytic cells into the vessel wall, and stimulates both vascular and inflammatory cell NADPH oxidases. The predominant source of reactive oxygen species, eg, vascular (endothelial, smooth muscle, adventitial) versus phagocytic NADPH oxidase, and the role of myelomonocytic cells in mediating arterial hypertension have not been defined yet.

Methods and Results—Angiotensin II (1 mg · kg⁻¹ · d⁻¹ for 7 days) increased the number of both CD11b⁺Gr-1lowF4/80⁺ macrophages and CD11b⁺Gr-1highF4/80⁺ neutrophils in mouse aorta (verified by flow cytometry). Selective ablation of lysozyme M-positive (LysM⁺) myelomonocytic cells by low-dose diphtheria toxin in mice with inducible expression of the diphtheria toxin receptor (LysM⁺DTR mice) reduced the number of monocytes in the circulation and limited ATII-induced infiltration of these cells into the vascular wall, whereas the number of neutrophils was not reduced. Depletion of LysM⁺ cells attenuated ATII-induced blood pressure increase (measured by radiotelemetry) and vascular endothelial and smooth muscle dysfunction (assessed by aortic ring relaxation studies) and reduced vascular superoxide formation (measured by chemiluminescence, cytochrome c assay, and oxidative fluorescence microtopography) and the expression of NADPH oxidase subunits gp91phox and p67phox (assessed by Western blot and mRNA reverse-transcription polymerase chain reaction). Adoptive transfer of wild-type CD11b⁺Gr-1⁺ monocytes into depleted LysM⁺DTR mice reestablished ATII-induced vascular dysfunction, oxidative stress, and arterial hypertension, whereas transfer of CD11b⁺Gr-1⁺ neutrophils or monocytes from gp91phox or ATII receptor type 1 knockout mice did not.

Conclusions—Infiltrating monocytes with a proinflammatory phenotype and macrophages rather than neutrophils appear to be essential for ATII-induced vascular dysfunction and arterial hypertension. (Circulation. 2011;124:1370-1381.)

Key Words: angiotensin II • blood vessels • hypertension • inflammation • oxidative stress

Arterial hypertension is the main risk factor for atherosclerosis, coronary heart disease, stroke, and chronic kidney disease and is the most prominent cause of death in the world.¹ Imbalanced prooxidant and antioxidant systems in the organism play a causative role in the development of arterial hypertension and other cardiovascular diseases. The major reason for the deleterious effect of reactive oxygen species (ROS; eg, superoxide anion [O₂⁻]) resides in the reaction with nitric oxide (NO), resulting in the highly potent oxidant peroxynitrite (ONOO⁻).²

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Primary sources of ROS in the cardiovascular system are the Nox-based, multisubunit enzymes NADPH oxidases.³ Several Nox isoforms are expressed and functional in phagocytic cells like monocytes, macrophages, and neutrophils (myelomonocytic cells); T cells; endothelial cells; vascular smooth muscle cells; and adventitial fibroblasts. All of these have been suggested to contribute to cardiovascular pathology,³,⁴ although the importance of individual cell types and their relative impact on the development of cardiovascular disease remain unclear.

The phagocytic NADPH oxidase is a major source of ROS elicited by angiotensin II (ATII).⁵ In ApoE⁻/⁻ mice fed a high-fat diet, the extent of atherosclerotic lesions is attenuated by limiting the burden of superoxide generated by myelomonocytes.⁶ Leukocyte activation by ATII involving the phagocyte-type NADPH oxidase had been demonstrated in...
human neutrophils, and ATII signaling had been described to be essential for monocyte migration and mobilization involving the ATII receptor type 1 (Agtr1). Antagonization of the gp91phox containing NADPH oxidase (Nox2) prevented ATII-induced vascular hypertrophy and infiltration of macrophages in rats and blood pressure increases in mice. In a mouse model of osteopetrosis, deficiency of macrophage colony stimulating factor in op/op mice limits ATII-induced vascular dysfunction and hypertension. Although these data suggest a role for infiltrating phagocytic cells in ATII-induced vascular pathology, direct evidence for the specific role of myelomonocytic cells in arterial hypertension is still lacking.

Myelomonocytic cells specifically express the major isoform of lysozyme, lysozyme M (LysM). LysM cells have been shown to be involved in the pathogenesis of atherosclerosis. To define the role of myelomonocytic cells in the pathogenesis of ATII-induced arterial hypertension and vascular dysfunction, we crossed LysM-Cre-mice with cre-inducible diphtheria toxin (DTX) receptor (iDTR) mice to generate LysM<i>DTR</i> mice, which allow ablation of myelomonocytic cells by low-dose DTX administration. The LysM promoter is very suitable for conditional gene targeting in macrophages and granulocytes; Cre-mediated gene deletion in LysM-Cre mice leads to a loss of the target gene in 95% of the F<sup>4</sup>80<sup>−</sup> macrophages. Here, we show, that (1) ablation of LysM<sup>−</sup> monocytes from the circulation and macrophages from the vasculature attenuates ATII-induced arterial hypertension, vascular dysfunction, and oxidative stress; (2) reconstitution of depleted mice with proinflammatory CD11b<sup>+</sup>Gr-1<sup>−</sup> monocytes, but not CD11b<sup>+</sup>Gr-1<sup>+</sup> neutrophils, reestablishes the pathophysiological response to ATII; and (3) monocytes require gp91phox and Agtr1 to act as mediators of ATII-induced hypertension.

**Methods**

A detailed description of the materials and methods used can be found in the online-only Data Supplement.

**Animals**

LysM<i>Cre</i>/Cre, ROSA<i>26<i>DTR</i></i>/<i>DTR</i>, and ROSA<i>26<i>lacZ</i></i>/<i>lacZ</i> (all backcrossed >10 generations to the C57Bl6 background) were crossed to generate male LysM<i>Cre</i>/<i>Cre</i>, ROSA<i>26<i>DTR</i></i>/<i>DTR</i>, and LysM<i>Cre</i>/<i>Cre</i>-<i>DTR</i>/<i>DTR</i>/<i>lacZ</i> mice (abbreviated LysM<i>n</i> = 96), LysM<i>DTR</i> (<i>n</i> = 125), and LysM<i>DTR</i>/<i>lacZ</i> (<i>n</i> = 28), respectively. In addition, male C57Bl6 (<i>n</i> = 36), Agrp<sub>1</sub><sup>−</sup> (<i>n</i> = 16), and hemizygous gp91<i>phox</i>−/− (<i>n</i> = 2) knockout, C57Bl6 background, (<i>n</i> = 16) mice were used. For DTX receptor–mediated cell ablation, LysM<i>DTR</i> and LysM<i>DTR</i>/<i>lacZ</i> mice along with their LysM controls received intraperitoneal injections with DTX (Sigma-Aldrich) once daily (solved in PBS; 25 μg/g from day 1 to 3, 5 ng/g thereafter; see Figure I in the online-only Data Supplement for the study protocol). Angiotensin II (1 mg·kg<sup>−1</sup>·d<sup>−1</sup> for 7 days) venous sodium (NaCl 0.9%) was delivered subcutaneously with minioptic pumps (model 1007D, ALZET, Cupertino, CA) from day 4 to 10. After day 10, mice were euthanized by exsanguination in isoflurane and blood, aorta, and heart were collected.

**Blood Pressure Recordings**

LysM and LysM<i>DTR</i> mice were equipped with carotid catheter implants for telemetric blood pressure measurements in freely moving animals using receiver platforms.

**Flow Cytometric Analysis of Blood and Aortic Lysates**

Monocytes and neutrophils were determined by flow cytometric analysis in erythrocyte-depleted blood stained with Mac1(CD11b)-PE, F4/80-PE, Gr-1-FITC, and CD115-APC monoclonal antibodies. Aortic vessels were cleaned of fatty tissue, minced, and digested. Single-cell suspensions were stained with CD11b-PE-Cy7, GR-1-APC, F4/80-FTTC, B220-APC-Cy7-Fluor, CD90.2 PERCP, and Po-Pro-1-Pacific Blue monoclonal antibodies. Cells (2.5 to 4.0×10<sup>5</sup>) were treated with Fc block, washed, and surface stained. Based on a live gate, events were acquired and analyzed.

**Reconstitution of Depleted Mice With Monocytes and Neutrophils**

CD11b<sup>+</sup>-Gr-1<sup>−</sup> monocytes were prepared from venous blood of C57Bl6, Agrp<sub>1</sub><sup>−</sup>−/−, and gp91<i>phox</i>−/− mice by negative selection using magnetic activated cell sorting after discarding granulocytes following Histopaque 1083 gradient. Neutrophils were magnetically isolated from bone marrow of C57Bl6 mice by positive selection using anti-Gr-1-biotin and anti-biotin microbeads. LysM<i>DTR</i>+DTX+ATII were reconstituted in vivo by single intravenous injection with 1.5×10<sup>6</sup> monocytes or neutrophils, respectively.

**Vascular Relaxation Studies**

Isolated aortas were cut into 4-mm segments and mounted on force transducers in organ chambers to perform concentration-relaxation curves in response to increasing concentrations of acetylcholine and glyceryl trinitrate.

**Reactive Oxygen Species Formation**

Oxidative burst of whole blood, superoxide formation of whole aortic rings, and NADPH oxidase activity in aortic and cardiac membrane fractions were assessed with L-012 (100 μmol/L) and lucigenin (5 μmol/L) enhanced chemiluminescence and the cytochrome c assay and dihydroethidine (1 μmol/L in PBS) staining of aortic cryosections.

**Protein and mRNA Expression**

Protein expression was assessed with SDS-PAGE and Western blotting. mRNA expression was analyzed by quantitative real-time reverse-transcription polymerase chain reaction.

**Histology and Immunohistochemistry**

For Sirius Red staining, nuclei were prestained with hemealaun, and stained with Sirius Red (0.1% w/v) staining of aortic cryosections. For TUNEL staining. TUNEL staining. To stain for Cre-expressing cells, aortic segments of LysM<i>DTR</i>/<i>lacZ</i> mice were subjected to X-Gal staining to assess β-galactosidase activity and quantified.

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical calculations were performed with GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA). The Mann-Whitney test, Kruskal-Wallis test, or Friedman test with a post hoc Dunn test was used as appropriate. Values of <i>P</i> < 0.05 were considered significant. It should be noted that the data presented here may be limited by small sample sizes; a nonsignificant difference cannot be interpreted as a lack of association.

**Results**

Angiotensin II (1 mg·kg<sup>−1</sup>·d<sup>−1</sup> for 7 days) venous sodium (NaCl 0.9%) was delivered subcutaneously with minioptic pumps (model 1007D, ALZET, Cupertino, CA) from day 4 to 10. After day 10, mice were euthanized by exsanguination in isoflurane anesthesia, and blood, aorta, and heart were collected.

Aortic vessels were cleaned of fatty tissue, minced, and digested. Single-cell suspensions were stained with CD11b-PE-Cy7, GR-1-APC, F4/80-FTTC, B220-APC-Cy7-Fluor, CD90.2 PERCP, and Po-Pro-1-Pacific Blue monoclonal antibodies. Cells (2.5 to 4.0×10<sup>5</sup>) were treated with Fc block, washed, and surface stained. Based on a live gate, events were acquired and analyzed.

Angiotensin II Induces Myelomonocytic Cell Infiltration and Inflammatory Gene Expression in the Vascular Wall

Angiotensin II treatment has been reported to induce an inflammatory response in the vascular wall. We analyzed aortic lysates to assess myelomonocytic infiltration after ATII by flow cytometry. Compared with sham-infused mice, the aorta of ATII-infused mice contained increased numbers of CD11b<sup>+</sup>Gr-1<sup>−</sup>F4/80<sup>−</sup> macrophages and CD11b<sup>+</sup>Gr-1<sup>+</sup>F4/80<sup>−</sup> neutrophils (Figure 1A). At the same time, ATII infusion increased the
expression of the inflammatory genes Cox-2, VCAM-1, CD68, iNOS, gp91phox (Nox2), and LysM in aortic tissue (Figure 1B and

Analysis of Circulating and Infiltrating LysM+ Cells in Angiotensin II–Infused Mice

As a powerhouse of ROS formation, myelomonocytic cells have been suspected to play a pathophysiological role in vascular dysfunction.5 A potential role of monocytes in arterial hypertension has been suggested by the observation of reduced endothelial dysfunction in monocytopenic macro-

Table. mRNA Expression of Inflammatory Genes in the Aorta of Angiotensin II–Infused C57B16 Mice

<table>
<thead>
<tr>
<th>Genes</th>
<th>C57B16 mRNA Expression, % of C57B16</th>
<th>C57B16 + ATII mRNA Expression, % of C57B16</th>
</tr>
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<tbody>
<tr>
<td>CD68</td>
<td>100.0 ± 19.6</td>
<td>175.2 ± 23.1*</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>100.0 ± 6.2</td>
<td>231.9 ± 30.1*</td>
</tr>
<tr>
<td>Cox-2</td>
<td>100.0 ± 7.8</td>
<td>174.1 ± 17.4*</td>
</tr>
<tr>
<td>iNOS</td>
<td>100.0 ± 15.9</td>
<td>833.6 ± 142.3*</td>
</tr>
<tr>
<td>Nox2</td>
<td>100.0 ± 14.5</td>
<td>167.2 ± 23.0*</td>
</tr>
</tbody>
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*P<0.05, Mann-Whitney test; n=4 to 6 animals per group.

phage colony stimulating factor–deficient osteopetrotic mice (op/op).13 As a result of fundamental physiological alterations, such as altered vascular development,22 reduced endothelial dysfunction in these mice cannot unequivocally be related to monocytopeny. To unambiguously address the role of inflammatory myelomonocytic cells, we used LysMiDTR mice as a mouse model of inducible myelomonocyte ablation (compare with Figure I in the online-only Data Supplement, the study protocol). Analyzing venous blood by flow cytometry revealed no significant changes in the population of monocytes or granulocytes based on light-scatter profiles in LysM mice control injected with DTX. In contrast, DTX application reduced the percentage of monocytes in the LysMiDTR but left the percentage of neutrophilic granulocytes unaltered (Figure II in the online-only Data Supplement). To assess the specificity and efficiency of our ablation regimen, we probed venous blood by staining with CD11b, Gr-1, F4/80, and macrophage colony stimulating factor receptor (CD115) antibodies and analyzed the cellular composition by flow cytometry. Neither DTX nor ATII caused a significant change in the overall percentage of monocytes or neutrophils in the LysM controls, although we observed a tendency of a population shift from CD11b+Gr-1low to CD11b+Gr-1high monocytes in response to ATII. However, in LysMiDTR, DTX nearly erased the number of CD11b+Gr-1+/CD115+ monocytes in venous blood regardless of the monocyte subtype. At the same time, the percentage of CD11b+ Gr-1highCD115+ neutrophils was significantly increased. Angiotensin II intensified the observed effects with regard to the neutrophil population, whereas it did not affect monocyte ablation (Figure 2).

To trace live LysM+ cells in the vasculature, we crossed the LysMiDTR to a LacZ reporter mouse,23 resulting in LysMiDTR/LacZ mice. In the LysMiDTR/LacZ, ATII infusion increased vascular infiltration of viable LysM+ cells with β-galactosidase activity, which was prevented by the ablation regimen (Figure 2B and Figure III in the online-only Data Supplement). Moreover, we observed a reduction of inflammatory markers in depleted LysMiDTR mice (Nox2, CD68, and F4/80; Figure IVA in the online-only Data Supplement), and cells infiltrating the adventitia in ATII-infused LysMiDTR mice were apoptotic, as revealed by TUNEL staining, indicating the effectiveness of the DTX receptor approach. It is noteworthy that ATII infusion per se and LysM mice lacking the iDTR control injected with DTX yielded a limited rate of apoptosis (Figure IV B in the online-only Data Supplement).
Figure 2. Ablation of lysozyme M (LysM)–positive cells reduces monocytes in the circulation and prevents angiotensin II (ATII)–induced vascular macrophage infiltration. A, Flow cytometric analysis of monocytes and neutrophils in venous blood. Blood cells were stained with anti-Gr-1-FITC, anti–CD11b-PE, anti–F4/80-PE, and anti–CD115-APC and gated for live cells based on forward- and side-scatter profiles. Top, Contour plots. Numbers indicate the percentage of gated cells within the total number (red, monocytes; blue, neutrophils). One representative plot of 4 to 7 independent experiments (4 to 7 animals) is shown. Bottom, Quantification of CD115^+H11001 Gr-1^low, CD11b^high Gr-1^low, and CD115^+H11001 F4/80^+ monocytes and of CD115^+H11002 Gr-1^high and CD11b^+H11001 Gr-1^high neutrophils. B, Cryosections of aortas from diphtheria toxin (DTX)– and sham (PBS)-injected LysMiDTR/lacZ with or without ATII were subjected to X-Gal staining to test for β-galactosidase activity at day 10 (see also Figure III in the online-only Data Supplement). IOD indicates integrated optical density. *P<0.05, Kruskal-Wallis test; n=4 to 7 animals per group.
Together, these data indicate a successful depletion of circulating monocytes in LysM<sup>DTR</sup> mice by DTX and a stimulation of neutrophil migration by ATII.24,25

Depletion of LysM<sup>+</sup> Cells Decreases Angiotensin II–Induced Vascular Macrophage Population

Angiotensin II significantly increased the number of live CD11b<sup>+</sup>Gr-1<sup>low</sup>F4/80<sup>+</sup> macrophages in the aorta of LysM<sup>DTR</sup> mice, reminiscent of our findings in C57Bl6 mice (Figure 3A). Depletion of LysM<sup>+</sup> cells did not change the number of macrophages and neutrophils at baseline, but attenuated ATII-induced macrophage increase compared with the LysM controls. In contrast, the aortic neutrophil count increased in LysM<sup>DTR</sup>+DTX in response to ATII. Flow cytometric analysis of venous blood samples from the same experimental animals with the identical set of antibodies revealed results that were analogous to the aorta. This suggests that the ATII-induced increase in aortic macrophages indeed arises from an infiltration of blood CD11b<sup>+</sup>F4/80<sup>+</sup> monocytes into the vessel and that this infiltration is prevented by DTX-mediated monocyte ablation (Figure V in the online-only Data Supplement). Likewise, mRNA expression of the proinflammatory genes CD68, Cox-2, VCAM-1, iNOS, Nox2, and LysM in aortic tissue increased in response to ATII in LysM+DTX, whereas this increase was attenuated or even absent in LysM<sup>DTR</sup>+DTX (Figure 3B).
Depletion of LysM+ Cells Prevents Angiotensin II–Induced Oxidative Burst and Vascular Oxidative Stress

To analyze the capacity of phagocytes to produce $\text{O}_2^-$, we used whole blood stimulated with the protein kinase C activator PDBu ex vivo. Angiotensin II infusion markedly increased L-012 enhanced chemiluminescence in LysM controls, which was drastically inhibited in depleted LysM:DTR mice (Figure 4A). This observation illustrates the critical importance of protein kinase C in oxidative burst of the phagocytic NADPH oxidase evoked by ATII and the functional effectiveness of the ablation regimen.

In aortic rings, lucigenin ECL increased in LysM controls infused with ATII, but was not significantly different in LysM:DTR+DTX and LysM:DTR+DTX+ATII as compared to the LysM controls (Figure 4B); these data were paralleled in cardiac membraneous fractions (Figure VI in the online-only Data Supplement).

Using oxidative microtopography, we assessed a drastic increase in the $\text{O}_2^-$ signal (red dihydroethidine-derived fluorescence) in all layers of the vascular wall of LysM controls infused with ATII. In LysM:DTR+DTX+ATII, the $\text{O}_2^-$ signal was reduced to levels comparable to sham-infused LysM+DTX mice (Figure 4C). Of note, changes in the $\text{O}_2^-$ signal were observed throughout all layers of the vascular wall, whereas infiltration and depletion of myelomonocytic cells in our experimental setup occurred mainly in the adventitial layer (compare Figure 2).

The changes in ROS formation were paralleled by the aortic levels of protein expression of the catalytic subunit of the phagocytic NADPH oxidase, Nox2 (gp91phox; Figure 4D), and of the important regulatory subunit of this enzyme, p67phox (inset, membranous [Mem] fraction) in aortic homogenates. Top, Densitometry. Bottom, One representative Western blot of 4 independent experiments. C indicates cytosolic fraction; M, membranous fraction. F, NADPH oxidase activity in aortic tissue lysates and aortic expression of Nox1, p47phox, and p22phox mRNA (G) measured by real-time reverse-transcription polymerase chain reaction. *$P<0.05$, Kruskal-Wallis test; n=4 to 7 animals per group.

Figure 4. Depletion of lysozyme M (LysM)–positive cells prevents angiotensin II (ATII)–induced oxidative burst and vascular oxidative stress. Respiratory burst in whole blood (A) in the presence or absence of the phorbol ester PDBu (100 nmol/L) measured by L-012 (100 μmol/L) enhanced chemiluminescence (ECL); aortic ring superoxide formation (B) measured by lucigenin (5 μmol/L) ECL. C, Oxidative fluorescence microtopography. Left, Photomicrographs of isolated aortic segments incubated with dihydroethidine (1 μmol/L; 30 minutes at 37°C). Autofluorescence of the laminae yields a green signal; red fluorescence reflects superoxide formation. Representative photomicrographs of 6 independent experiments (6 animals) are shown. E indicates endothelium; M, media; A, adventitia; and DTX, diphtheria toxin. Right, Densitometric analysis. Protein expression of Nox2 (D) and p67phox (E; inset, membranous [Mem] fraction) in aortic homogenates. Top, Densitometry. Bottom, One representative Western blot of 4 independent experiments. C indicates cytosolic fraction; M, membranous fraction. F, NADPH oxidase activity in aortic tissue lysates and aortic expression of Nox1, p47phox, and p22phox mRNA (G) measured by real-time reverse-transcription polymerase chain reaction. *$P<0.05$, Kruskal-Wallis test; n=4 to 7 animals per group.
Depletion of LysM+ Cells Attenuates Angiotensin II–Induced Vascular Dysfunction, Disturbance of Nitric Oxide Bioactivity, and Arterial Hypertension

Endothelial dysfunction in response to ATII (Figure 5A and Table I in the online-only Data Supplement) as assessed by concentration-relaxation curves in response to acetylcholine (endothelium dependent) was significantly attenuated in the depleted LysM−/DTXTR mice. LysM+ cell ablation restored ATII-mediated inhibition of cGMP-dependent kinase activity, resulting in increased aortic levels of serine 239–phosphorylated vasodilator-stimulated phosphoprotein indicative of increased vascular NO bioavailability26 and soluble guanylyl cyclase activity (Figure 5B). At the same time, LysM+ cell ablation completely normalized the dose-response relationship to the endothelium-independent vasodilator glyceryl trinitrate. This indicates that ATII-induced reduction of vascular NO bioavailability and vascular smooth muscle dysfunction can be prevented by ablation of O2−-producing LysM+ cells. This effect can be explained at least in part by the reduction of ROS within endothelial and smooth muscle cells, which will lead to an improvement in the redox sensitive bioactivation of glyceryl trinitrate by the mitochondrial aldehyde dehydrogenase-2,27,28.

To assess the levels of peroxynitrite (ONOO−), a potent inhibitor of the soluble guanylyl cyclase,29 immunohistochemistry for 3-nitrotyrosine was performed in isolated aortic segments. The ATII-infused LysM control mice showed the strongest increase in positive staining, whereas the LysM−/DTX mice showed much less evidence for ONOO− formation in response to ATII (Figure 5C).

Most important, depletion of LysM+ cells attenuated blood pressure increase in response to ATII in LysM−/DTXTR. In contrast, blood pressure levels were increased by ATII in nonablated LysM−/DTXTR mice and unaltered in sham-infused DTX-injected LysM or LysM−/DTXTR. Similarly, Sirius Red staining revealed an increase in vascular fibrosis and hypertrophy in response to ATII in the LysM+/DTX but not in the LysM−/DTXTR (Figure 6).

Reconstitution of Depleted LysM−/DTXTR Mice With Gr-1+CD11b+ Monocytes, but Not Gr-1+CD11b+ Neutrophils, Reestablishes Vascular Dysfunction, Oxidative Stress, and Arterial Hypertension in Response to Angiotensin II

Although in our study DTX-mediated cell ablation in LysM−/DTXTR mice preferentially removed monocytes from the bloodstream and macrophages from the aorta but not neutrophils, neutrophils may attract inflammatory monocytes30 and might give rise to macrophages under certain conditions.31 To differentiate the roles of monocytes and neutrophils in ATII-induced vascular dysfunction, we adoptively transferred isolated CD11b+Gr-1+ monocytes or CD11b+Gr-1+ neutrophils into ATII-infused LysM−/DTXTR mice. Reconstitution of ablated LysM−/DTXTR mice with wild-type monocytes, but not with neutrophils, reestablished an increase in systolic arterial blood pressure in response to ATII and worsened the endothelium-dependent and-inhibitor of the soluble guanylyl cyclase,29 immunohistochemistry for 3-nitrotyrosine (arrows) in aortic sections. One representative image of 3 independent experiments (3 animals) is shown. E indicates endothelium; M, media; A, adventitia; and DTX, diphtheria toxin.

Figure 5. Depletion of lysozyme M (LysM)–positive cells prevents angiotensin II (ATII)–induced vascular dysfunction and disturbance of nitric oxide bioactivity. A, Concentration-relaxation curves of endothelium-dependent (acetylcholine [ACH]) and endothelium-independent (glyceryl trinitrate [GTTN]) vasodilators. *P<0.05, Friedman test; n=5 to 10 animals per group. B, Activity of nitric oxide/soluble guanylyl cyclase/cyclic GMP pathway assessed by quantification of serine 239–phosphorylated vasodilator-stimulated phosphoprotein (P-VASP). Top, Densitometry. Bottom, One representative Western blot of 4 independent experiments. *P<0.05, Kruskal-Wallis test; n=5 animals per group. C, Immunohistochemistry of nitrotyrosine (arrows) in aortic sections. One representative image of 3 independent experiments (3 animals) is shown. E indicates endothelium; M, media; A, adventitia; and DTX, diphtheria toxin.
cardiac NADPH oxidase activity in monocyte-reconstituted mice. In contrast, neutrophil-reconstituted mice were not significantly different from their buffer-reconstituted controls (Figure 7A through 7D and Table II in the online-only Data Supplement).

To reveal whether ATII-induced vascular dysfunction depends on NADPH oxidase activity and Agtr1 signaling in monocytes, we performed adoptive transfers with monocytes from Agtr1<sup>-/-</sup> and gp91<sub>phox</sub><sup>-/-</sup> mice. After reconstitution of ATII-infused depleted LysMiDTR mice with Agtr1<sup>-/-</sup> and gp91<sub>phox</sub><sup>-/-</sup> monocytes, we could not observe a significant change in systolic blood pressure, in the efficacy of endothelium-dependent or -independent vasodilators, in the oxidative burst of whole blood, or in the ROS formation in the vascular wall (Figure 7E through 7H and Table III in the online-only Data Supplement).

**Discussion**

We present here experimental evidence supporting a strong causative role of CD11b<sup>+</sup>Gr-1<sup>-</sup> monocytes in the pathophysiology of ATII-induced arterial hypertension. Monocytes need to contain gp91<sub>phox</sub> and Agtr1 to mediate ATII-induced arterial hypertension, vascular dysfunction and inflammation, oxidative stress, and depression of NO/soluble guanylyl cyclase/cGMP activity.

Our findings suggest that the phagocytic NADPH oxidase of infiltrating proinflammatory monocytes drives the inflammatory and oxidative signaling cascade in ATII-induced vascular dysfunction. Consecutively, they stimulate O₂·⁻ production in the smooth muscle and in the endothelial layer, derived from various cellular sources, further amplifying overall ROS formation.

We found that the amount of CD11b<sup>+</sup>Gr-1<sup>low</sup>F4/80<sup>+</sup> macrophages in the aorta almost tripled in response to ATII. Although vascular infiltration of monocytes<sup>32</sup> and macrophages<sup>33</sup> and their recruitment to inflammatory sites from a splenic reservoir<sup>9</sup> in response to ATII have been described, there is also evidence that ATII leads to neutrophil migration both in vitro<sup>24</sup> and in vivo.<sup>25,30</sup> To the best of our knowledge, an explicit aortic infiltration of neutrophils in ATII-induced hypertension has not been shown so far. Interestingly, we were able to detect a population of CD11b<sup>+</sup>Gr-1<sup>high</sup>F4/80<sup>-</sup> neutrophils in the aorta after ATII infusion that was clearly distinct from the macrophage population. These data support a role for vascular phagocytic cell infiltration in ATII-induced hypertension and raise the question of the pathophys-

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**Figure 6.** Depletion of lysozyme M (LysM)<sup>+</sup>-positive cells attenuates angiotensin II (ATII)-induced arterial hypertension. A, Summary of 240 hours of telemetrically recorded systolic blood pressures. *P<0.05, Friedman test; n=3 to 9 animals per group. B, Representative original blood pressure recordings. C, Sirius red staining of aortic tissue was performed to test for vascular fibrosis and hypertrophy. Representative images of 4 independent experiments (4 animals) are shown. E indicates endothelium; M, media; A, adventitia; and DTX, diphtheria toxin.

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Figure 7. Reconstitution of depleted LysM\textsuperscript{DTR} mice with wild-type monocytes, but not with neutrophils or Nox2- or angiotensin II (ATII) receptor type 1–deficient monocytes, reestablishes vascular dysfunction, oxidative stress, and arterial hypertension in response to ATII. For in vivo reconstitution, LysM\textsuperscript{DTR} mice were treated with diphtheria toxin (DTX) and ATII and injected intravenously with 1.5\times10^6 neutrophils from C57Bl6 or with 1.5\times10^6 monocytes from C57Bl6, Agtr1\textsuperscript{−−}, or gp91phox\textsuperscript{−−} mice vs buffer alone on day 4; DTX application was continued until day 10. A and E, Summary of 240 hours of telemetrically recorded systolic blood pressures. *\(P<0.05\), Friedman test; n=3 to 9 animals per group. B and F, concentration-relaxation curves of endothelium-dependent (acetylcholine [ACh]) and endothelium-independent (glyceryl trinitrate [GTN]) vasodilators. *\(P<0.05\), Friedman test; n=4 to 9 animals per group. C and G, Respiratory burst in whole blood assessed by L-012 (100 \text{mol/L}) enhanced chemiluminescence. D and H, Oxidative fluorescence microtopography. Left, Representative photomicrographs of 4 independent experiments are shown. Right, Densitometric analyses. E indicate endothelium; M, media; and A, adventitia. *\(P<0.05\), Kruskal-Wallis test; n=4 to 5 animals per group.
iological role of monocytes/macrophages versus neutrophils in the pathogenesis of this disease.

Because LysM<sup>DTTR</sup> mice<sup>19</sup> have successfully been used to perform DTX-mediated ablation of macrophages vivo in a mouse model of wound healing,<sup>18</sup> we used this animal model to test the relevance of LysM<sup>+</sup> cells in ATII-induced hypertension. Application of DTX in sham- and ATII-infused LysM<sup>DTTR</sup> mice induced a significant reduction in the monocytocyte count in the peripheral blood, whereas the neutrophil count was not changed and even increased in response to ATII (Figure 2A).

Aortic lysates showed an increase in both live macrophages and, to a lesser extent, neutrophils in the aorta of LysM<sup>+</sup>DTX in response to ATII. Although the ablation strategy of LysM<sup>+</sup> cells caused a reduction in aortic macrophages, we observed a population shift of myelomonocytic cells with a marked increase in neutrophil count. This result is parallel to the immunohistochemistry and TUNEL staining data and points toward a differential functional role of these cell types in ATII-induced arterial hypertension and vascular inflammation.

The majority of LysM expression in tissue arises from newly recruited myelomonocytes, and it is an inducible marker of activation of these cells.<sup>34</sup> In contrast to neutrophils, which secrete predominantly preformed lysozyme, mature macrophages maintain their ability to synthesize lysozyme de novo.<sup>35</sup> Additionally, the LysM gene is functionally involved in the myelomonocytic lineage development and is essential for both monocyte and granulocyte differentiation.<sup>36</sup> In light of the literature, our data indicate increased activation of vascular macrophages<sup>34</sup> and increased neutrophil turnover<sup>37,38</sup> in ATII-induced vascular inflammation in the aorta, possibly accentuated by the rate of DTX-induced apoptosis.

Neutrophils are responsible mainly for respiratory burst activity in whole blood<sup>39</sup> and outnumber the monocyte count by far; therefore, the inhibition of the oxidative burst observed in whole blood may point to an at least functional impairment of neutrophils in our study.

Angiotensin II–induced vascular oxidative stress was markedly reduced in deleted mice, although ATII has been demonstrated to activate the NADPH oxidase not only in inflammatory leukocytes but also in vascular cells.<sup>3</sup>

Dihydrorhodamine and protein tyrosine nitration staining of aortic segments showed a significant increase of the O<sub>2</sub>− and the ONOO<sup>−</sup> signal in the vessel wall caused by ATII with maximum ROS production located in the adventitial layer (Figure 4C). This observation was supported by X-Gal staining (Figure 2B) and immunohistochemistry data showing an infiltration of CD68<sup>+</sup>F4/80<sup>+</sup>Nox2<sup>+</sup>LysM<sup>+</sup> cells mainly into the adventitia (Figure II in the online-only Data Supplement), consistent with previous observations that ATII leads to an NADPH oxidase–dependent infiltration of macrophages in the adventitia.<sup>11</sup> On depletion of LysM<sup>+</sup> cells, the ROS signal was greatly reduced through all vessel layers (Figure 3C), accompanied by reduced activity and expression of iNOS and NAPDH oxidase subunits in aortic lysates (Figures 2B and 4D through 4G), indicating that ATII-induced vascular oxidative stress in total may result at least in part from the activation of CD11b<sup>+</sup>Gr-1<sup>+</sup> monocytes entering the vessel wall.<sup>11,32,33,39</sup> The reduction of Nox1 expression is of particular interest because this subunit is smooth muscle specific and Nox1<sup>−/−</sup> mice are protected from ATII-induced increase in blood pressure.<sup>40</sup>

Telemetric blood pressure recordings revealed that ablation of LysM<sup>+</sup> cells not only improved vascular dysfunction but also nearly abolished the increase in systolic blood pressure in response to ATII, paralleled by a strikingly decreased rate of vascular fibrosis and medial hypertrophy (Figure 6). However, not only monocytes/macrophages but also neutrophils might contribute to blood pressure regulation<sup>41</sup> and, via their myeloperoxidase activity, to blood pressure increase in response to ATII.<sup>42</sup> Additionally, LysM<sup>+</sup> neutrophils play a role in the progression of early atherosclerotic lesions, as demonstrated in monocyte-depleted ApoE<sup>−/−</sup>LysM<sup>Gr-1<sup>±</sup></sup><sub>Gfp/egfp</sub> mice.<sup>15</sup> Importantly, these mice are identical to the ApoE<sup>−/−</sup>mice in terms of lipid profile and plaque quality and are therefore valid models to study the role of LysM<sup>+</sup> cells in atherosclerosis.<sup>16</sup>

In our present study, we provide strong evidence that monocytes rather than neutrophils are responsible for mediating ATII-induced arterial hypertension and vascular dysfunction. First, in blood and aortic lysates of depleted LysM<sup>DTTR</sup> mice, which showed attenuated ATII response, CD11b<sup>+</sup>Gr-1<sup>−</sup> monocytes and F4/80<sup>−</sup>CD11b<sup>+</sup> macrophages, but not CD11b<sup>+</sup>Gr-1<sup>−</sup> neutrophils, were effectively reduced. Second, adoptive transfer of CD11b<sup>+</sup>Gr-1<sup>−</sup> monocytes, but not CD11b<sup>+</sup>Gr-1<sup>−</sup> neutrophils, reestablished systolic blood pressure increase, endothelial and smooth muscle vascular dysfunction, and oxidative stress in response to ATII infusion. In addition, monocytes defective of Agtr1 and gp91<sup>Phox</sup> failed to reestablish vascular dysfunction and oxidative stress and, most compelling, ATII-mediated systolic blood pressure increase. This finding indicates the importance of intact ATII signaling and of a functional NADPH oxidase activity in monocytes not only for ROS formation but also for the capacity of monocytes to mediate arterial hypertension.

Interestingly, monocytes and neutrophils have been shown to exert differential effects in another experimental model of vascular inflammation: reconstitution of heart transplanted CD11b<sup>−/−</sup> mice with wild type monocytes, but not with neutrophils determined graft survival and exacerbation of graft vasculopathy.

Our data nevertheless indicate that neutrophils are involved in the orchestrated inflammatory response induced by ATII without being the myelomonocytic subset responsible for the functional and sustained effects of ATII on the vasculature. In the setting of atherosclerosis, the functional coherence between neutrophils and monocytes has already been suggested; plaques contain both LysM<sup>+</sup> neutrophils and monocytes/macrophages in a ratio of ∼1:3. However, neutrophils might outnumber monocytes in the plaque shoulder and contribute to instability of this particular region.<sup>17</sup> These data are comparable to the findings of our study; we found an expansion of both CD11b<sup>+</sup>Gr-1<sup>−/−</sup>F4/80<sup>+</sup> macrophages and CD11b<sup>+</sup>Gr-1<sup>−/−</sup>F4/80<sup>−</sup> neutrophils in the inflamed vessel wall of ATII-infused mice. Neutrophils might be important to initially activate or fully stimulate monocytes, which later
may give rise to proinflammatory macrophages in the vessel wall. In a model of peritoneal cavity inflammation, secretion products of neutrophils were shown to pave the way for the development of proinflammatory monocytes/macrophages.\textsuperscript{30} Whether this relationship exists in ATII-induced arterial hypertension must be investigated in future studies.

**Conclusion**

We conclude that selective ablation of proinflammatory Gr-1\textsuperscript{+}CD11b\textsuperscript{+} monocytes might represent a novel therapeutic principle to treat arterial hypertension. However, further characterization of hypertension-mediating monocytes, their modes of activation, and their potential targets for pharmacological intervention is required before a cell-based antiinflammatory therapy of high blood pressure can be developed.

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**Disclosures**

Dr Wenzel, Waisman, and Münzel have applied for a patent on the use of ablation of myelomonocytic cells in the treatment of arterial hypertension (09014209.2/EP09014209). The other authors report no conflicts.

**References**


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CLINICAL PERSPECTIVE

Arterial hypertension represents the most important risk factor for cardiovascular disease and death. Activation of the renin-angiotensin-aldosterone system is central to the pathomechanism of hypertension, and the vasoconstrictor angiotensin II very potently initiates an inflammatory and an oxidative stress response within the vasculature. With the present studies, we provide experimental evidence that proinflammatory monocytes and macrophages are mediators of angiotensin II–induced vascular dysfunction and may be causally involved in the development of high blood pressure in this particular animal model. Ablation of lysozyme-positive myelomonocytic cells markedly attenuates angiotensin II–induced blood pressure increases and vascular dysfunction in vivo. These experimental data highlight the important role of angiotensin II as a proinflammatory promoter of atherosclerosis and provide incremental mechanistic evidence for the positive vascular effects of angiotensin-converting enzyme inhibitors and angiotensin II type I receptor blockers in the prevention of cardiovascular diseases. Identification of the molecular targets of the proinflammatory monocytes/macrophages that are specific for the angiotensin II–induced effects and that are accessible to pharmacotherapy might potentially open up new therapeutic options to treat arterial hypertension and atherosclerosis.
Lysozyme M–Positive Monocytes Mediate Angiotensin II–Induced Arterial Hypertension and Vascular Dysfunction


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Supplemental Material

Lysozyme M positive monocytes mediate angiotensin II-induced arterial hypertension and vascular dysfunction

First Author: Wenzel; Short title: Monocytes and arterial hypertension

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Supplemental Materials and Methods

**Chemicals:** All chemicals used were of highest analytical grade and obtained from either Sigma, Fluka, or Merck.

**Animals:** LysM\(^{\text{Cre/Cre}}\), ROSA26\(^{\text{iDTR/iDTR}}\) and ROSA26\(^{\text{lacZ/lacZ}}\) (all backcrossed more than 10 times to a C57Bl6 background) were bred and crossed to generate male LysM\(^{\text{Cre/wt}}\), LysM\(^{\text{Cre/wt}/\text{ROSA26}^{\text{iDTR/wt}}}\) and LysM\(^{\text{Cre/wt}/\text{ROSA26}^{\text{iDTR/lacZ}}}\) mice (abbreviated LysM (n=96), LysM\(^{\text{iDTR}}\) (n=125) and LysM\(^{\text{iDTR/lacZ}}\) (n=28), respectively) as experimental animals, in addition to male C57Bl6 (n=36), Agtr1\(^{-/-}\) (n=16) and hemizygous gp91\(^{\text{phox}^{-/-}}\) mice (Nox 2 knockout, C57Bl6 background, n=16). Mice received i.p. injections with diphtheria toxin (DTX, Sigma-Aldrich) once daily (solved in PBS; 25ng/g from day 1-3, 5ng/g thereafter)\(^{2,6}\). ATII (1mg/kg/d/ for 7 days) vs. sham (NaCl 0.9%) was delivered s.c. using miniosmotic pumps (model 1007D, ALZET, Cupertino, USA) from day 4-10. On day 10, mice were killed by exsanguination in isoflurane anesthesia (5% inhalant in room air). Blood was collected by right ventricular puncture. Heart and aorta were rapidly excised, transferred to 4 °C Krebs-Hepes-solution (pH 7.35, containing 99.01mM NaCl, 4.69mM KCl, 2.5mM CaCl\(_2\), 1.2mM MgSO\(_4\), 25.0mM NaHCO\(_3\), 1.03mM K\(_2\)HPO\(_4\), 20.0 mM Na-Hepes, 11.1mM D-glucose) and cleaned of adhesive tissue. Aortas were carefully rinsed prior to further handling. A scheme of the experimental protocol is depicted in supplemental data (Fig. S1).

Animal treatment was in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and was granted by the local Ethics Committee.

**Blood pressure recordings:** LysM and LysM\(^{\text{iDTR}}\) mice were equipped with carotid catheter implants for telemetric blood pressure measurements (TA-PA11C10, Data Science
International (DSI, Tilburg, Netherlands). Surgery was carried out under sterile conditions after anesthesia and analgesia with intraperitoneal ketamin/xylazin. After 2 weeks of recovery, DTX and ATII treatment regimen was initiated as described above and blood pressure was continuously recorded in freely moving animals using receiver platforms (DSI); measurements were taken using the DataQuest system (DSI).

**Flow cytometric identification of monocytes and neutrophils in peripheral blood:**

Ablation and reconstitution of Mφ were determined by flow cytometric analysis of monocytes and neutrophils. Erythrocyte-depleted blood was stained with Mac1-PE, F4/80-PE mAb, Gr-1-FITC and CD115-APC mAb. To block nonspecific Fc receptor-mediated binding, cells were preincubated with unlabeled mAb against CD16/CD32. Based on a life gate, 30,000 events were acquired using the BD FACScalibur (BD, Heidelberg, Germany). Data were analyzed using the Summit software (Dako cytomation, Glostrup, Denmark). Mac1highGr-1low, CD115+ Gr-1low and CD115+ F4/80+ were considered to be monocytes, Mac1+Gr-1high and CD115- Gr-1high were considered to be neutrophils.

**Flow cytometry analysis of aortic vessel:**

Aortic vessels were cleaned of fatty tissue, reduced to small pieces and digested with liberase TM (Roche, Basel, Switzerland). Single-cell suspensions were prepared and stained with CD11b-PE-Cy7, GR1-APC, F4/80-FITC, B220-APC-Cy7-Fluor, CD90.2 PERCP, Po-Pro-1-Pacific Blue monoclonal antibodies. 2.5 to 4x10⁵ cells were treated with Fc-block (eBioscience, Frankfurt, Germany), washed and surface-stained. Based on a live gate, events were acquired on FACSCanto II (BD), and analyzed with FlowJo (Tree Star, Ashland, USA). Po-Pro-1’CD90.2’B220’CD11b’(=Mac-1’ )Gr1highF4/80’ were considered to be neutrophils, Po-Pro-1’CD90.2’B220’CD11b’(=Mac-1’ )Gr1lowF4/80’ were considered to be macrophages.
Reconstitution of depleted mice with monocytes and neutrophils:

Monocytes were prepared by negative selection using magnetic activated cell sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Venous blood was drawn from C57Bl6, Agtr1⁻/⁻ and gp91phox⁻/⁻ mice, citrated and pooled. Resulting buffy coats were subjected to 5 ml of ACK-Lysis-buffer to eliminate remaining erythrocytes. Gr-1⁺CD11b⁺ monocytes were selected according to the manufacturer’s instructions with a purity >85% after discarding granulocytes following Histopaque 1083 (Sigma-Aldrich) gradient.

Neutrophils were prepared from bone marrow of C57Bl6 mice by positive selection using MACS. Fresh bone marrow cells were flushed out of the tibia and femurs with PBS-1% FCS. For separation, neutrophils were indirectly labeled with Anti-Gr-1-Biotin (a kind gift of Dr. Radsak) and Anti-Biotin Microbeads (Miltenyi). Magnetic separation was performed following the manufacturer’s instructions with a purity of CD11b⁻Gr-1⁺ cells of >90%. For in vivo reconstitution, LysMⁱDTR mice were treated with DTX and ATII as described above and injected intravenously with 1.5x10⁶ monocytes or neutrophils, respectively, on day 4 and DTX application was continued until day 10.

Vascular relaxation studies: Isolated aortas were cut into 4mm segments and mounted on force transducers (Kent scientific corporation, Torrington, USA; Powerlab, ADInstruments, Spechbach, Germany) in organ chambers filled with Krebs-Henseleit solution (37°C, pH 7.35, containing 118.3mM NaCl, 4.69mM KCl, 1.87mM CaCl₂, 1.2mM MgSO₄, 1.03mM K₂HPO₄, 25mM NaHCO₃, 11.1mM D-Glucose bubbled with carbogen gas (95% O₂, 5%CO₂) and containing 10µM indomethacin to prevent endogenous synthesis of prostaglandins. To test for vasorelaxation in response to acetylcholine (ACh) and nitroglycerin (glycerol trinitrate, GTN, from G.Pohl-Boskamp, Hohenlockstedt, Germany), aortic segments were stretched gradually over one hour to reach a resting tension of 3.0 grams. Following preconstriction with
prostaglandin F$_2$ (3nM) to reach 50-80% of maximal tone induced by KCl, concentration-relaxation curves in response to increasing concentrations of ACh and GTN were recorded.

**Reactive oxygen species formation using enhanced chemiluminescence**

Enhanced chemiluminescence (ECL) was assessed using a Lumat LB9507 single photon counter from Berthold Techn. (Bad Wildbad, Germany).

*Oxidative burst of whole blood:* Venous blood was drawn into 0.1 vol of 3.8% sodium citrate. The blood was kept at room temperature and diluted 1:50 in Dulbecco’s PBS (without Mg$^{2+}$, Ca$^{2+}$, and bicarbonate). The L-012 (100µM) ECL signal was counted in 0.2ml samples in the absence or presence of PDBu (10µM) at intervals of 30s for 10min$^9$. ECL was expressed as counts per minute after incubation for 10min.

*Superoxide formation of whole aortic rings:* The ECL signal of intact isolated aortic rings (length approximately 1cm) were counted in PBS buffer after addition of lucigenin (5µM). The ECL of each ring was counted at intervals of 1min over a period of 20min using a Lumat LB9507. The results are expressed as counts per minute per milligram of aortic tissue (dry weight).

*NADPH oxidase activity in cardiac membrane fractions:* Membrane fractions were obtained after differential centrifugation up to 100,000g (60 min, 4°C) as described $^9, ^{10}$. The 100,000g pellet was resuspended and adjusted to a concentration of 0.2mg/ml protein in PBS. NADPH oxidase activity (200µM NADPH) of the membrane suspensions was measured by lucigenin (5µM) ECL. Results were normalized for protein content and expressed as counts/mg/min after 5min.
**Aortic NADPH oxidase activity**

Sample preparation was performed by glass/glass homogenization, centrifugation at 8,000g for 20min and subsequent protein determination using the Bradford assay. The resulting particulate fraction was used for measurement of NADPH oxidase activity in aortic tissue as described\textsuperscript{11} by using the absorption spectra of reduced cytochrome c at 550nm (peak) and 100 μmol/L NADPH. NADPH oxidase activity was expressed as difference of optical density at 550nm per µg protein of lysed aorta (ΔE\textsubscript{550}/µg).

**Fluorescence oxidative microtopography**: Isolated aorta was cut into 3 mm rings and incubated in Krebs-Hepes-solution for 15 min at 37°C, embedded in aluminium cups of about 1ml of OCT resin (Tissue Tek, USA) and frozen in liquid nitrogen. Cryosections (8 µm) were stained with the superoxide-sensitive dye dihydroethidine (DHE, 1μM in PBS) and incubated for 30 min at 37°C. Green autofluorescence and red DHE fluorescence was detected using a Zeiss Axiovert 40 CFL microscope, Zeiss lenses (LD A-plan 40x/o.50Ph2) and Axiocam MRm camera (Zeiss, Oberkochen, Germany) at room temperature. Sections of all 4 study arms were analyzed in parallel with identical imaging parameters using the AxioVision data acquisition software (Zeiss).

**Protein and mRNA expression**

*Western Blotting*: Aortas were cleaned of adhesive adipose tissue, rinsed and snap frozen. To determine translocation of p67\textsuperscript{phox}, protein homogenates of aortic tissue were divided by ultracentrifugation (1h, 100,000g, 4°C) to obtain cytosolic (supernatant) and membrane fractions (pellet, resuspended in buffer\textsuperscript{12} containing 1% Triton X; re-centrifuged for 0.5h, 100,000g, 4°C to remove debris). Protein suspensions from homogenized aortic tissue was submitted to SDS PAGE and immunoblotting (BioRad, Hercules, USA), using the following antibodies: vasodilator activated phosphoprotein phosphorylated at serine 239 (P-VASP,
dilution 1.5µg/ml, Upstate, Lake Placid, NY, USA), β-actin (rabbit polyclonal, dilution 1:5000, Sigma-Aldrich, Seelze, Germany), alpha-actinin (rabbit polyclonal, dilution 1:5000, Sigma-Aldrich), Nox 2 (gp91phox, dilution 1:500, Transduction Laboratories, Lexington, USA) and p67phox (mouse monoclonal, dilution 1:500, Transduction Laboratories).

We used secondary antibodies directed towards mouse, goat and rabbit IgG (anti-mouse-, anti-goat and anti-rabbit-IgG peroxidase-labelled, Vector, Burlingame, USA). Immunodetections were accomplished with either SuperSignal Substrate (Pierce, Rockford, USA) or ECL Reagent (Amersham, Piscataway, USA). The bands were evaluated by densitometry.

**RT-PCR:** mRNA expression was analyzed by quantitative real-time RT-PCR using an 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Briefly, total RNA from mouse aorta was isolated according to the manufacturer's protocol of the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany). 0.5 µg of total RNA was used for real-time RT-PCR analysis with the QuantiTect™ Probe RT-PCR kit (Qiagen). TaqMan® Gene Expression assays for TATA-box binding protein (TBP; Mm00446973_m1), lysozyme M (LysM; Mm001318119_m1), Nox 2 (gp91phox; Mm00432775_m1), V-CAM-1 (Mm00449197_m1), inducible nitric oxide synthase (iNOS; Mm00440485_m1), cyclooxygenase-2 (cox-2; Mm00478374_m1), CD68 (Mm00839636_m1), Nox 1 (Mm00549170_m1), p47phox (Mm00447921_m1) and p22phox (Mm00514478_m1) were purchased as probe and primer sets (Applied Biosystems).

The comparative Ct method was used for relative mRNA quantification. Gene expression was normalized to the endogenous control, TBP mRNA, and the amount of target gene mRNA expression in each sample was expressed relative to that of control.

**Histology and immunohistochemistry**
**Sirius red staining:** Paraffin-embedded samples of aortic tissue were de-paraffinated and then nuclei were pre-stained with hemeauna. Then samples were stained for 1 hour in 0.1% sirius red solution containing saturated picric acid (1.2%). Finally, tissue samples were dehydrated with ethanol and coverslipped with entellan.

**Immunohistochemistry:** Aortic segments were fixed in paraformaldehyde (4%) and paraffin-embedded. Sections were stained for nitrotyrosine made in mouse (upstate, Lake Placid, USA) diluted 1:100 in REAL antibody diluent, CD68 made in mouse (DAKO, Glostrup, Denmark) diluted 1:100 in diluent (M.O.M.-Kit, Vector, Burlingame, CA), F4/80 made in rat (e-bioscience, San Diego, CA) diluted 1:100 in REAL antibody diluent (DAKO) and Nox 2 (BD Pharmingen, gp91phox, Heidelberg, Germany) made in mouse diluted 1:100 in REAL antibody diluent. Following the species of primary mAb appropriate biotinylated secondary antibodies were used after dilution following the manufacturer’s instructions for anti-mouse (Vector) and for F4/80 anti-rat (1:100). For immunochemical detection ABC reagent (Vector) and then DAB (peroxidase substrate Kit, Vector) reagent as substrate were used.

Apoptosis was assessed using TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining applying the Cell-Death detection kit following the manufacturer’s instructions (AP, Roche).

**Beta-galactosidase activity staining:** To stain for Cre-expressing cells, aortic segments of LysM^{DTR}/lac^Z mice were subjected to X-Gal staining to assess beta-galactosidase activity. Aortic segments were embedded in OCT resin (tissue tek, USA), cut in cryostat sections (8µm) and mounted on silane-coated slides. Sections were fixed to slides in 0.2% glutaraldehyde in PBS for 10-15 minutes at 4°C and rinsed three times for 5 minutes in LacZ washing buffer (PBS (-Mg2,-Ca), 0.002M MgCl2 10% Igepal (Nonidet-P40), 10% Na-deoxycholate) at room temperature. Slides were stained in X-gal reaction buffer (containing
0.6 M potassium ferricyanid, 0.6 M potassium ferrocyanid, 0.002 M MgCl₂, 10% Igepal (Nonidet-P40), 10% Na-deoxycholate, X-Gal Stock-solution (0.5mg/ml), 1 x PBS) overnight at 37°C.

After rinsing in PBS 3 times, for 5 minutes each, slides were dehydrated 2 minutes with 70% ethanol and counterstained for 3 sec with eosin/phloxin (10% in PBS). After complete ethanolic dehydration the slides were mounted in entellan and photographed.

To quantify beta-glactosidase activity staining, images of all 4 study arms were analyzed by the amount of blue color signal. Blue color stimulus specifications were automatically identified, selected and copied to a new sheet by using Adobe Photoshop CS2 (Adobe systems, San Jose, USA). Selection of all blue color specifications was counterchecked by deleting the selected spots in the original image. All copied spots were transferred into adjusted black and white mode, densitometrically quantified and normalized on the respective area of the original image using identical parameters for each section.

In all histology and immunohistochemistry methods, images were acquired using a Olympus IX71 microscope, Olympus lenses (plan 20x/0.40/oo/0.17 and plan 40x/0.65/oo/0.17) and Olympus colorView U-TVO.5XC-2 camera (Olympus, Tokyo, Japan) at room temperature. Sections of all 4 study arms were analyzed in parallel with identical imaging parameters using the Cell B data acquisition software (Olympus)

**Statistical Analysis:** Data are expressed as mean+/−SEM. Statistical calculations were performed with GraphPad Prism 5 (GraphPad Software Inc, San Diego, Calif). Mann-Whitney test, Kruskal-Wallis test or Friedman test with posthoc Dunn test was used as appropriate. Values of \( P \) 0.05 were considered significant. It should be noted that the data presented here may be limited by small sample sizes; a nonsignificant difference cannot be interpreted as a lack of association.
Supplemental tables

Supplemental Table 1: Efficacy of the concentration-relaxation curves in isolated aortic rings of LysM vs. LysM^{iDTR} mice infused with ATII or sham.

<table>
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<th>LysM +DTX</th>
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<th>LysM^{iDTR} +DTX</th>
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</tbody>
</table>

* P<0.05 vs. LysM +DTX and # P<0.05 vs. LysM +DTX +ATII.

Supplemental Table 2: Efficacy of the concentration-relaxation curves in isolated aortic rings of LysM vs. LysM^{iDTR} mice infused with ATII reconstituted with buffer, neutrophils or wild type monocytes.

<table>
<thead>
<tr>
<th></th>
<th>LysM^{iDTR} +DTX</th>
<th>LysM^{iDTR} +DTX +ATII</th>
<th>LysM^{iDTR} +DTX +ATII</th>
</tr>
</thead>
<tbody>
<tr>
<td>+DTX</td>
<td>+ATII</td>
<td>+ATII</td>
<td></td>
</tr>
<tr>
<td>+ATII</td>
<td>+ monocytes</td>
<td>+ neutrophils</td>
<td></td>
</tr>
<tr>
<td>+buffer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACh Efficacy [%]</td>
<td>62.55±4.30</td>
<td>33.86±3.34</td>
<td>66.34±3.40</td>
</tr>
<tr>
<td>(n=4)</td>
<td>(n=5) *</td>
<td>(n=5)</td>
<td></td>
</tr>
<tr>
<td>GTN Efficacy [%]</td>
<td>82.75±2.47</td>
<td>53.99±5.70</td>
<td>79.46±3.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>relaxation, %]</th>
<th>(n=4)</th>
<th>(n=4) *</th>
<th>(n=5)</th>
</tr>
</thead>
</table>

* P<0.05 vs. LysMiDTR+DTX+ATII+buffer
Supplemental Table 3: Efficacy of the concentration-relaxation curves in isolated aortic rings of LysM vs. LysM\textsuperscript{iDTR} mice infused with ATII reconstituted with buffer, Agtr1\textsuperscript{−/−} or gp91\textsuperscript{phox−/−} monocytes.

<table>
<thead>
<tr>
<th></th>
<th>LysM\textsuperscript{iDTR}</th>
<th>LysM\textsuperscript{iDTR}</th>
<th>LysM\textsuperscript{iDTR}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+DTX</td>
<td>+DTX +ATII</td>
<td>+DTX +ATII</td>
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<tr>
<td>+ATII</td>
<td></td>
<td>+ Agtr1\textsuperscript{−/−}</td>
<td>+ gp91\textsuperscript{−/−} monocytes</td>
</tr>
<tr>
<td>+buffer</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>ACh Efficacy [max. relaxation, %]</th>
<th>GTN Efficacy [max. relaxation, %]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=8) 63.94±3.77</td>
<td>(n=4) 81.05±2.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(n=4) 86.78±2.60</td>
</tr>
<tr>
<td></td>
<td>* P&lt;0.05 vs. LysM\textsuperscript{iDTR} +DTX +ATII +buffer</td>
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</tbody>
</table>
Supplemental figures and figure legends

**Figure S1: Scheme of the experimental protocol.** After surgery (carotid implants for telemetric blood pressure recordings), mice were allowed to recover for 2 weeks. DTX, diphtheria toxin; ATII, angiotensin II.
Figure S2: DT mediated ablation of LysM$^+$ cells in the circulation of LysM$^{IDTR}$ mice. Flow-cytometric analysis of monocytes and granulocytes in venous blood from LysM and LysM$^{IDTR}$ mice ± ATII and with/without DTX at d10 of the protocol. Light scatter parameters (monocytes gated in red, granulocytes gated in blue) Numbers indicate percentage of gated cells within total number. 1 representative plot of 6 independent experiments (6 animals) is shown. SSC, sideward scatter; FSC, forward scatter.
Figure S3: Beta galactosidase activity staining. Cryosections of aortas from DTX- and sham (PBS)-injected LysM^{DTR/lacZ} were subjected to X-Gal staining to test for β-galactosidase activity at d10. Original sections, dark blue indicates X-Gal staining. 1 representative image of 4 independent experiments (4 animals) is shown.
Figure S4: DTX-mediated ablation of LysM⁺ cells in the circulation of LysM⁺DTR mice reduces AT-II induced phagocytic cell infiltration into the vascular wall. A, Aortic sections from LysM and LysM⁺DTR mice treated or untreated with ATII (1mg/kg/d/7d) at d10 probed with antibodies against F4/80, CD68 and Nox2. Brown staining indicates positive immunohistochemistry. 1 out of 3 independent experiments (3 animals per group) shown. On each image, the adventitia is located on the farthest right and the endothelial layer on the farthest left of the tissue section. B, TUNEL staining (purple colour) for apoptotic cell detection. Aortic sections from LysM and LysM⁺DTR mice treated or untreated with ATII (1mg/kg/d/7d) at d10. 1 out of 3 independent experiments (3 animals per group) is shown. On each image, the adventitia is located on the farthest right and the endothelial layer on the farthest left of the tissue section.
Figure S5: Depletion of LysM+ cells reduces the CD11b+Gr-1lowF4/80+ monocytes population in venous blood. Flow-cytometric analysis of CD11b+Gr-1lowF4/80+ monocytes and CD11b+Gr-1highF4/80- neutrophils in venous blood from LysM and LysMIDTR mice+DTX±ATII at d10 of the protocol, assessed with the same settings as with aortic lysates. Representative original plots of 4-6 independent experiments.
Figure S6: Depletion of LysM$^+$ cells prevents ATII induced cardiac NADPH oxidase activity. NADPH oxidase activity in membraneous fractions of homogenates of cardiac tissue in the presence of 200µM NADPH measured by lucigenin (5µM) ECL *, p<0.05; all data are mean ± SEM of n=5-7 animals per group.
Supplemental References


