Aims
Recent publications have shed new light on the role of the adaptive and innate immune system in the pathogenesis of hypertension. However, there are limited data whether receptors of the innate immune system may influence blood pressure. Toll-like receptor 4 (TLR4), a pattern recognition receptor, is a key component of the innate immune system, which is activated by exogenous and endogenous ligands. Hypertension is associated with end-organ damage and thus might lead to the release of damage-associated molecular patterns (DAMPs), which are endogenous activators of TLR4 receptors. The present study aimed to elucidate whether TLR4 signalling is able to modulate vascular contractility in an experimental model of hypertension thus contributing to blood pressure regulation.

Methods and results
NG-nitro-L-arginine methyl ester (L-NAME)-induced hypertension was blunted in TLR4-/- when compared with wild-type mice. Treatment with L-NAME was associated with a release of DAMPs, leading to reactive oxygen species production of smooth muscle cells in a TLR4-dependent manner. As oxidative stress leads to an impaired function of the NO-sGC-cyclic GMP (cGMP) pathway, we were able to demonstrate that TLR4-/- was protected from sGC inactivation. Consequently, arterial contractility was reduced in TLR4-/-.

Conclusions
Cell damage-associated TLR4 signalling might act as a direct mediator of vascular contractility providing a molecular link between inflammation and hypertension.

Keywords
Hypertension • Innate immunity • Toll-like receptor • Reactive oxygen species • Vascular contractility

1. Introduction
Hypertension is a major healthcare concern because of its high prevalence and its effect on the development of stroke, ischaemic heart disease, and other vascular diseases, thus substantially contributing to morbidity and mortality in the general population.1

Recent studies demonstrated the impact of the adaptive immune system in the development of vascular dysfunction and hypertension.2 These studies support the role of inflammation in the pathogenesis of this prevalent disease. Besides adaptive immunity, it has been demonstrated that macrophages3 and Toll-like receptor 4 (TLR4) expressed on contractile cells are associated with hypertension.4 Macrophages and TLR4 represent key components of the innate immune system.5 Therefore, the role of the innate immune system in blood pressure regulation and thus its impact on the development of hypertension need to be further elucidated.

The accumulation of endothelial and subendothelial macrophages is a common finding during chronic administration of NG-nitro-L-arginine methyl ester (L-NAME), which is a dose-dependent model of hypertension and progressive vascular lesions.6,7 Additionally, infiltration of
predominantly macrophages has been shown in perivascular areas in different models of hypertensive rats. In line with this finding, the depletion of macrophages under hypertensive conditions results in reduced endothelial dysfunction, vascular remodelling, and oxidative stress. This suggests a critical role of macrophages and pro-inflammatory mediators in vascular injury.

L-NAME is an inflammatory stimulus and leads to production of reactive oxygen species (ROS) via either leucocytes or endothelial cells. ROS, in turn, cause organ damage on a cellular basis resulting in the release of various molecules, such as high-mobility group box-1 (HMGB-1) and heat shock proteins. Those so-called damage-associated molecular patterns (DAMPs) are well-known endogenous ligands of TLR4. Furthermore, besides initiating the production of cytokines, TLR4 signalling leads directly to the production of ROS thus potentially affecting blood pressure. In keeping with the association of innate immune cells on L-NAME-induced hypertension, constitutive TLR4 signalling has been associated with impaired baseline cyclic GMP (cGMP) production in thrombocytes. In vascular smooth muscle cells (VSMCs), cGMP constitutes a major second messenger, responsible for vascular dilation.

Interestingly, L-NAME administration not only leads to cellular damage, but also causes an up-regulation of TLR4 expression in contractile cells. Consequently, chronic administration of L-NAME results not only in the activation and homing of macrophages as typical representatives of the innate immune system, but also could additionally stimulate the TLR4 signalling in vascular SMCs by DAMPs.

However, the relevance of TLR4 signalling under hypertensive conditions on vascular function and blood pressure remains uncertain. Our study demonstrates that TLR4−/− mice are protected from a blood pressure increase after induction of L-NAME-induced hypertension. We confirm that L-NAME leads to cellular damage and DAMP release, which, in turn, leads to TLR4-dependent vascular ROS production. As a consequence, vascular contractility is reduced in TLR4−/− mice, which we explain by an effect of reduced vascular ROS on the NO–sGC–cGMP pathway. Thus, we propose a novel mechanism involving signalling of components of the innate immune system in the pathogenesis of L-NAME-induced hypertension.

2. Methods

2.1 Mice strains
TLR4-deficient (TLR4−/−) mice with a C57BL/6 background were bred in our own facility. C57BL/6 (wild-type, WT) mice were purchased from Charles River (Sulzfeld, Germany) and used as control animals. Approval to perform in vivo animal experiments was granted by the local public authorities of the state of Bavaria (Approval reference number: AZ 55.2-1-54-2531-19-09). This investigation conforms with the principles of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication, 8th Edition, 2011), and the German Law on the Protection of Animals was followed.

2.2 Intra-arterial blood pressure measurement of L-NAME treatment
The insertion of an intra-arterial pressure transducer was performed under inhalation anaesthesia with isoflurane (1.5–2.5% added to breathing air). Adequacy of anaesthesia was controlled by monitoring heart rate and breathing frequency. Mice were fixed on a heat-controlled plate, and the left carotid artery was exposed. The tip of an intra-arterial pressure transducer (Model TA11PA-C10, Data Sciences International, St. Paul, USA) was inserted into the left carotid artery, and the telemetry device was positioned in the neck of mice under sterile conditions. After a recovery period of 5 days, the blood pressure was assessed with radiotelemetry for consecutive 3 days prior to treatment with L-NAME and during 14 consecutive days of treatment with L-NAME over 24 h. L-NAME was applied via tap water at a dose of 50 mg/kg/day.

2.3 Wire myograph and contractility studies
Vascular contractility studies were performed according to the technique described by Mulvany and Halpern. In brief, animals were sacrificed by an incision into the left ventricle under isoflurane anaesthesia (2%). After median laparotomy, the mesenteric bed was exposed. The superior mesenteric arcade was removed and transferred into a dissecting dish containing 3-(N-Morpholino)propanesulfonic acid (MOPS)-buffered saline (in mmol/L: NaCl 145; KCl 4.7; CaCl2 3; MgSO4 1.17; NaH2PO4 1.2; glucose 5; pyruvate 2; MOPS 3; EDTA 0.2; Sigma-Aldrich, Munich, Germany). Second- and third-generation branches of the mesenteric artery (internal diameter 255–390 μm) were cleaned of periadventitial fat and connective tissue, and two stainless steel wires were insertedatraumatically into the lumen of each artery. Two arteries were mounted in a Mulvany wire myograph Model 500A (Danish Myo Technology, Aarhus, Denmark) in ambient MOPS-buffered saline. After a resting period of 10 min, vessels were slowly heated to 37°C and pre-stretched to 90% of maximal diameter. Subsequently, the organ bath solution was changed for a fresh MOPS solution and vascular functions were analysed.

2.4 Experimental protocol
After a resting period of 30 min, vessels were subjected to viability testing using ascending concentrations of noradrenaline (100 nmol/L to 1 μmol/L; Fluka, Munich, Germany) and acetylcholine (ACh, 100 nmol/L to 10 μmol/L, Fluka). After washout of vasoactive substances and a resting period of 5 min, vessels were first calcium depleted by addition of a calcium-free MOPS-buffered saline and afterwards the relaxed vessels were depolarized using a saline solution containing 125 mmol/L of potassium, allowing to constrict arteries by a stepwise increase of extracellular calcium concentration from 0.25 to 3 mmol/L. Finally, endothelium-independent vasorelaxation was assessed by addition of ascending concentration of sodium nitroprusside (SNP, 100 nmol/L to 100 μmol/L, Fluka) under depolarizing conditions in the presence of 3 mmol/L of calcium. In a second set of experiments, 3-isobutyl-1-methylxanthine (IBMX, Tocris, Bristol, UK), a phosphodiesterase inhibitor, was added to mesenteric arteries pre-contracted by 3 mmol/L of extracellular calcium in ascending concentration (10 μmol/L to 10 μmol/L).

2.5 Primary aortic smooth muscle cells and vascular tissue
Tissue of mice was obtained after sacrificing animals by an incision into the left ventricle under isoflurane anaesthesia (2%). VSMCs from mouse aorta were isolated using an adapted protocol according to Kobayashi et al. Briefly, after endothelial cells were removed by treatment with collagenase II for 45 min, aortas were cut lengthwise into small 2 mm cubes and placed onto a 60 mm dish coated with gelatin. Subsequently, Dulbecco’s modified eagle’s medium (DMEM) (Biochrom, Berlin, Germany) containing 10% FBS (Sigma-Aldrich) and 2 mmol/L of l-glutamine (Biochem) was added and the explants were cultivated for 3–4 weeks. After the cells achieved locally high density, the explants were removed and VSMCs were trypsinized using 0.25% Trypsin/EDTA (Gibco, Darmstadt, Germany) solution. Homogeneity of the culture was assessed by the presence of a ‘spindle-shape’ morphology and also by expression of a-smooth muscle actin, calponin, and smoothelin.

From untreated primary aortic VSMCs of WT and TLR4−/− mice, RNA was isolated with an RNasy Mini Kit (Qiagen, Hilden, Germany) and transcribed into cDNA with an iScript cDNA synthesis kit (Bio-rad, Munich,
samples were submitted to collagenase digestion to get a single cell suspension. After sacrificing WT and TLR4 (Biovision, CA, USA), cultured VSMCs were transferred to 60 mm dishes and treated with either 1 μg/mL of lipopolysaccharide (LPS) or 100 ng/mL of HMGB-1 for 48 h. Afterwards, cells were treated with 10 mmol/L of DCFH-DA for 45 min. Cells were lysed using Cellytic M reagent (Sigma-Aldrich), centrifuged, and the supernatant was transferred to a 96-well plate. Fluorescence was then measured at 485 nm excitation and at 530 nm emission wavelength. ROS concentration was normalized to the total protein content of lysed cells. The measurement of ROS production in aortic tissue was quantified by CellRox Green staining using confocal microscopy. In brief, isolated aortic rings were placed in DMEM supplemented with 2% FBS and incubated for 18 h either in the presence of L-NAME (500 μmol/L) or without. After incubation, aortic rings were washed with phosphate-buffered saline and stained with 5 μmol/L of CellRox Green (Life Technologies, Darmstadt, Germany) following manufacturer’s instructions. Primers against sGCα (forward: CGTCAAGGTATGATGATCTCA, reverse: GGGGTATGAGATTGGGATGT) and sGCβ (forward: ACTGAGCAAGGAGAGG GTT; reverse: TTGGTGACAGGAGGAGG) were amplified and detected in the MyQ Real-Time PCR detection system using iQ Sybr Green Supermix (Biorad). Data were relate to the expression level of GAPDH and were evaluated in the Biorad IQ5 software.

Measurement of ROS production in VSMCs was performed using 2’,7’-dichlorofluorescein diacetate (DCFH-DA) (BMG Labtechnologies, Offenbach, Germany) according to the manufacturer’s instructions. In brief, cultured VSMCs were transferred to 60 mm dishes and treated with either 1 μg/mL of lipopolysaccharide (LPS) or 100 ng/mL of HMGB-1 for 48 h. Afterwards, cells were treated with 10 mmol/L of DCFH-DA for 45 min. Cells were lysed using Cellytic M reagent (Sigma-Aldrich), centrifuged, and the supernatant was transferred to a 96-well plate. Fluorescence was then measured at 485 nm excitation and at 530 nm emission wavelength. ROS concentration was normalized to the total protein content of lysed cells. The measurement of ROS production in aortic tissue was quantified by CellRox Green staining using confocal microscopy. In brief, isolated aortic rings were placed in DMEM supplemented with 2% FBS and incubated for 18 h either in the presence of L-NAME (500 μmol/L) or without. After incubation, aortic rings were washed with phosphate-buffered saline and stained with 5 μmol/L of CellRox Green (Life Technologies, Darmstadt, Germany) for 1 h. Aortic rings were transferred into a 4-well slide chamber (Millipore, Darmstadt, Germany). About 1 μg/mL of Hoechst 33342 (Life Technologies) was added and slide chambers were placed on a LSM 710 confocal laser microscope (Carl Zeiss, Oberkochen, Germany) with a 40-fold magnification objective. Emission was recorded at 460 and 520 nm, respectively. Data were analysed using the MetaMorph software (Molecular Devices, Downingtown, USA). Total nuclei and CellRox Green-positive nuclei were determined simultaneously and an overlap of staining of nuclei was performed. CellRox-positive nuclei were only counted in the case of a positive overlap.

HMGB-1 measurements in serum of WT mice without and after treatment with L-NAME (50 μg/kg/day) were performed using a commercially available ELISA (IBL International, Hamburg, Germany) following manufacturer’s instructions. cGMP concentration after L-NAME treatment was performed directly after L-NAME treatment and TL4R-/- mice. For the determination of cGMP concentration after L-NAME treatment, isolated aortas were weighed, placed in 50 μmol/L of FACS buffer and placed on ice. Data were analysed using the MetaMorph software (Molecular Devices, Downingtown, USA). Total nuclei and CellRox Green-positive nuclei were determined simultaneously and an overlap of staining of nuclei was performed. CellRox-positive nuclei were only counted in the case of a positive overlap.

Nuclear factor kappa B (NF-κB) activation was assessed using PathScan® Phospho-NF-κB p65 (Ser536) Sandwich ELISA (Cell Signaling, Danvers, USA) according to the manufacturer’s instructions. In brief, after 2 weeks of L-NAME treatment, aortas were isolated and protein isolation was performed using cell lysis buffer 9803 (Cell Signaling). Fifteen microgram of protein per aorta (four mice per group) was used for each individual experiment. The magnitude of optical density is proportional to the quantity of phospho-NF-κB p65 (Ser536).

### 2.6 Fluorescence-activated cell sorting analysis of leucocyte infiltration of periarterial tissue

Two-colour flow cytometric analysis (fluorescence-activated cell sorting (FACS)) was used to identify leucocyte infiltration in perivascular aortic tissue by detection of CD45 expression as described previously. In brief, samples were submitted to collagenase digestion to get a single cell suspension. Cells were re-suspended in 300 μL of FACS buffer and placed on ice. Fluorescein isothiocyanate anti-rat CD45R (eBioscience, San Diego, CA, USA) was added. Analysis was done on a Beckman Coulter flow cytometer (Beckman Coulter, Krefeld, Germany). Before running the samples on the flow cytometer, propidium iodide (1:1000 of a 1 mg/mL stock; Sigma Chemicals, Munich, Germany) was added to label dead cells. Data were analysed using the FlowJo Software (Tree Star, Inc., Ashland, USA). Dead cells were excluded using propidium iodide staining. A positive control for anti-rat CD45 antibody was performed on rat spleen cells.

### 2.7 Statistics

Vasorelaxation was calculated as the reversal of the preceding constriction elicited by 3 mmol/L of Ca²⁺:

\[
(F_s - F_{basal})/(F_{basal} - F_{Ca^{2+}}) \times 100
\]

where \(F_s\) force at a given Ca²⁺ concentration (0–3 mmol/L); \(F_{basal}\) force under baseline conditions; and \(F_{Ca^{2+}}\) maximal force at Ca²⁺ concentration of 3 mmol/L.

Student’s t-test, analysis of variance (ANOVA) test with post hoc Bonferroni correction, and Kruskal–Wallis test were used to analyse significance, as appropriate. A P-level of <0.05 was considered significant.

### 3. Results

#### 3.1 Role of TLR4 in modulation of blood pressure

Using blood pressure telemetry as the gold standard method of blood pressure monitoring in mice, we found no difference in baseline mean arterial blood pressure (baseline MBP) between TL4R-/- and WT mice. However, in an experimental model of hypertension caused by chronic L-NAME supplementation (50 mg/kg/day) in drinking water leading to an inhibition of NO synthase, we observed strain-specific differences. Whereas WT mice exhibited a steady increase in MBP from 106.9 mmHg (Day 0) to 123.4 mmHg (Day 14), TL4R-/- mice were protected against blood pressure increases (Figure 1A). This increase in MBP was reflected by both systolic and diastolic blood pressure (Figure 1B and C). The heart rate increase was more apparent in WT than in TL4R-/- mice, yet this finding failed to reach statistical significance (Figure 1D).

#### 3.2 L-NAME leads to DAMP-mediated ROS production

Chronic administration of L-NAME (50 mg/kg/day) leads to a similar systemic HMGB-1 release in WT and TL4R-/- mice (Figure 2A). To investigate whether LPS and HMGB-1 lead to divergent ROS production in VSMCs of WT and TL4R-/- mice, we measured the concentration of DCFH-DA, a marker of intracellular H₂O₂ production, in aortic SMC lysates. Baseline ROS production had the tendency to be lower in TL4R-/- when compared with WT mice, yet this finding failed to reach statistical significance (\(P = 0.06\)). However, upon exogenous stimulation of VSMCs with TL4R ligands, TL4R-/- mice showed a significantly lower ROS production (Figure 2B) when compared with WT mice. To further strengthen the role of ROS in this setting, we analysed vascular ROS production as a response to L-NAME treatment. Staining of VSMC with the ROS-sensitive dye CellRox Green allows the detection of ROS production in intact tissue by staining nuclei. Intact aortic rings exhibited only low levels of CellRox Green-positive nuclei after incubation in DMEM without L-NAME. Treatment of
Figure 1  Role of TLR4 in L-NAME-induced hypertension. WT and TLR4<sup>−/−</sup> mice were treated for 14 days with 50 mg/kg/day L-NAME. Blood pressure and heart rate were assessed in freely moving WT and TLR4<sup>−/−</sup> mice by radiotelemetry (n = 8–12 each). (A) Baseline mean blood pressure (baseline MBP) in untreated WT and TLR4<sup>−/−</sup> mice showed no difference between the strains. During L-NAME treatment, MBP rose from 106.9 to 123.4 mmHg in WT mice, whereas TLR4<sup>−/−</sup> mice showed no increase in MBP (107.6–99.8 mmHg). All data are expressed as mean ± SD; *P < 0.05 vs. WT Days 3–14. (B) Systolic blood pressure (SBP) rose from 129.9 to 146.6 mmHg in WT. TLR4<sup>−/−</sup> did not develop an increase in SBP (130.9–122.8 mmHg); *P < 0.05 vs. WT Days 2–14. (C) Diastolic blood pressure (DBP) rose from 94.9 to 111.4 mmHg in WT. No increase in DBP was observed in TLR4<sup>−/−</sup> (95.5–85.8 mmHg); *P < 0.05 vs. WT Days 2–14. (D) A maximal heart rate increase did not differ between WT and TLR4<sup>−/−</sup>. All data are expressed as mean ± SD (repeated-measures ANOVA with Bonferroni correction).

Figure 2  Influence of L-NAME treatment on DAMP release and ROS production. (A) Fourteen days of treatment with L-NAME (50 mg/kg/day) lead to a significant increase of systemic serum concentration of HMGB-1 in WT and TLR4<sup>−/−</sup> (n = 6 and 9 each group). Data are expressed as ng/mL ± SD; *P < 0.05 as indicated (two-way ANOVA with Bonferroni correction). (B) ROS production in cultured vascular SMCs was significantly lower in TLR4<sup>−/−</sup>. Treatment of cultured SMCs with HMGB-1 (100 ng/mL) and LPS (1 μg/mL) for 48 h leads to a significant increase of ROS production, yet ROS production in TLR4<sup>−/−</sup> remained significantly lower as in WT (n = 3 each). Data are expressed as the percentage of ROS production with untreated WT being normalized to 100% ± SD; *P < 0.05 as indicated (Kruskal–Wallis test).
aortic rings with menadione (100 μmol/L) as a positive control leads to a significant increase in CellRox Green-positive nuclei. Similarly, the treatment of aortic rings with L-NAME (500 μmol/L) for 18 h leads to a significant increase in CellRox Green-positive nuclei (Figure 3A–D).

Finally, activation of NF-κB was assessed in order to analyse the significance of ROS-mediated signalling. Treatment of mice with L-NAME leads to significant augmentation of NF-κB signalling as expressed by an increase in phosphorylation of Ser536 of NF-κB p65 (Figure 4A).

3.3 sGC dependency

As ROS increase vascular contractility via inactivation of sGC, we further investigated the influence of TLR4 on its function in L-NAME-induced hypertension. Determining sGC activity by measuring cGMP concentration in aortic lysates, we found a significantly higher activity in TLR4−/− when compared with WT mice after treatment with L-NAME (Figure 4B).

To further investigate the role of TLR4 on sGC activity, we measured cGMP production after treatment with LPS. cGMP concentration in aortic lysates was significantly lower after incubation of aortas with LPS when compared with untreated. Co-incubation of aortas with LPS and TEMPOL, an inhibitor of ROS production, restored vascular cGMP production, yet this finding failed to reach statistical significance (P = 0.06) (Figure 4C). cGMP degradation did not differ between mice strains as determined by measuring vasorelaxation in the presence of ascending concentrations of IBMX, an inhibitor of phosphodiesterases (Figure 4D). Expression of sGC subunits was not higher in TLR4−/− when compared with WT mice (data not shown).

3.4 Influence of TLR4 on vascular function

No significant difference in internal diameter was detected in TLR4−/− when compared with WT mice under baseline conditions (335 ± 8 vs. 329 ± 5 μm; P = ns). As reflected by in vivo blood pressure measurements, assessment of vasoconstriction of isolated mesenteric arteries revealed no significant difference under baseline conditions (data not shown). However, vasoconstriction was significantly reduced in TLR4−/− mice after treatment with L-NAME when compared with WT mice (Figure 5).

Since reduced endothelial-dependent vasodilation is the main mechanism of L-NAME-induced hypertension, we investigated the dilative response to ACh in mesenteric arteries. However, we found no significant difference in ACh-induced endothelium-dependent vasodilation in either untreated or L-NAME-treated WT and TLR4−/− mice (data not shown). To assess the endothelial-independent vasodilation in mesenteric arteries, we tested the dilative properties as elicited by administration of exogenous NO using SNP. Dilation was significantly augmented in TLR4−/− when compared with WT mice. This observation
was evident under baseline conditions and after treatment with L-NAME (Table 1).

### 3.5 Role of TLR4 on perivascular inflammation in L-NAME-induced hypertension

As shown, immune cells can contribute to vascular dysfunction and the development of hypertension caused by angiotensin II.2 Similar to the angiotensin II model, L-NAME-induced hypertension reflects a state of chronic low-grade inflammation.20 TLR4 is a major component of the innate immune system and is able to modulate the perivascular recruitment of immune cells.21,22 Therefore, we investigated the perivascular infiltration in arterioles of epicardial tissue using haematoxylin–eosin staining and quantified leucocytes in aortic lysates by CD45 FACS analysis. As shown in Figure 6A, perivascular infiltration in epicardial arterioles was similar in both WT and TLR4^{−/−} mice under resting conditions. Chronic L-NAME treatment increased perivascular infiltration. However, no difference between WT and TLR4^{−/−} mice was detected.

Similarly, infiltration of perivascular aortic tissue with CD45-positive immune cells was detectable in WT and TLR4^{−/−} mice. However, infiltration was low (1.1% of gated cells in WT and 1.5% in TLR4^{−/−}) and no significant difference between WT and TLR4^{−/−} mice was detectable (Figure 6B).

### 4. Discussion

In this study, we show that the innate immune system plays an important role in the development of L-NAME-induced hypertension. This model is characterized by a pronounced perivascular infiltration and is associated with the induction of an inflammatory response.23 In this model, TLR4^{−/−} mice demonstrated a full blood pressure protection. As an underlying mechanism, our data suggest that L-NAME leads to a release of DAMPs, which in terms induce ROS formation via TLR4 signalling, leading to an impaired sGC function. After L-NAME treatment, TLR4^{−/−} mice exhibit reduced vascular constriction and enhanced dilative properties when compared with WT mice. We suggest that an abrogated TLR4 signalling leads to reduced ROS production upon DAMP stimulation. As a consequence, sGC activity remains intact after L-NAME administration in TLR4^{−/−} mice.
Administration of L-NAME induces inflammation and tissue damage. Damaged tissue releases unspecific molecules such as heat shock proteins, fibrinogen, or HMGB-1, which become detectable in blood. Those so-called DAMPs are known endogenous ligands of pattern recognition receptors such as TLR4. We were able to demonstrate that L-NAME administration, indeed, leads to a release of DAMPs, namely HMGB-1. We speculated that those DAMPs are able to induce the production of ROS via TLR4 signalling. Indeed, VSMCs responded with an up-regulation of ROS production upon stimulation with DAMPs. This is in line with observations in an experimental model of ischaemia/reperfusion, where DAMPs are known to induce the production of ROS in a TLR4-dependent manner. Consequently, VSMCs of mice with an abrogated TLR4 signalling exhibited reduced ROS production in this setting.

Figure 5 Influence of TLR4 signalling on vascular function. Vasoconstriction in mesenteric arteries was assessed in depolarized resistance arteries (125 mM K⁺) in response to increases in extracellular Ca²⁺ (Ca²⁺ ex.: 0–3 mM) after 14 days of L-NAME treatment (50 mg/kg/day). Calcium-induced vasoconstriction was reduced in TLR4⁻/⁻ mice when compared with WT mice (n = 8 and 12). Data are expressed as means ± SEM; *p < 0.05 vs. WT (repeated-measures ANOVA with Bonferroni correction).

 ROS production is a key component in the pathogenesis of hypertension by increasing vascular contractility and impairing dilation. Several mechanisms of ROS have been proposed in this context. One aspect includes the modulation of vasoconstriction by inactivation of sGC, an enzyme that has been evidenced to play a critical role in vascular contractility and blood pressure regulation. sGC activity is critically linked to the presence of a Fe²⁺–haem residue. ROS lead to the oxidation of this haem, leading to a reduction of sGC activity. In keeping with the blunted ROS production in TLR4⁻/⁻ mice, we were able to detect a higher sGC activity as measured by cGMP production in the vasculature of this mouse strain. Higher levels of cGMP can be the result of either a higher activity of sGC, a higher expression of sGC, or alternatively of a reduced degradation by phosphodiesterases. We were able to exclude a higher expression of sGC. Furthermore, we excluded an impaired degradation of cGMP. Thus, the higher cGMP concentration in VSMCs observed in TLR4⁻/⁻ are the result of a higher sGC activity. Reduction of sGC activity seems to be mediated by TLR4-dependent ROS production rather than by L-NAME-induced NO synthase uncoupling since treatment with LPS, a key TLR4 ligand acting independent of L-NAME, decreased cGMP concentration. Contrary, treatment with TEMPOL, a ROS scavenging substance, was able to restore vascular cGMP production. This observation is in line with previous observation of a blood pressure-lowering effect by systemic treatment with TEMPOL. Interestingly, cGMP concentration was higher after treatment with L-NAME. sGC activity can be enhanced by allosteric modification in an NO-independent manner. It is speculative that, in our model in the absence of endogenous NO, this might account for the unexpected higher activity of sGC after treatment with L-NAME. However, it is important to notice that cGMP concentration still remained significantly higher in TLR4⁻/⁻ mice. Taken together, those results point to the importance of TLR4-mediated ROS production as a response to L-NAME-induced DAMP release, which in term influences vascular contractility by modifying sGC activity.

Blood pressure is controlled by modulating the resistance of arteries via constriction and dilation. Enhanced vascular contractility leading to a rise in total peripheral resistance is characteristic in the setting of hypertension. Corresponding to the blood pressure protection and higher sGC activity under treatment with L-NAME, we found an attenuated vascular contractility in TLR4⁻/⁻ mice. The finding that TLR4⁻/⁻ mice exhibited an augmented dilative capacity in response to exogenous

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Data are represented in % reversal of constriction ± SEM.

*p < 0.05 WT vs. TLR4⁻/⁻ without L-NAME treatment.

**p < 0.05 WT vs. TLR4⁻/⁻ with L-NAME treatment.
NO supplementation even under baseline conditions is further evidence that TLR4 signalling interacts with the ROS–sGC–cGMP pathway.

Since baseline blood pressure did not differ between TLR4^{−/−} and WT mice, we speculate that constitutional TLR4 signalling is not sufficient to modulate blood pressure. However, after induction of cellular damage by L-NAME treatment, which leads to a predominantly perivascular inflammation, TLR4 increases blood pressure. This suggests that, in our experimental model, L-NAME, which increases TLR4 ligands via tissue damage, is sufficient to contribute to blood pressure elevation in a TLR4-dependent manner. As hypertension itself is associated with chronic low-grade inflammation causing cell damage,39 we speculate that DAMP-dependent TLR4 signalling might be an additive factor in the pathogenesis of this disease.

Recent investigations have highlighted the importance of the adaptive immune system in the pathogenesis of hypertension. Given the close interaction between the innate and adaptive immune system, induction of TLR4 signalling by tissue damage might constitute the initiation of the adaptive immune system. Furthermore, since chronic inflammation caused by adaptive immunity leads to further cell damage and release of DAMPs, TLR4 signalling might perpetuate immune-mediated hypertension. A recent study on the role of TLR4 in spontaneous hypertensive rats, in which inhibition of TLR4 signalling leads to an impaired vascular contractility, supports this concept.30

There are certain limitations to our study. Most importantly, we cannot demonstrate that treatment with TEMPOL does actually lead to a lower ROS production in aortic VSMCs as a response to L-NAME. As it is difficult to scavenge ROS successfully, we had to resort to an indirect way of demonstrating the importance of ROS signalling in L-NAME-induced hypertension. In this regard, we were able to demonstrate that L-NAME treatment, indeed, leads to an increase of phosphorylated NF-κB p65 as a marker of an activation of ROS signalling. An additional limitation of this study is that we were not able to directly measure ROS production in vivo as a response to L-NAME. Staining of intact aortic tissue with the ROS-sensitive dye CellRox Green

Figure 6 Influence of L-NAME on vascular infiltration. Perivascular infiltration was analysed in haematoxylin–eosin-stained sections of epicardial arterioles in untreated and L-NAME-treated WT and TLR4^{−/−} mice, respectively. (A) Histomorphology (representative of four experiments). (B) Quantification of CD45-positive immune cells in perivascular aortic tissue of WT and TLR4^{−/−} mice by FACS analysis (representative of four experiments).
demonstrates that L-NAME leads to an augmented ROS production. Yet, this finding is only semi-quantitative.

In summary, in this study, we provide a novel pathway in which L-NAME-induced tissue damage causes a TLR4-dependent inactivation of sGC. Furthermore, we were able to demonstrate that an abrogated TLR4 signalling is protective against the deleterious effects of L-NAME on blood pressure. To the best of our knowledge, this is the first study to demonstrate that TLR4, as a key element of the innate immune system, has direct effects on vascular contractility and blood pressure.

Considering the potential implications of DAMP provoked signalling of components of the innate immune system in the pathogenesis of hypertension, the role of TLR4 needs to be further addressed in different experimental models of hypertension. Furthermore, the idea of cell damage as a contributor to vascular contractility may link other factors, such as metabolic disorders, also causing cell damage to hypertension and to an increased cardiovascular risk.

Funding
This work was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG BA 2173/6-1) and of the Technical University Munich (KKF A7-09).

Conflict of interest: none declared.

References