Angiotensin II–Induced Vascular Dysfunction Depends on Interferon-γ–Driven Immune Cell Recruitment and Mutual Activation of Monocytes and NK-Cells

Sabine Kossmann,* Melanie Schwenk,* Michael Hausding,* Susanne H. Karbach, Maria I. Schmidgen, Moritz Brandt, Maike Knorr, Hanhan Hu, Swenja Kröller-Schön, Tanja Schönfelder, Stephan Grabbe, Matthias Oelze, Andreas Daiber, Thomas Münze,* Christian Becker,* Philip Wenzel*

**Objective**—Immune cells contribute to angiotensin II (ATII)–induced vascular dysfunction and inflammation. Interferon-γ (IFN-γ), an inflammatory cytokine exclusively produced by immune cells, seems to be involved in ATII-driven cardiovascular injury, but the actions and cellular source of IFN-γ remain incompletely understood.

**Approach and Results**—IFN-γ−/− and Tbx21−/− mice were partially protected from ATII-induced (1 mg/kg per day of ATII, infused subcutaneously by miniosmotic pumps) vascular endothelial and smooth muscle dysfunction, whereas mice overexpressing IFN-γ showed constitutive vascular dysfunction. Absence of T-box expressed in T cells, the IFN-γ transcription factor encoded by Tbx21, reduced vascular superoxide and peroxynitrite formation and attenuated expression of nicotinamide adenosine dinucleotid phosphate oxidase subunits as well as inducible NO synthase, monocyte chemoattractant protein 1, and interleukin 12 in aortas of ATII-infused mice. Compared with controls, IFN-γ−/− and Tbx21−/− mice were characterized by reduced ATII-mediated vascular recruitment of both NK1.1+ NK-cells as the major producers of IFN-γ and CD11b+Gr-1low interleukin-12 secreting monocytes. Selective depletion and adoptive transfer experiments identified NK-cells as essential contributors to vascular dysfunction and showed that T-box expressed in T cells+LysM+ myelomonocytic cells were required for NK-cell recruitment into vascular tissue and local IFN-γ production.

**Conclusions**—We provide first evidence that NK-cells play an essential role in ATII-induced vascular dysfunction. In addition, we disclose the T-box expressed in T cells–IFN-γ pathway and mutual monocyte–NK-cell activation as potential therapeutic targets in cardiovascular disease. *(Arterioscler Thromb Vasc Biol. 2013;33:00-00.)*

**Key Words:** angiotensin II ■ inflammation ■ interferon-γ ■ natural killer cells ■ oxidative stress ■ vascular function

Recruitment of immune cells into the vessel wall has been recognized as an important early step in angiotensin II (ATII)–induced vascular dysfunction and arterial hypertension.1 Interferon γ (IFN-γ), an inflammatory cytokine exclusively produced by immune cells, mainly by NK-cells,2 but also NKT cells,3 Th0 and Th1 T cells, macrophages,4 and dendritic cells,5 is well known to promote inflammatory reactions, such as activation of macrophages,6 to increase chemokine and adhesion molecule expression and to foster recruitment of immune cells to inflammatory sites.7,8 Reflecting its inflammatory activity, IFN-γ is upregulated in the spleen of hypertensive rats9 and in the kidney of ATII-infused mice.10 Recently, it was shown that cardiac IFN-γ expression and IFN-γ–driven tissue injury in the heart are increased by ATII11 and that IFN-γ–receptor deficient mice are partially protected from ATII-driven cardiac damage and renal injury.12 These observations strongly support a role of IFN-γ in ATII-driven pathology, however its cellular source and specific influence on vascular function and inflammation are incompletely defined. Analyzing ATII-mediated vascular inflammation and dysfunction in IFN-γ−/− mice and mice lacking the transcription factor T-box expressed in T cells (T-bet), which directs IFN-γ transcription in immune cells,13 we identified the crucial role of the T-bet–IFN-γ axis in vascular monocyte recruitment and inflammation, reactive oxygen species (ROS) production,
and vascular endothelial and smooth muscle dysfunction. Additionally, we revealed a central role for IFN-γ–producing NK-cells and the mutual activation of NK-cells and monocytes in the vasculature in ATII-induced vascular injury.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement. Male C57BL/6, IFN-γ−/−, Rag-1 −/− (all from Jackson Laboratories, Bar Harbor, Maine), as well as SAP–IFN-γ, Tbx21 −/−, LysMCre/Cre, ROSA26LysMCre/iDTR (crossed to generate male LysMCre/wt and LysMCre/iDTR all on the C57BL/6J background) were used as experimental animals. Mice were treated with ATII-loaded miniosmotic pumps (1 mg/kg per day for 7 days) or sham-treated, partially equipped with carotid catheter implants for telemetric blood pressure measurements. In selected experiments, C57BL/6 mice were depleted of NK-cells by injecting depleting anti-NK1.1 antibody, or LysMiDTR mice were depleted of monocytes by diphtheria-toxin receptor–mediated cell ablation. After 7 days of ATII treatment, mice were killed, and blood and aorta were collected. Tissue was subjected to vascular relaxations studies, ROS measurements (lucigenin-enhanced chemiluminescence and fluorescence oxidative microtopography), flow cytometry analysis of inflammatory cells and cytokine production, and mRNA and protein expression analysis. Data are expressed as mean±SEM or median with box plot and whiskers (min to max).

**Results**

**IFN-γ Promotes ATII-Induced Vascular Inflammation and Dysfunction**

ATII-induced vascular endothelial and smooth muscle dysfunction in wild-type (WT) mice was accompanied by increased aortic IFN-γ and T-bet expression (Figure 1A and 1B). IFN-γ−/− and Tbx21−/− mice (deficient in the gene encoding for T-bet) remained largely protected from ATII-induced vascular dysfunction (Figure 1C and 1D; Tables I and II in the online-only Data Supplement), whereas transgenic SAP–IFN-γ mice with systemic IFN-γ overproduction showed constitutive endothelial dysfunction and nox2 and p67phox upregulation in aortic lysates in the absence of ATII (Figure 1A and IB in the online-only Data Supplement). Indicative of a proinflammatory and pro-oxidant role of IFN-γ in ATII-induced vascular injury expression levels and membrane translocation of the nicotinamid adenosin dinucleotid phosphate oxidase subunits Nox2 (gp91phox), p22phox, p67phox, and p47phox increased in response to ATII in WT controls but remained significantly dampened in Tbx21−/− mice (Figure 2A–2D; Figure IIA and IIB in the online-only Data Supplement). Likewise, ATII-infused Tbx21−/− mice showed less superoxide formation in aortic rings and whole blood and drastically less aortic peroxynitrite formation than controls, paralleled by blunted ATII-induced aortic inducible NO synthase mRNA expression (Figure 2E–2H; Figure IIC in the online-only Data Supplement). In contrast to differences in ATII-induced vascular dysfunction and except for a slight delay in blood pressure increase in Tbx21−/− mice, blood pressure levels evaluated by continuous telemetric recording at baseline and during 7 days of ATII infusion (at 336 hours) showed no significant difference in systolic blood pressure between Tbx21−/− (systolic: 121.1±1.79 versus 157.9±11.48 mm Hg) and WT mice (120.9±1.02 versus 167.1±1.00 mm Hg; Figure IID in the online-only Data Supplement).

**Depletion of NK-Cells Protects From ATII-Induced Vascular Dysfunction**

ATII-induced vascular dysfunction in WT mice was marked by a drastic recruitment of NK1.1+TCRβ− NK-cells into the aortic wall virtually absent in Tbx21−/− and IFN-γ−/− mice (Figure 3A). Remarkably, depletion of NK-cells by injection of a depleting monoclonal NK1.1-specific antibody before ATII treatment significantly reduced endothelial and smooth-muscle vascular dysfunction in WT mice (Figure 3B and 3C; Figure 3D in the online-only Data Supplement).
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Table III in the online-only Data Supplement) to the level observed in IFN-γ−/− and Tbx21−/− mice. In contrast, depletion of CD4+ T cells by injection of a depleting monoclonal anti-CD4 antibody did not significantly influence ATII-induced vascular dysfunction (not shown). These observations indicate that NK-cells are required for the initiation of ATII-induced vascular inflammation.

ATII-Induced Vascular Inflammation Depends on Mutual NK-Cell Monocyte Activation

In addition to NK-cell infiltration, ATII-induced aortic infiltration of CD11b+Gr-1low monocytes was significantly attenuated in Tbx21−/− and IFN-γ−/− mice (Figure 3D) and consecutively accompanied by reduced upregulation of monocyte chemotactic protein-1, macrophage inflammatory protein 1α, and P-selectin ligand in ATII-infused Tbx21−/− mice (Figure 3E; Figure IIIA–IIIC in the online-only Data Supplement).

NK-cells are driven to full maturation by CD11b+ monocyte–derived interleukin (IL)-12.19 Interestingly, ATII increased vascular IL-12 mRNA expression in the aortic wall of WT mice (Figure 3F and 3G), underlining the important role for T-bet directing IL-12 formation by CD11b+ cells. Confirming the interconnection to NK-cell–derived IFN-γ in ATII-induced vascular inflammation, aortic NK1.1+ NK-cells produced IFN-γ in response to IL-12/IL-18 ex vivo stimulation (Figure 3H). Because the latter suggested a role of T-betCD11b+ myelomonocytic cells in NK-cell recruitment and local activation, we depleted LysM+ cells and reconstituted them with Tbx21−/− or WT monocytes. Depletion of LysM+ cells attenuated the increase of IFN-γ+NK1.1+ NK-cells in response to ATII (Figure 4A). Reconstitution of depleted LysM+ mice with WT, but not with T-bet−/− monocytes,18 re-establishes ATII-induced vascular endothelial or smooth muscle dysfunction (Figure 4B; Table IV in the online-only Data Supplement) and vascular oxidative stress (Figure 4C). Together these findings reveal a crucial role of monocytic T-bet expression in vascular NK-cell recruitment and mutual NK-cell and monocyte activation in ATII-induced vascular dysfunction.

Discussion

We show here that ATII-induced vascular dysfunction depends on vascular entry and IFN-γ production by NK-cells. Despite representing a major component of the innate immune system and playing an important role in tissue inflammation, NK-cells have surprisingly not been considered in ATII-induced vascular dysfunction earlier.

NK-cells are poised for immediate effector function and are powerful producers of various inflammatory cytokines and growth factors, such as IFN-γ, tumor necrosis factors-α, and granulocyte-macrophage colony-stimulating factor. On
recruitment to inflammatory sites; NK-cells engage with monocytes in a reciprocal program of activation. Within this mutual activation, NK-cell–derived IFN-γ plays an important role in propagating the activation and maturation of monocytes into macrophages and dendritic cells that produce IL-15, IL-12, and IL-18.20–22 In turn, IL-12 synergizes with IFN-γ in stimulating IL-18 production in NK-cells, resulting in a positive feedback loop that represents an important amplifying mechanism in the early innate inflammatory response. In accordance with this mechanism, we find that the accumulation of NK-cells and monocytes is paralleled by an increase not only of IFN-γ, but also of IL-12 in aortic tissue in response to ATII, a mechanism drastically reduced in Tbx21−/− mice as compared with ATII-infused C57BL/6 mice.31 Consistent with this concept, MCP-1 and macrophage inflammatory protein 1 α (MCP-1) mRNA expression in aortic lysates, Kruskal-Wallis test, n= 2 to 5. G, IL-12 formation of CD11b+ cells with or without interferon (IFN)-γ-stimulation. Representative flow cytometry dot plot. H, Flow-cytometric analysis of IFN-γ–producing NK1.1+ NK-cells in aortic tissue of sham-treated and ATII-treated C57BL/6 mice. Left, Representative contour plots of IL-12/IL-18–stimulated aortic cells. Right, Quantification. t test (Wilcoxon-matched pairs test). n= 4 to 5.

Indeed, monocytes have been shown to depend on T-bet to activate NK-cells via IL-12, and when we depleted monocytes in vivo, the amount of IFN-γ–competent NK-cells in the aorta of mice challenged by ATII was drastically reduced (Figure 4). Thus, the T-bet/IFN-γ axis participates in both sides of the reciprocal monocyte NK-cell activation, initiating vascular inflammation independent from blood pressure changes, which were unaltered in Tbx21−/− mice. This observation is in line with previous studies on ATII-induced cardiac inflammation and cardiac damage in mice deficient in IFN-γ–signaling, revealing an unaltered blood pressure response to ATII compared with WT mice. Thus, although IFN-γ seems to be required in the initiation of vascular inflammation, it does not affect blood pressure changes, indicating that the T-bet/IFN-γ–driven proinflammatory action of ATII on the vasculature is independent of hemodynamics.

In summary, we identify IFN-γ formed locally by NK-cells in the aortic wall as a critical initiator of vascular oxidative stress, inflammatory cell recruitment, and reciprocal innate immune cell activation in the vessel wall and show that NK-cell depletion largely protects from ATII-induced vascular dysfunction.

Acknowledgments

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Disclosures

None.

References

3. Wang X, Mosmann T. In vivo priming of CD4 T cells that produce interleukin (IL)-2 but not IL-4 or interferon (IFN)-gamma, and can subsequently differentiate into IL-4- or IFN-gamma-secreting cells. J Exp Med. 2001;194:1069–1080.
Inflammatory monocytes and macrophages are essential for angiotensin II–induced vascular dysfunction. Interferon-γ is one of the most prominent proinflammatory cytokines and seems to play a role in angiotensin II–induced cardiovascular injury and inflammation. We here show that mice deficient in interferon-γ or its transcription factor, T-box expressed in T cells, are largely protected from vascular oxidative stress, endothelial dysfunction, and from vascular infiltration of monocytes and NK-cells in response to angiotensin II. We uncover a hitherto unrecognized role of natural killer cells in driving angiotensin II–induced vascular inflammation and reveal that NK-cells and monocytes undergo a reciprocal pattern of activation that depends on T-box expressed in T cells, NK-cell–derived interferon-γ, and mono
cyte-derived interleukin-12 within the vascular wall. These findings could have broad implications for our understanding of atherogenesis and provide potential new therapeutic targets in the treatment of arterial hypertension and cardiovascular disease.
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Supplemental Material

Angiotensin II-induced vascular dysfunction depends on interferon gamma driven immune cell recruitment and mutual activation of monocytes and natural killer cells

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1 2nd Medical Clinic, 2 Center for Thrombosis and Hemostasis (CTH), Department for Dermatology, University Medical Center of the Johannes Gutenberg-University, Mainz, Germany; * and #: equal contribution

running title: NK cells, IFN-γ and vascular dysfunction

Corresponding author: Philip Wenzel MD, Universitätsmedizin der Johannes-Gutenberg-Universität Mainz, 2. Medizinische Klinik and Junior Group „Vascular Biology“, Center for Thrombosis and Hemostasis, Langenbeckstr. 1, 55131 Mainz, Germany. email: wenzelp@uni-mainz.de Tel: 0049 6131 170, Fax: 0049 6131 179960
Supplemental figures and figure legends

**Supplementary figure I:** A, Concentration-relaxation curve of endothelium-dependent vasodilator ACh. *P<0.05, n=5-6 animals, t-test of maximal relaxation. B, Enhanced protein expression of nox2 and p67phox in SAP-IFN-γ mice compared to wild-type mice. Top: Representative Western blot. Bottom: Densitometry. *P<0.05, Mann-Whitney, n=4.

**Supplementary figure II:** Oxidative stress and associated parameters are diminished in Tbx21−/− mice after ATII-treatment compared to C57BL/6 mice. A, Translocation of p67phox (A) and p47phox (B) from the cytosolic to the membrane fraction in response to ATII was assessed by SDS-PAGE and Western blot in aortic lysates. Kruskal-Wallis-test, n=3. C, Lucigenin enhanced chemiluminescence of aortic rings. Kruskal-Wallis-test, n=4 animals. D, Summary of 336 hours of telemetrically recorded systolic blood pressures. ATII treatment started after 168 hours. n=3 animals per group.
Supplementary figure III: ATII-induced chemokine and adhesion molecule expression is decreased in Tbx21-/- mice compared to wild-type mice.

Vascular protein expression of monocyte chemotactic protein-1 (MCP-1, A) and macrophage inflammatory protein-1α (MIP-1α, B) was assessed by SDS-PAGE and Western blot of aortic lysates. Top: Quantification, Bottom: Representative original blot of two independent experiments. Kruskal-Wallis-test, n=4. mRNA expression of P-Selectin ligand (C) in aortic lysates was measured by realtime RT-PCR. Kruskal-Wallis-test, n=3.
Supplementary figure IV: Transfer of T-bet+ NK cells or monocytes could not restore ATII-induced vascular dysfunction and ROS production in Tbx21-/- mice.

A, About 10x10^6 NK cells from WT mice were transferred intravenously into Tbx21-/- mice. ATII-infusion started at the same day. After 7 days aortas were isolated as described in the methods. Concentration-relaxation curves of endothelium-dependent (ACh) and endothelium-independent vasodilators (GTN) were measured. 1-way-ANOVA of maximal relaxation n=3-10. About 1x10^6 isolated monocytes from WT mice were transferred intravenously into Tbx21-/- mice. ATII-infusion started at the same day. After 7 days aortas were isolated as described in the methods. Concentration-relaxation curves of endothelium-dependent (ACh) and endothelium-independent vasodilators (GTN). n=2-10 animals, t-test of maximal relaxation. B, C, Dihydroethidine staining of aortic cryosections showed no enhanced ROS production after T-cell or NK cell transfer. Mann-Whitney-test n=4-10. A, adventitia; M, media; E, endothelium.
Supplemental tables

Supplemental Table I: Efficacy and potency of the concentration-relaxation curves in isolated aortic rings of C57BL/6 vs. IFN-γ−/− mice infused with ATII or sham.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C57BL/6</th>
<th>C57BL/6 +ATII</th>
<th>IFN-γ+/−</th>
<th>IFN-γ−/− +ATII</th>
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<tbody>
<tr>
<td>ACh Efficacy [%]</td>
<td>74.98±2.45 (n=11)</td>
<td>36.21±4.90 (n=13) *</td>
<td>62.33±4.81 (n=14) #</td>
<td>53.26±5.28 (n=11) *</td>
</tr>
<tr>
<td>GTN Efficacy [%]</td>
<td>88.76±1.85 (n=16)</td>
<td>57.94±4.86 (n=14) *</td>
<td>82.14±3.67 (n=14) #</td>
<td>71.63±2.40 (n=15) *#</td>
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*P<0.05 vs. C57BL/6 and # P<0.05 vs. C57BL/6 +ATII.

<table>
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<th>Parameter</th>
<th>C57BL/6</th>
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<th>IFN-γ+/−</th>
<th>IFN-γ−/− +ATII</th>
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<tr>
<td>ACh Potency [−log M]</td>
<td>7.06±0.078 (n=11)</td>
<td>6.55±0.094 (n=13) *</td>
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<td>6.58±0.073 (n=12) *</td>
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<td>GTN Potency [−log M]</td>
<td>7.18±0.115 (n=17)</td>
<td>6.41±0.237 (n=14) *</td>
<td>6.86±0.191 (n=15)</td>
<td>7.02±0.136 (n=15)</td>
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*P<0.05 vs. C57BL/6 and # P<0.05 vs. C57BL/6 +ATII.
Supplemental Table II: Efficacy and potency of the concentration-relaxation curves in isolated aortic rings of C57BL/6 vs. Tbx21<sup>−/−</sup> mice infused with ATII or sham.

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<th>Parameter</th>
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<th>C57BL/6 +ATII</th>
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<th>Tbx21&lt;sup&gt;−/−&lt;/sup&gt; +ATII</th>
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<tr>
<td>ACh Efficacy [max. relaxation, %]</td>
<td>65.69±2.71 (n=21)</td>
<td>29.99±3.57 (n=21) *</td>
<td>58.17±2.28 (n=19) #</td>
<td>48.45±4.91 (n=11) *#</td>
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<td>GTN Efficacy [max. relaxation, %]</td>
<td>83.95±1.95 (n=23)</td>
<td>55.82±4.64 (n=23) *</td>
<td>81.90±2.28 (n=19) #</td>
<td>70.84±4.45 (n=19) #</td>
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*P<0.05 vs. C57BL/6 and # P<0.05 vs. C57BL/6 +ATII.

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<th>Parameter</th>
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<th>Tbx21&lt;sup&gt;−/−&lt;/sup&gt; +ATII</th>
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<td>ACh Potency [EC&lt;sub&gt;50&lt;/sub&gt;, -log M]</td>
<td>7.29±0.010 (n=21)</td>
<td>6.36±0.154 (n=20) *</td>
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<td>GTN Potency [EC&lt;sub&gt;50&lt;/sub&gt;, -log M]</td>
<td>7.53±0.090 (n=23)</td>
<td>6.95±0.162 (n=23) *</td>
<td>7.40±0.108 (n=19) #</td>
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*P<0.05 vs. C57BL/6, †P<0.05 vs. C57BL/6 +ATII and †P<0.05 vs. Tbx21<sup>−/−</sup>.
Supplemental Table III: Efficacy and potency of the concentration-relaxation curves in isolated aortic rings of C57BL/6 infused with ATII or sham and treated with anti-Nk1.1 antibody or sham treated.

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<td>69.11±3.85</td>
<td>33.02±5.49</td>
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<td>(n=15) #</td>
<td>(n=14) *#†</td>
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<tr>
<td>GTN Efficacy [%]</td>
<td>86.74±1.82</td>
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<td>(n=16) *</td>
<td>(n=15) #</td>
<td>(n=15) *#†</td>
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*P<0.05 vs. C57BL/6, † P<0.05 vs. C57BL/6 +ATII and † P<0.05 vs. C57BL/6 +antiNK1.1.

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<td>GTN Potency [EC50, -log M]</td>
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<td>(n=16)</td>
<td>(n=15) *</td>
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*P<0.05 vs. C57BL/6, † P<0.05 vs. C57BL/6 +ATII and † P<0.05 vs. C57BL/6 +antiNK1.1.
Supplemental Table IV: Efficacy and potency of the concentration-relaxation curves in isolated aortic rings of LysM vs. LysM<sup>IDTR</sup> all treated with DTX, infused with ATII reconstituted with T-bet<sup>neg</sup> monocytes or sham reconstituted.

<table>
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<th>Parameter</th>
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<td></td>
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<td>Monos</td>
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<table>
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<tr>
<th>ACh Efficacy [max. relaxation, %]</th>
<th>55.06±4.09 (n=13)</th>
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<tbody>
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<td>GTN Efficacy [max. relaxation, %]</td>
<td>75.81±3.60 (n=13)</td>
<td>77.40±3.93 (n=4)</td>
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<td>+Tbet&lt;sup&gt;neg&lt;/sup&gt;</td>
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<td>Monos</td>
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<th>ACh Potency [EC&lt;sub&gt;50&lt;/sub&gt;, -log M]</th>
<th>6.63±0.151 (n=13)</th>
<th>6.59±0.119 (n=4)</th>
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<tr>
<td>GTN Potency [EC&lt;sub&gt;50&lt;/sub&gt;, -log M]</td>
<td>7.08±0.062 (n=13)</td>
<td>7.29±0.033 (n=4)</td>
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Supplemental Materials and Methods

Chemicals: All chemicals were of highest analytical grade and either from Sigma-Aldrich (Seelze, Germany) or Merck (Whitehouse Station, NJ).

Mice: Male C57BL/6, IFN-γ−/−, Rag-1−/− (all from Jackson Laboratories, Bar Harbor, Maine, USA), as well as SAP-IFN-γ−/−, Tbx21−/−, LysMCre/Cre, ROSA26IDTR/IDTR (crossed to generate male LysMCre+/+ and LysMCre+/+, abbreviated LysM and LysMIDTR+/+ 5) all on the C57BL/6J background were used as experimental animals. 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 University Medical Center Mainz Ethics Committee. Mice were treated with Angiotensin II (ATII, 1mg/kg/d/7d)-loaded mini-osmotic pumps (model 1007D, ALZET, Cupertino, USA). Mice receiving NaCl-loaded pumps served as control (sham treatment). After 7 days of ATII treatment mice were killed by exsanguination under isoflurane anesthesia and blood was collected by right ventricular puncture. Aorta was transferred to 4°C Krebs-Hepes-solution (pH 7.35, containing 99.01mM NaCl, 4.69mM KCl, 2.50mM CaCl2, 1.20mM MgSO4, 25.0mM NaHCO3, 1.03mM KHPO4, 20.0mM Na-Hepes, 11.1mM D-glucose), cleaned of adhesive tissue and carefully rinsed prior to further handling.

Vascular relaxation studies: Isolated aortas were cut into 4 mm 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 118.3 mM NaCl, 4.69 mM KCl, 1.87 mM CaCl2, 1.2 mM MgSO4, 1.03 mM K2HPO4, 25 mM NaHCO3, 11.1 mM 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), nitroglycerin (GTN) and NONOate (DEA), aortic segments were stretched gradually over one hour to reach a resting tension of 3.0 grams. Following preconstriction with prostaglandin F2 (3nM) to reach 50-80% of maximal tone induced by KCl, concentration-relaxation curves were recorded in response to increasing concentrations of ACh, GTN and NONOate.

Blood pressure recordings: C57BL/6 and Tbx21−/− mice were equipped with carotid catheter implants for telemetric blood pressure measurements (TA-PA11C10, Data Science International (DSI), Tilburg, the Netherlands). Surgery was carried out under sterile conditions under anesthesia and analgesia with intraperitoneal ketamin/xylazin. After 2 weeks of recovery, ATII treatment regimen was initiated as described above and blood pressure was continuously recorded in freely moving animals using receiver platforms (DSI) for 2 weeks, the second week with ATII infusion. Measurements were taken using the DataQuest system (DSI).

Reactive Oxygen Species Formation using lucigenin-enhanced chemiluminescence
Oxidative burst of whole blood: Venous blood was drawn into an 0.1ml volume of 3.8% sodium citrate. Diluted blood was kept at room temperature and further diluted 1:50 in Dulbecco’s PBS (without Mg2+ and Ca2+). L012-enhanced chemiluminescence (ECL) signals was counted in 0.5ml samples in the absence or presence of PdBU (10µM) at intervals of 30s using a Lumat LB9507 from Berthold Technologies (Bad Wildbad, Germany). CL was expressed as counts per minute after incubation for 10, 15, 20 and 25min.

Superoxide formation of whole aortic rings: ECL signals of intact isolated aortic rings (length approximately 5 mm) 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).

Fluorescence oxidative microtopography: Isolated aorta were 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 dihydroethidium (DHE, 1µM in PBS) and incubated for 30 min at 37°C. Green autofluorescence derived from aortic lamina and red ethidium fluorescence inside the ROS producing cells was detected using a Zeiss Axiovert 40 CFL Camera (Zeiss, Oberkochen, Germany). Sections of all study arms were analyzed in parallel with identical imaging parameters.

**Protein expression:** Aortas were cleaned of adhesive adipose tissue, rinsed and snap frozen. Protein suspensions from homogenized aortic tissue were submitted to SDS PAGE and immunoblotting (BioRad, Hercules, USA), using antibodies against α-actinin (mouse, monoclonal, dilution 1:2000, Sigma-Aldrich, Seelze, Germany), β-actin (rabbit, polyclonal, dilution 1:1000, Sigma-Aldrich, Seelze, Germany), p22phox (rabbit, polyclonal, dilution 1:200; Santa Cruz; Santa Cruz, USA), p47phox (rabbit polyclonal antibody, 1:1000, Millipore; Temecula, CA), p67phox (mouse, monoclonal, dilution 1:500, BD Biosciences, Franklin Lakes, NJ), nitrotyrosine (mouse, monoclonal, dilution 1:1000, Millipore, Temecula, CA), MCP-1 (rabbit, polyclonal, dilution 1:5000, AbD Serotec; Oxford, UK), MiP-1α (rabbit polyclonal, dilution 1:5000, AbD Serotec; Oxford, UK) and Nox2 (gp91phox, mouse, monoclonal, dilution 1:500, BD Biosciences, Franklin Lakes, NJ) followed by peroxidase-labelled secondary antibodies against mouse, goat and rabbit IgG (Vector, Burlingame, CA). Immunodetection was accomplished with either SuperSignal Substrate (Pierce, Rockford, IL) or ECL Reagent (Amersham, Piscataway, NJ). Bands were evaluated by densitometry.

**mRNA expression:** mRNA expression was analyzed by quantitative real-time RT-PCR using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Briefly, total RNA from mouse aorta and heart 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, Hilden, Germany). TaqMan® Gene Expression assays (Applied Biosystems, Foster City, CA) for TATA-box binding protein (TBP; Mm00446973_m1), P-Selectin Ligand (Selplg; Mm01204501_m1), IFN-γ (ifng; Mm99999071_m1), inducible nitric oxide synthase (iNOS; Mm00440485_m1), MCP-1 (Ccl2; Mm00441242_m1) were purchased as probe and primer sets.

The comparative DCt 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.

For IL-12 mRNA expression total RNA from snap-frozen aortic tissue was isolated according to the method described by the modified guanidine isothiocyanate method of Chomczynski and Sacchi. After determining quality and purity of the RNA, the cDNA was synthesized from 1 µg of total RNA by reverse transcription using the high capacity RNA-to-cDNA Master Mix from Applied Biosystems (Foster City, CA). The primers in the following PCR used for analysis of IL-12p40 were 5’-CAGAAGCTAACCATCTCCTGTTTG-3’ (sense) and 5’-TCCGGAGTAAATTGTTGTTCACAC-3’ (antisense) and for mouse L-32 5’-CCTCTTGGTGAACCAGCAGATC3’ (forward) and 5’-TCTGGGTCTCCGCCAGTGT-3’ (reverse). All PCR amplifications were performed in a 25 µl reaction mixture using the REDTaq Ready Mix PCR Reaction Mix from Sigma-Aldrich (Seelze, Germany). The reaction was pursued for 30 cycles in a thermal cycler (Eppendorf, Hamburg, Germany) with denaturation at 94 °C for 1 minute, annealing at 55 °C for 2 minutes and extension at 72 °C for 3 minutes. The PCR products were loaded onto a 1.5% agarose gel containing GelRed Nucleic Acid Stain and visualized by GelDOC EZ Imager (BioRad, Hercules, USA). Bands were densitometric analysed with Gel Pro Analyzer software (Media Cybernetics, Bethesda, USA).

**Flow cytometric identification of NK-cells and monocytes in aorta:** The presence of NK cells and myelomonocytic cells in whole peripheral blood and aorta was determined by flow cytometric analysis of NK1.1 positive NK cells, CD11b positive and Gr-1 positive.
monocytes and granulocytes in WT, IFN-γ−/− and Tbx21−/− mice. Blood erythrocytes were hemolysed by ACK-buffer. For analysis of cells in mouse aorta, the total aorta was digested by using collagenase II (1 mg/ml) and DNAse I (50 µg/ml) for 20 minutes at 37°C. To block nonspecific Fc receptor-mediated binding, cells were pre-incubated with unlabeled antibody against CD16/CD32 for 10 minutes. A quantity of at least 1.4 x 10^5 aortic cells were stained for 20 minutes with CD11b PE-Cy7, Gr-1 FITC (BD Pharmingen, San Diego, USA) and F4/80 APC or with TCRβ PE-Cy5 and NK1.1 PE-Cy7 and for gating of B and dead cells additionally with CD45.2 APC-eFluor780, B220-Pacific Blue, and Fixable Viability Dye eFluor506 (dead cell marker, all from eBioscience, San Diego, CA). IL-12 PE or IFN-γ FITC or PE (eBioscience, San Diego, CA) was stained after fixation with Fixation/Permeabilization solution from eBioscience or BD Pharmingen. A minimum of 100 000 events was acquired using the BD FACS Canto II or the BD FACS Calibur (Becton Dickinson, Heidelberg, Germany), viable CD45+ cells gated for analysis on the basis of forward- and side-scatter signals or Fixable Viability Dye and data were analyzed with BD FACSDiva or Summit v4.0 (Dako, Glostrup, Denmark) software and quantified as number of cells per aorta.

Ex vivo stimulation of blood cells and aortic cells and detection of intracellular IFN-γ or IL-12 production: Isolated cells from aorta were stimulated in vitro with 20 ng/ml IL-12 (R&D, Minneapolis, MN) and 20 ng/ml IL-18 (MBL, Aichi, Japan) for 4-5 hours. After 30 minutes 1.4 µl (1:10) / 200µl sample volume BD GolgiStop was added. For analysis of IL-12 expression isolated cells were stimulated with 10 ng/ml mIFN-γ overnight followed by 5 hours of stimulation with 1 µg/ml LPS (Sigma-Aldrich, Seelze, Germany) in the presence of 10 µg/ml Brefeldin A (eBioscience, San Diego, CA). After stimulation and surface staining, cells were fixed and stained for intracellular IFN-γ FITC or PE or IL-12 PE (eBioscience, San Diego, CA) with the BD Cytofix/Cytoperm Kit as indicated in the manufacturer’s instructions (BD Pharmingen, San Diego, CA).

In vivo Depletion of NK cells or myelomonocytic cells: For NK cell depletion mice were intraperitoneally and subcutaneously injected with the anti-NK1.1 mouse monoclonal antibody PK136 (30 µg/animal) at day 1-3, followed by intraperitoneal injections (15 µg/animal) at day 4 and 7. Angiotensin II-treatment started at day 4 in some mice. NK and T cell depletion in the blood were analyzed by flow cytometry. LysMCre and LysMiDTR mice were used for monocyte depletion experiments. For DTX receptor-mediated cell ablation mice received intraperitoneal injections with DTX once daily (solved in PBS; 25 ng/g from day 1 to 3, 5 ng/g thereafter); for reconstitution of depleted LysMCre+ mice, CD11bGr-1+ monocytes were prepared from venous blood of Tbx21−/− by positive selection using magnetic activated cell sorting after discarding granulocytes following Histopaque 1083 gradient 5.

Reconstitution of Tbx21−/− mice with NK1.1+ cells and CD11bGr-1+ monocytes: For in vivo reconstitution experiments, Tbx21−/− mice were injected intravenously with 0.5x10⁷ NK1.1+ cells isolated from isogenic rag1−/− mice or 1x10⁶ CD11bGr-1+ monocytes isolated from venous blood of isogenic C57BL/6 mice in 0.9% NaCl vs. 0.9% NaCl alone (100µl final volume) on d1 of the protocol, and ATII application (1mg/kg/d/7d) by using osmotic mini-pumps was continued from d2 until d8. In every protocol, cells were isolated by positive selection with magnetic assisted cell sorting.

Statistical Analysis. Data are expressed as mean±/SEM or median with box plot and whiskers (min to max). Statistical calculations were performed with GraphPad Prism 5 (GraphPad Software Inc, San Diego, Calif). D’Agostino-and-Pearson normality test was first performed, and t-test, Wilcoxon matched pair test, Mann-Whitney test, Kruskal-Wallis test, 1-way-ANOVA or Friedman test with posthoc Dunn or Bonferroni test was used as appropriate. n indicates number of animals studied. One asterisk indicates P-values <0.05, two asterisks p<0.01 and three p<0.001, considered to be statistically significant.
Kossmann et al. NK cells, IFN-γ and vascular dysfunction- Supplemental material and methods

References


