Objective—The purpose of this study was to determine whether myeloid differentiation factor 88 (MyD88) and its related Toll-like receptors (TLRs) 2 and 4 contributed to the development of angiotensin II (AngII)-induced abdominal aortic aneurysms (AAAs) and atherosclerosis.

Methods and Results—AngII was infused into either apoE−/− or LDL receptor (LDLR)−/− male mice that were either MyD88+/+ or −/−. MyD88 deficiency profoundly reduced AngII-induced AAAs and atherosclerosis in both strains. To define whether deficiency of specific TLRs had similar effects, AngII was infused into LDLR−/− mice that were also deficient in either TLR2 or TLR4. TLR2 deficiency had no effect on AAA development but inhibited atherosclerosis. In contrast, TLR4 deficiency attenuated both AAAs and atherosclerosis. To resolve whether MyD88 and TLR4 exerted their effects through cells of hematopoietic lineage, LDLR−/− mice were lethally irradiated and repopulated with bone marrow-derived cells from either MyD88 or TLR4 strains. MyD88 deficiency in bone marrow-derived cells profoundly reduced both AngII-induced AAAs and atherosclerosis. However, TLR4 deficiency in bone marrow-derived cells had no effect on either pathology.

Conclusion—These studies demonstrate that MyD88 deficiency in leukocytes profoundly reduces AngII-induced AAAs and atherosclerosis via mechanisms independent of either TLR2 or TLR4. (Arterioscler Thromb Vasc Biol. 2011;31:2813-2819.)

Key Words: aneurysms ■ angiotensin II ■ atherosclerosis ■ myeloid differentiation factor 88 ■ toll-like receptors

Abdominal aortic aneurysms (AAAs) in humans are characterized by permanent dilations that have an increasing propensity to rupture with expansion. Despite the devastating impact of the disease in an increasingly larger number of people, the mechanistic basis for the initiation, progression, and rupture of AAAs has not been defined. Aneurysmal tissues are highly heterogeneous, which is manifested as accumulation of several types of leukocytes, substantial fragmentation of extracellular matrix, and atherosclerosis.1 Notably, cells of the innate and adaptive immune systems accumulate in human AAA tissue and have been linked to the development of the disease.2

Several animal models of AAAs have become widely used to gain insight into mechanisms of the human disease.3 One of the most commonly used AAA models generates the disease by chronic subcutaneous infusion of angiotensin II (AngII) into mice.4 AngII infusion promotes aneurysmal disease in both normo- and hypercholesterolemic mice, although AAAs occur more frequently in hypercholesterolemic mice.5,6 In addition to AAAs, infusion of AngII to hypercholesterolemic mice also augments atherosclerosis.7,8 Like the human disease, AAAs developed during AngII infusion are characterized by progressive leukocyte accumulation, extracellular matrix degradation, lumen expansion, thrombus, and atherosclerosis.4

Both innate and adaptive immunities have proposed effects on AAA formation,9 although many facets are unknown regarding the relative contribution and mechanism. Myeloid differentiation factor 88 (MyD88) is a mediator of signaling cascades that directly influences leukocytes involved in innate immunity and has indirect effects on adaptive immunity.10 MyD88 was initially identified as a myeloid differentiation marker and was subsequently defined as an adaptor protein for signaling.11 It was first demonstrated to act as an adaptor protein in signaling mechanisms that follow the engagement of interleukin-1 with interleukin-1 receptors.12 Later, MyD88 was identified as a critical adaptor protein engaged by the majority of the Toll-like receptors (TLRs). MyD88 deficiency abolishes signaling of most TLRs, with the exception of the TLR3 and TLR4 MyD88-independent
signaling pathways.\textsuperscript{13} Stimulation of MyD88 activates several signaling pathways including nuclear factor \( \kappa \)B and mitogen-activated protein kinase that trigger transcription of numerous inflammatory cytokines to promote leukocyte recruitment.\textsuperscript{15}

MyD88 and its association with TLRs have been studied in hypercholesterolemia-induced atherosclerosis.\textsuperscript{14–18} However, a role for MyD88 in development of AAAs is currently unknown. Therefore, we determined the role of MyD88 in AngII-induced AAAs and atherosclerosis in both apolipoprotein E deficient (apoE\textsuperscript{-/-}) and low-density lipoprotein receptor deficient (LDLR\textsuperscript{-/-}) mice. MyD88 deficiency led to profound reductions on AngII-induced AAAs and atherosclerosis in both strains. In addition, the effects of MyD88 on these two vascular pathologies were predominantly mediated by cells of the hematopoietic lineage. Although TLR4 also contributed to AngII-induced AAA formation and atherosclerosis, this TLR was not responsible for the MyD88-dependent signaling in hematopoietic cells that promoted these 2 vascular pathologies.

**Methods**

A detailed description of all methods is presented in the Supplemental Materials, available online at http://atvb.ahajournals.org.

**Experimental Animal Models**

MyD88\textsuperscript{-/-},\textsuperscript{14} TLR2\textsuperscript{-/-}\textsuperscript{,16} or TLR4\textsuperscript{-/-}\textsuperscript{,19} mice were bred to either apoE\textsuperscript{-/-} or LDLR\textsuperscript{-/-} background as described in detail in the Supplemental Materials. AngII (1,000 ng/kg/min) was infused into male mice to develop AAAs and atherosclerosis as described previously.\textsuperscript{4} Bone marrow transplantsations were performed as described previously.\textsuperscript{20} All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

**Genotyping by Polymerase Chain Reaction**

All mice were genotyped by polymerase chain reaction (PCR) as described in detail in the Supplemental Materials (Supplemental Table I).

**Measurements of Vascular Pathologies**

AAAs and atherosclerosis were quantified as described previously.\textsuperscript{21–23}

**Statistics**

All statistical analyses were performed using either version 3.5 of SigmaStat or version 8.2 of SAS. All measurements are represented as the mean\( \pm \)SEM. Analyses were performed using tests that were appropriate for the number of groups and the parametric versus nonparametric nature of the data.

**Results**

**Deficiency of MyD88 Reduced AngII-Induced AAAs and Atherosclerosis**

To determine the contribution of MyD88 to AngII-induced AAAs and atherosclerosis, initial studies were performed in male apoE\textsuperscript{-/-} mice that were either MyD88\textsuperscript{+/+} or \textsuperscript{-/-}. These mice were infused with AngII (1,000 ng/kg/min) for 28 days. The absence of MyD88 profoundly reduced AAA development, with external aortic diameters of 2.0\( \pm \)0.2 mm versus 0.97\( \pm \)0.08 mm in \textsuperscript{+/+} and \textsuperscript{-/-} mice, respectively \((P<0.001; \text{Figure 1A})\). MyD88 deficiency also led to significant reductions in death due to aorta rupture \((P<0.01; \text{Supplemental Table II})\). The absence of MyD88 markedly reduced AngII-induced atherosclerosis in both aortic arches and thoracic aortas (82% and 79% reductions, respectively, \(P<0.001; \text{Figure 1B})\). These reductions in AngII-induced AAAs and atherosclerosis occurred in the absence of MyD88 exerting any effects on plasma cholesterol concentrations, lipoprotein-cholesterol concentrations, or systolic blood pressure (Supplemental Table III and Supplemental Figure I).
TLR2 and TLR4 Deficiencies Exerted Differential Effects on AngII-Induced AAAs and Atherosclerosis

TLR2 signaling occurs via a MyD88-dependent signaling pathway. Therefore, to determine whether TLR2 deficiency mimicked the effects of MyD88 deficiency, male LDLR−/− mice that were either TLR2+/+ or −/− were fed the saturated fat-enriched diet for 5 weeks and also infused with AngII (1,000 ng/kg/min) during the final 4 weeks. Surprisingly, TLR2 deficiency exerted no significant effect on AngII-induced expansion of suprarenal aortic diameter, AAA incidence, or death due to aortic rupture (Figure 2A and Supplemental Table II). Similar to reported effects in hypercholesterolemic mice,16–18 TLR2 deficiency reduced AngII-induced atherosclerosis in both aortic arches and thoracic aortas to a similar extent as observed in MyD88 deficient mice (73% and 87%, respectively, *P<0.001; Figure 2B). TLR2 deficiency had no effect on plasma cholesterol concentrations, systolic blood pressure, or body weight (Supplemental Table III). These data demonstrate that reductions in AAA formation in MyD88 deficient mice are independent of TLR2-MyD88 signaling.

TLR4 signaling is mediated through either MyD88 dependent or independent mechanisms. We determined whether TLR4 deficiency mimicked the effects of MyD88 deficiency on the AngII-induced vascular pathologies. Unlike TLR2 deficiency, but comparable to MyD88 deficiency, TLR4 deficiency nearly ablated AngII-induced increases in external diameters of suprarenal aortas and AAA incidence (P<0.001; Figure 3A and Supplemental Table II). Deficiency of TLR4 in male LDLR−/− mice also resulted in reductions of atherosclerotic lesion size in both aortic arches and thoracic aortas (55% and 66%, respectively, P<0.001; Figure 3B). TLR4 deficiency had no effect on plasma cholesterol concentrations (Supplemental Table III) but a modest effect in reducing systolic blood pressure. Because AngII-induced increases in systolic blood pressure have minimal impact on AAAs and atherosclerosis, these changes in systolic blood pressure are not likely to have contributed to reduced vascular pathologies in TLR4 deficient mice.5,27 These data were generally consistent with TLR4 being the major receptor for MyD88 in the regulation of AngII-induced vascular pathologies.

MyD88 Expression in Bone Marrow-Derived Cells Determined AngII-Induced AAAs and Atherosclerosis Independent of TLR4 in Hematopoietic Cells

Given the critical roles of MyD88 and TLR4 in the development of AngII-induced vascular pathologies, we used bone marrow transplantation to define whether the effect of MyD88 or TLR4 deficiency on AngII-induced AAAs and atherosclerosis was mediated by cells of the hematopoietic lineage. Recipients with MyD88+/+ or −/− donor cells had no change in plasma cholesterol concentrations, systolic blood pressure, body weight, and white blood cells (Supplemental Tables IV and V). At the termination of each experiment, bone marrow was harvested and genotyping was performed to confirm chimerism.

Similar to the results of whole body deletion, deficiency of MyD88 in bone marrow-derived cells significantly attenuated...
suprarenal aortic width (Figure 4A) and atherosclerosis (Figure 4B). AngII infusion into LDLR\(^{-/-}\) mice repopulated with MyD88\(^{-/-}\) bone marrow-derived cells resulted in a 68% incidence of AAAs versus only 9% (\(P<0.001\)) in mice repopulated with MyD88\(^{+/+}\) bone marrow-derived cells. Furthermore, atherosclerotic lesions were significantly attenuated in both aortic arches and thoracic aortas (\(P<0.006\); Figure 4B) of mice repopulated with MyD88\(^{-/-}\) bone marrow-derived cells, compared to those repopulated with MyD88\(^{+/+}\) donor cells.

Chimerism of LDLR\(^{-/-}\) mice repopulated with TLR4\(^{+/+}\) or \(^{-/-}\) donor cells was confirmed by genotyping of bone marrow DNA at the end of the experiment and no effect was found on plasma cholesterol concentrations, systolic blood pressure, and body weight (Supplemental Table IV). Although the overall numbers of WBCs were not changed, there was a small reduction of platelets in mice repopulated with TLR4\(^{-/-}\) donor cells (\(P=0.04\); Supplemental Table V). Surprisingly, TLR4 deficiency in bone marrow-derived cells had no effect on either the expansion of suprarenal aortas (Figure 5A) or the development of atherosclerosis in aortic arches and thoracic aortas (Figure 5B) of LDLR\(^{-/-}\) recipient mice infused with AngII. These data imply that unlike MyD88, the effects of TLR4 on AAAs and atherosclerosis are mediated by nonhematopoietic cells.

**MyD88 Deficiency Attenuated AngII-Induced Monocytosis and Redistribution of Ly-6C Populations**

Chronic AngII infusion for 28 days significantly increased leukocytes, neutrophils, and monocytes (\(P<0.001\)) in peripheral blood of LDLR\(^{-/-}\) mice. Although MyD88\(^{+/+}\) and \(^{-/-}\) mice had no differences on leukocyte numbers prior to AngII infusion, MyD88 deficiency completely attenuated the AngII-induced increases of leukocyte numbers, with equivalent effects on neutrophils and monocytes (Supplemental Figure III). However, TLR4 deficiency did not change leukocyte and its subtype numbers.

Redistribution of Ly-6C populations has been detected in AngII-induced AAAs and implicated in the progression of atherosclerosis in apoE\(^{-/-}\) mice.\(^{28,29}\) Therefore, we investi-
whereas TLR4 deficiency showed no significant effect on macrophage elicitation to peritoneal cavities. All of these findings infer differential effects of MyD88 and TLR4 on monocyte/macrophage behaviors.

We also examined whether AngII had differential effects on MyD88 and TLR4 in smooth muscle cells isolated from the aneurysmal prone suprarenal aortic region. While AngII had no effect on the expression of MyD88 in smooth muscle cells, it greatly increased the abundance of TLR4 mRNA in these cells that was comparable to the TLR4 agonist, lipopolysaccharide (P<0.001; Supplemental Figure VII).

### Discussion

AngII infusion into hypercholesterolemic mice has been used in many studies to induce AAAs and augment the development of atherosclerosis.\textsuperscript{4,8} These diseases are frequently comorbid, and therefore the model permits simultaneous evaluation of both vascular pathologies. Previous studies have demonstrated distinctions between mechanisms that influence the processes of AAAs versus atherosclerosis.\textsuperscript{31–34} In the present study, MyD88 and TLR4 deficiencies had equivalent effects on AngII-induced AAAs and atherosclerosis. However, although TLR2 deficiency reduced AngII-induced atherosclerosis, we were not able to discern any effect on the development of AAAs.

Deficiency of MyD88 was studied on the effects of AngII-induced AAAs in both apoE\textsuperscript{−/−} mice fed a normal laboratory diet and LDLR\textsuperscript{−/−} mice fed a saturated fat-enriched diet. There are marked differences in plasma cholesterol concentrations and lipoprotein characteristics between the 2 strains. There is also evidence that apoE has direct effects on innate and adaptive immune responses independent of lipoproteins, which has the potential to lead to different responses between apoE\textsuperscript{−/−} and LDLR\textsuperscript{−/−} mice.\textsuperscript{24–26} Despite these differences, aneurysms that form during AngII infusion in the 2 strains are similar in size and characteristics. Consistently as demonstrated in this study, deficiency of MyD88 had a similar effect in attenuating AngII-induced AAAs in both strains.

Infusion of AngII leads to medial accumulation of macrophages in regions that are prone to aneurysmal formation.\textsuperscript{35} Following luminal dilation, there is enhanced macrophage accumulation and the presence of other leukocyte types, such as lymphocytes.\textsuperscript{35} Macrophages are the most abundant leukocyte type that infiltrates aortic tissue at all stages of aneurysmal formation.\textsuperscript{35,36} Despite this abundance, the function of macrophages in aneurysmal pathology has not been determined. Our previous study using osteopetrotic mice that have marked reductions in circulating monocytes\textsuperscript{37} was confounded by several defects in this strain and a low incidence of AngII-induced AAAs in the genetic background-matched wild-type mice. A later study demonstrated that substantial reductions of circulating monocytes by clodronate-liposomes decreased severity of AngII-induced AAAs.\textsuperscript{6} In the present study, we found that the striking effect of MyD88 on AAA formation was attenuated by selective deletion of MyD88 in hematopoietic cells, strongly suggesting a role for MyD88 signaling in leukocytes in promoting AAAs. Interestingly, the same effect did not occur for TLR4 deficiency in hematopoietic cells.
etic cells, despite the pronounced attenuation of AAA formation in mice with whole body TLR4 deficiency.

Ly-6C<sup>hi</sup> monocytes are the predominant population of macrophages accumulating in AngII-induced AAAs. We demonstrated that MyD88 deficiency ablated AngII-induced Ly-6C<sup>hi</sup> monocyte switching in both peripheral blood and spleens. These results are consistent with the previous report that AngII mediates monocyte recruitment from the spleen to peripheral tissues after injury. It is possible that MyD88 deficiency blunts macrophage infiltration into the aortic wall via abolishing the ability of AngII-induced Ly-6C<sup>hi</sup> monocyte switching.

AngII exerts its bioactive effects predominantly through binding AT1a receptors. AT1a receptors are ubiquitously present on many cell types including macrophages and resident cell types of the aorta. Comparable to whole body deficiency of AT1a receptors, the whole body deficiency of TLR4 also profoundly reduced AngII-induced AAAs. However, although MyD88 deficiency in bone marrow-derived cells also profoundly reduced AngII-induced AAAs, AT1a receptor deficiency on bone marrow-derived cells failed to influence AngII-induced AAAs. These findings infer that AngII induces changes in cells of nonhematopoietic origins that subsequently promote AAAs through a mechanism based on MyD88 of hematopoietic origin.

The role of MyD88 and its link to TLRs in the development of atherosclerosis have been previously studied in apoE<sup>−/−</sup> mice. Consistent with the previous studies, we demonstrated that deficiency of MyD88 also decreased atherosclerotic lesions that were augmented by AngII infusion in apoE<sup>−/−</sup> mice. We also demonstrated that deficiency of MyD88 had a comparable effect on the reduction of atherosclerosis in LDLR<sup>−/−</sup> mice. The effect of MyD88 deficiency on AngII-induced atherosclerosis occurred in both mouse strains, despite profound differences in both concentrations and characteristics of plasma lipoprotein cholesterol distributions. Our study advances the previous understanding of the role of MyD88 in atherosclerosis by demonstrating that MyD88 in bone marrow-derived cells accounts for the predominant effect of this adaptor protein in the development of atherosclerosis. Deficiency of TLR2 or TLR4 decreases atherosclerosis in both apoE<sup>−/−</sup> and LDLR<sup>−/−</sup> mice. There is compelling evidence for the similarities of the effects of MyD88, TLR2, and TLR4 in the development of atherosclerosis. However, it was unclear whether MyD88 served as a required adaptor signaling molecule for TLR2 or TLR4 to promote atherosclerosis. Our findings using bone marrow transplantation in the present study clearly demonstrate that reductions in atherosclerosis in mice with complete deficiency of TLR4 were attributable to MyD88-independent mechanisms. This conclusion is consistent with the recent demonstration that TRIF deficiency reduced atherosclerosis by a mechanism not involving hematopoietic cells.

In conclusion, this study demonstrates that MyD88 deficiency in hematopoietic cells has a profound effect in reducing AngII-induced AAAs and atherosclerosis. Whereas whole body deficiency of TLR4 had a similar ability to reduce AngII-induced vascular pathologies, the use of bone marrow transplantation clearly demonstrated that these effects were unrelated to MyD88 mediated signaling in hematopoietic cells. Future studies will systematically evaluate other MyD88-linked receptors, such as for interleukin-1β and interleukin-18, in AngII-induced AAA formation and atherosclerosis.

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Disclosures

None.

References


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Supplemental Methods and Data

MyD88 Deficiency Attenuates Angiotensin II-induced Abdominal Aortic Aneurysm Formation Independent of Signaling Through Toll-like Receptors 2 and 4

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MATERIALS AND METHODS

Experimental Animals and Diet

MyD88-/- x apoE-/- mice were obtained from Drs. Freeman and Moore.1 MyD88-/- mice were also bred into LDLR-/- background (N10 to C57BL/6 strain) using mice from The Jackson Laboratory. LDLR-/- mice that were either TLR2+/+ or -/-, or TLR4+/+ or -/- were obtained from Drs. Curtiss and Tobias (Scripps Institute)2 and Dr. Akira (Osaka University).3 All mice were bred as littermate controls, and housed in a pathogen-free barrier facility. All MyD88+/+ and -/- mice were given autoclaved sterile water ad libitum containing sulfatrim (0.2%). ApoE-/- mice were fed a normal laboratory diet throughout experimentation. LDLR-/- mice were fed a diet enriched with saturated fat (milk fat 21% wt/wt) and cholesterol (0.2% wt/wt; Diet number TD.88137, Harlan Teklad) for 1 week prior to AngII infusion and throughout the duration of this infusion. All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Genotyping by Polymerase Chain Reaction

The primers used to genotype mice for apoE, LDLR, MyD88, TLR2, and TLR4 are listed in online Table I. Genotypes were verified by two independent investigators.

Osmotic Minipump Implantation

At 8 to 12 weeks of age, male mice were implanted with Alzet osmotic minipumps (Model 2004, Durect Corporation) subcutaneously into the right flank. Infusion of AngII (1,000 ng/kg/min; Catalog number H-1705, Bachem) continued for 28 days, as described previously.4

Irradiation and Bone Marrow-derived Cell Repopulation

This procedure was performed as described previously.5 Male LDLR-/- mice (6 to 8 weeks old) were lethally irradiated with a total of 900 rads divided into two doses (450 rads/dose, 3 hours apart) from a cesium γ source. Bone marrow-derived cells were harvested from MyD88+/+ or -/- and TLR4+/+ or -/- male mice (6-8 weeks old) and injected into irradiated recipient LDLR-/- mice (1x10^7 donor cells per animal).

Four weeks after irradiation, recipient mice were fed the saturated fat-enriched diet. AngII infusion was started 1 week later, while mice were continuously fed this special diet. At termination, DNA was isolated from bone marrows of the recipient mice and PCR was performed to verify the successful repopulation of donor cells.

Blood Pressure Measurements

Systolic blood pressure (SBP) was measured on conscious mice using either BP-2000 (Visitech Systems) or Coda 8 (Kent Scientific Corp) tail-cuff system as described previously.6 SBP was measured for 5 consecutive days prior to pump implantation, and during the last week of AngII administration.
Aortic Tissue and Plasma Collection
Twenty-eight days after pump implantation, mice were terminated, and blood was drawn from right ventricles for plasma collection. Aortas were perfused with saline, extracted, and placed into formalin (10% wt/vol). These aortas were then carefully cleaned free of adventitia for abdominal aortic aneurysm (AAA) and atherosclerosis analyses.

White Blood Cell Analysis
White blood cells and the subtypes in whole blood were immediately counted at the time of termination on a Hemavet 950 LV veterinary multi-species hematology system (Drew Scientific).

Measurement of Plasma Lipids
Plasma cholesterol concentrations and lipoprotein cholesterol distribution were analyzed as described previously.7

Measurements of Abdominal Aortic Aneurysms
Maximal external diameters of suprarenal aortas were measured to define AAAs as described previously.8

Quantification of Atherosclerosis
Atherosclerotic lesions were measured on the intimal surface of aortic arches and thoracic aortas using an en face technique, as described previously.9,10

Histology and Immunostaining
Abdominal aortas or spleens were embedded in OCT and sectioned in a cryostat. Staining of hematoxylin and eosin was performed in aortic sections. Immunostaining of CD68 was determined in sections of spleens using a rat anti-CD68 antibody (FA-11; Catalog number MCA1957, AbD Serotec), as described previously.11

Blood Monocyte and Gr-1/Ly-6C Determination with Flow Cytometry
MyD88-/- x LDLR-/-, TLR4-/- x LDLR-/-, and LDLR-/- mice (n=8/genotype) were fed the saturated fat-enriched diet for 3 weeks (started 1 week prior to AngII infusion) and infused with AngII for 2 weeks. Peripheral blood was collected into EDTA (1.8 mg/ml) at the termination and mononuclear cells were isolated via lysis of red blood cells with an ammonium chloride solution. In a separate experiment, spleens from MyD88+/+ x LDLR-/- and MyD88-/- x LDLR-/- male mice were harvested (n=4/genotype) and split into 4 equal sections. All sections were incubated in RPMI 1640 with fetal bovine serum (10% vol/vol), glucose (1% wt/vol), penicillin/streptomycin (1% vol/vol of each), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 1mM), and sodium pyruvate (1 mM) with either saline or AngII (1 μM) for 24 hours. Subsequently, splenocytes were isolated by crushing the spleen sections into 100 μM cell strainers and all red blood cells were lysed using the ammonium chloride solution. Peripheral blood and splenic monocyte populations were determined via fluorescence-activated-cell sorting (FACSTM)
on a Becton Dickson LSRII using antibodies against CD115 (phycoerythrin labeled; Catalog number 12-1162-83, eBioscience), F4/80 (biotinylated; Catalog number MCA497B, AbD Serotec), and Gr-1 (PerCp-Cy5.5 labeled; Catalog number 552093, BD Pharmingen). The biotinylated antibody was detected using streptavidin-APC (eBioscience). Monocyte populations were determined as being CD115 and F4/80 double positive. These double positive monocytes were then classified as Ly-6C low (Ly-6C\textsuperscript{low}), intermediate (Ly-6C\textsuperscript{int}), and high (Ly-6C\textsuperscript{hi}), as described previously.\textsuperscript{12}

**Thioglycollate Elicitation of Peritoneal Macrophages**

MyD88\textsuperscript{-/-} x LDLR\textsuperscript{-/-} (n=10), TLR4\textsuperscript{-/-} x LDLR\textsuperscript{-/-} (n=5), and LDLR\textsuperscript{-/-} (n=10) mice were fed the saturated fat-enriched diet for 1 week and subsequently injected intraperitoneally with thioglycollate broth medium (4% wt/vol). Four days after injection, mice were killed, and peritoneal macrophages were extracted, as described previously.\textsuperscript{13} Red blood cells were lysed using the ammonium chloride solution. Cell numbers were calculated using a hemacytometer and normalized to CD68 positive cell population, as stained with a fluorescein isothiocyanate-labeled CD68 antibody (Catalog number MCA1957F, AbD Serotec) and analyzed via flow cytometry.

**Real-time Polymerase Chain Reaction**

Vascular smooth muscle cells (VSMCs) were harvested from aortas of C57BL/6 mice as described previously.\textsuperscript{14,15} These cells were incubated with saline (24 hours), AngII (1 \textmu M for 24 hours), or lipopolysaccharide (1 \textmu g/ml for 6 hours; Invitrogen). Cells were washed and RNA was harvested using the Qiagen All Prep RNA/protein kit. All samples were incubated with Turbo DNA-free (Ambion) to remove DNA contamination. Real-time PCR was performed as described previously.\textsuperscript{16} Taqman probes were utilized for detection of TLR4 (AB catalog Mm00445273_m1) and MyD88 (AB catalog Mm00440339_g1). Real-time PCR of 18S rRNA was used as the endogenous control (Catalog number 4352930E, Applied Biosystems). Negative controls were performed as described previously.\textsuperscript{16}

**Statistics**

All statistical analyses were performed using either version 3.5 of SigmaStat (SPSS Inc.) or version 8.2 of SAS (SAS Institute). All measurements are represented as the mean±SEM. Two-group comparisons on quantitative variables were performed parametrically (Student’s t test) or nonparametrically (Mann-Whitney Rank Sum test) according to the results of preliminary tests for within-group normality and equality of variances. SBP data were analyzed by fitting a linear mixed model with explanatory variables of genotypes and time (prior to and during AngII infusion) along with random effects for individual mice. One Way ANOVA was performed, where indicated, with an appropriate post hoc test. Values with \( P<0.05 \) were considered statistically significant.
<table>
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<th>Gene</th>
<th>Primers</th>
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<tr>
<td>ApoE</td>
<td>5'-GCCGCCCCGACTGCATCT 5'-TGTGACTTGGAGCTGAGC 5'-GCCTAGCCGAGGGAGGAGCCG</td>
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<td>LDLR</td>
<td>5'-AGGTGAGATGACAGGAGATC 5'-AGGATGACTTCCGATGCCAG 5'-GCAGTGCTCCTCATCTGACTTG</td>
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<td>MyD88</td>
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Table II. Incidence and Rupture Rate of AngII-induced AAAs in Whole Body Deficient Mice and their Relative Wild Type Controls

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<th>Mouse Genotype</th>
<th>Number of Mice</th>
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<td></td>
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<tr>
<td>MyD88&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>42</td>
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<td>1/27 (4%)*</td>
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<td>LDLR&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>TLR2&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>9</td>
<td>8/9 (89%)</td>
<td>2/9 (22%)</td>
</tr>
<tr>
<td>TLR2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>11</td>
<td>9/11 (82%)</td>
<td>4/11 (36%)</td>
</tr>
<tr>
<td>TLR4&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>10</td>
<td>14/14 (100%)</td>
<td>4/14 (29%)</td>
</tr>
<tr>
<td>TLR4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>20</td>
<td>5/20 (25%)*</td>
<td>4/20 (20%)</td>
</tr>
</tbody>
</table>

* denotes \( P < 0.01 \) for comparisons of MyD88<sup>−/−</sup> or TLR4<sup>−/−</sup> versus their relative wild type controls using Fisher Exact Test.
### Table III. Characteristics of Study Mice with Genotypes of MyD88, TLR2, or TLR4

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Number of Mice</th>
<th>Plasma Cholesterol Concentration (mg/dL)</th>
<th>Systolic Blood Pressure (mmHg)</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Baseline</td>
<td>AngII Infusion</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>MyD88+/+</td>
<td>42</td>
<td>320 ± 32</td>
<td>112 ± 5</td>
</tr>
<tr>
<td></td>
<td>MyD88−/−</td>
<td>27</td>
<td>306 ± 23</td>
<td>116 ± 3</td>
</tr>
<tr>
<td>LDLR−/−</td>
<td>MyD88+/+</td>
<td>19</td>
<td>1174 ± 57</td>
<td>127 ± 2</td>
</tr>
<tr>
<td></td>
<td>MyD88−/−</td>
<td>23</td>
<td>1250 ± 72</td>
<td>135 ± 10</td>
</tr>
<tr>
<td></td>
<td>TLR2+/+</td>
<td>9</td>
<td>1334 ± 57</td>
<td>115 ± 2</td>
</tr>
<tr>
<td></td>
<td>TLR2−/−</td>
<td>11</td>
<td>1275 ± 62</td>
<td>118 ± 4</td>
</tr>
<tr>
<td></td>
<td>TLR4+/+</td>
<td>14</td>
<td>1257 ± 62</td>
<td>142 ± 2†</td>
</tr>
<tr>
<td></td>
<td>TLR4−/−</td>
<td>19</td>
<td>1180 ± 50</td>
<td>131 ± 2</td>
</tr>
</tbody>
</table>

* denotes $P<0.001$ for comparisons of AngII infusion versus baseline within genotypes using repeated measures analysis with Bonferroni post hoc test.
† denotes $P=0.005$ for comparisons of MyD88−/− versus +/+ using Mann-Whitney Rank Sum test.
‡ denotes $P=0.03$ for comparisons of TLR4+/+ versus −/− at baseline and AngII-infusion, respectively, using two-way ANOVA with repeated measures.
Table IV. Characteristics of LDLR<sup>−/−</sup> Mice that were Chimeric for either MyD88 or TLR4

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Number of Mice</th>
<th>Plasma Cholesterol Concentrations (mg/dL)</th>
<th>Systolic Blood Pressure (mmHg)</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Baseline</td>
<td>AngII Infusion</td>
</tr>
<tr>
<td>MyD88&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>22</td>
<td>982 ± 63</td>
<td>122 ± 5</td>
<td>165 ± 15*</td>
</tr>
<tr>
<td>MyD88&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>22</td>
<td>831 ± 53</td>
<td>126 ± 3</td>
<td>154 ± 5*</td>
</tr>
<tr>
<td>TLR4&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>22</td>
<td>963 ± 65</td>
<td>123 ± 4</td>
<td>154 ± 6*</td>
</tr>
<tr>
<td>TLR4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>23</td>
<td>977 ± 58</td>
<td>126 ± 3</td>
<td>152 ± 6*</td>
</tr>
</tbody>
</table>

* denotes P<0.001 for comparisons of AngII infusion versus baseline within genotypes using repeated measures analysis with Bonferroni post hoc test.
Table V. Cell Numbers in Peripheral Blood of LDLR⁻/⁻ Mice that were Chimeric for either MyD88 or TLR4

<table>
<thead>
<tr>
<th>Genotype of Donors</th>
<th>Number of Mice</th>
<th>Cell Number (10³ cells/µl)</th>
<th>White Blood Cells</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyD88⁺/⁺</td>
<td>22</td>
<td></td>
<td>3.42 ± 0.3</td>
<td>1.57 ± 0.3</td>
<td>1.54 ± 0.3</td>
<td>0.15 ± 0.02</td>
<td>663 ± 43</td>
</tr>
<tr>
<td>MyD88⁻/⁻</td>
<td>22</td>
<td></td>
<td>4.09 ± 0.5</td>
<td>1.26 ± 0.2</td>
<td>2.48 ± 0.4</td>
<td>0.19 ± 0.03</td>
<td>598 ± 63</td>
</tr>
<tr>
<td>TLR4⁺/⁺</td>
<td>22</td>
<td></td>
<td>4.25 ± 0.5</td>
<td>1.19 ± 0.1</td>
<td>2.63 ± 0.3</td>
<td>0.26 ± 0.03</td>
<td>661 ± 48</td>
</tr>
<tr>
<td>TLR4⁻/⁻</td>
<td>23</td>
<td></td>
<td>4.12 ± 0.5</td>
<td>1.25 ± 0.2</td>
<td>2.40 ± 0.3</td>
<td>0.23 ± 0.03</td>
<td>507 ± 44*</td>
</tr>
</tbody>
</table>

* denotes $P=0.04$ for comparisons of TLR4⁻/⁻ versus⁺/⁺ by Mann-Whitney Rank Sum analysis.
Figure I. Lipoprotein cholesterol distribution determined by size exclusion chromatography. (A) ApoE<sup>-/-</sup> and (B) LDLR<sup>-/-</sup> mice. Symbols represent means of 5-10 plasma samples from individual mice, and bars are SEMs.
Figure II. Examples of aortas from AngII-infused MyD88^{+/+} and ^{}^-/- mice in an LDLR^{+/+} background. AngII infusion into MyD88^{+/+} mice promoted elastin rupture in the suprarenal aortic region and led to profound luminal expansion. In contrast, aortas maintained normal appearance during AngII infusion in MyD88^-/- mice. The upper panels show entire aortas and magnified images of AAA-prone region. The lower panels show hematoxylin and eosin-stained tissue sections from the suprarenal aortas. Tissue sections were derived approximately from the regions noted with purple lines, respectively. Red line indicates the location of the last intercostal arteries.
**Figure III.** MyD88 deficiency attenuated AngII-induced leukocytosis. White blood cells (WBCs), neutrophils (Neut), and monocytes (Mono) in peripheral blood were counted in AngII-infused MyD88^+/+ (n=19) and ^-/- (n=23) mice (LDLR^- background) on a Hemavet analyzer. * denotes \( P<0.01 \) for comparisons of MyD88^-/- versus ^+/+ by Mann-Whitney Rank Sum analysis.
Figure IV. **MyD88 deficiency ablated AngII-induced redistribution of Ly-6C monocyte populations in peripheral blood.** Peripheral blood monocytes (CD115+ and F4/80+) were divided into Ly-6C low (Ly-6C<sub>low</sub>), Ly-6C intermediate (Ly-6C<sub>int</sub>), and Ly-6C high (Ly-6C<sub>hi</sub>) populations in MyD88<sup>+/+</sup> (n=8/group for saline and AngII) or <sup>-/-</sup> (n=8/group for saline and AngII) mice in an LDLR<sup>−/−</sup> background. * denotes P<0.001 for comparisons with MyD88<sup>+/+</sup> saline infusion, MyD88<sup>-/-</sup> saline infusion, and MyD88<sup>-/-</sup> AngII infusion, respectively, within Ly-6C monocyte populations by one way ANOVA on Ranks.
Figure V. MyD88 deficiency ablated AngII-induced redistribution of Ly-6C monocyte populations in spleen. Monocytes (CD115+ and F4/80+) in spleens were divided into Ly-6C low (Ly-6C\text{low}) , Ly-6C intermediate (Ly-6C\text{int}), and Ly-6C high (Ly-6C\text{hi}) populations in MyD88\text{+/+} or \text{--} (n=4/genotype) mice in an LDLR\text{--} background. * denotes \textit{P}<0.001 for comparisons with MyD88\text{+/+} saline, MyD88\text{--} saline, and MyD88\text{--} AngII, respectively, within Ly-6C monocyte populations by one way ANOVA on ranks.
Figure VI. **MyD88 deficiency attenuated macrophage elicitation.** Thioglycollate-elicited CD68⁺ peritoneal macrophages were quantified in wild type (WT; n=10), MyD88⁻/⁻ (n=10), and TLR4⁻/⁻ (n=5) mice in an LDLR⁻/⁻ background. * denotes $P<0.001$ for comparisons with WT and TLR4⁻/⁻ groups.
Figure VII. **AngII increased mRNA abundance of TLR4 but not MyD88 in SMCs.**
mRNA abundance of MyD88 and TLR4 in SMCs isolated from suprarenal aortic regions of C57BL/6 male mice were determined by real-time PCR. Lipopolysaccharide (LPS; 1 μg/ml) was used as TLR4 agonist. * denotes $P<0.001$ for comparisons with cells incubated with saline.
References


