Dual Activation of TRIF and MyD88 Adaptor Proteins by Angiotensin II Evokes Opposing Effects on Pressure, Cardiac Hypertrophy, and Inflammatory Gene Expression

Madhu V. Singh, Michael Z. Cicha, David K. Meyerholz, Mark W. Chapleau, François M. Abboud

Abstract—Hypertension is recognized as an immune disorder whereby immune cells play a defining role in the genesis and progression of the disease. The innate immune system and its component toll-like receptors are key determinants of the immunologic outcome through their proinflammatory response. Toll-like receptor–activated signaling pathways use several adaptor proteins of which adaptor proteins myeloid differentiation protein 88 (MyD88) and toll-interleukin receptor domain–containing adaptor protein–inducing interferon-β (TRIF) define 2 major inflammatory pathways. In this study, we compared the contributions of MyD88 and TRIF adaptor proteins to angiotensin II (Ang II)–induced hypertension and cardiac hypertrophy in mice. Deletion of MyD88 did not prevent cardiac hypertrophy and the pressor response to Ang II tended to increase. Moreover, the increase in inflammatory gene expression (Tnfa, Nos2, and Agtr1a) was significantly greater in the heart and kidney of MyD88-deficient mice when compared with wild-type mice. Thus, pathways involving MyD88 may actually restrain the inflammatory responses. However, in mice with nonfunctional TRIF (Trif−/− mice), Ang II–induced hypertension and cardiac hypertrophy were abrogated, and proinflammatory gene expression in heart and kidneys was unchanged or decreased. Our results indicate that Ang II induces activation of a proinflammatory innate immune response, causing hypertension and cardiac hypertrophy. These effects require functional adaptor protein TRIF-mediated pathways. However, the common MyD88-dependent signaling pathway, which is also activated simultaneously by Ang II, paradoxically exerts a negative regulatory influence on these responses. (Hypertension. 2015;66:647-656. DOI: 10.1161/HYPERTENSIONAHA.115.06011.)

Key Words: angiotensin II ▪ gene expression ▪ hypertension ▪ MyD88 protein ▪ TICAM-1 protein ▪ toll-like receptors

In recent years, hypertension has increasingly been recognized as an immunologic disorder, whereby the components of the immune system play an important role in determining 2 key aspects of the disease: blood pressure and end organ damage.1-4 Greater understanding of the immune system itself has accelerated the progress in defining the cell populations of the adaptive immune system that play a role in hypertension5-15 and the neuroimmune axis that may activate these cells.16-20 More recently, the innate immune system, specifically the toll-like receptors (TLRs), has been shown to be the mediators of neuroinflammatory response that can be modulated by angiotensin II (Ang II).16,17,19 The goal of this study was to determine the innate immune mechanisms involved in Ang II–induced hypertension and cardiac hypertrophy.

Ang II is a major product of the renin–angiotensin system that plays a crucial role in the pathology and treatment of hypertension. It causes vasoconstriction, promotes sodium retention in the kidneys, acts as a trophic factor in the myocardium, activates the sympathetic nervous system, and induces a proinflammatory state that results in organ damage.21-24 Ang II promotes inflammation by inducing cytokine release, immune cell infiltration in the kidney, expression of genes in kidneys and the heart that are proinflammatory, profibrotic, and profibrotic, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, and transforming growth factor-β.1,25

TLRs are a major class of innate immune receptors that constitute a diverse family of pattern recognizing receptor. They bind to exogenous pathogen-associated molecular patterns or endogenous damage-associated molecular patterns to initiate intracellular signaling cascades culminating in expression of proinflammatory genes as well as to regulate the activity of adaptive immune cells.26 TLR signaling is dependent on MyD88 but not TRIF.27-29 MyD88 is the major pathway used for Ang II induction of proinflammatory cytokine gene expression.30,31 MyD88-dependent induction of cytokine gene expression is important for the development of hypertension and cardiac hypertrophy.32,33 However, deletion of MyD88 did not prevent Ang II–induced hypertension, but the pressor response to Ang II showed a trend toward an increase.34 MyD88 does not appear to be required for Ang II–induced cardiac hypertrophy.34,35 It is possible that the pressor response to Ang II is not affected because the rats used in that study did not develop hypertension.34 MyD88 deficiency has been shown to slow down the development of Ang II–induced hypertension in mice.34 In a recent study, we compared the contributions of MyD88 and TRIF adaptor proteins to Ang II–induced hypertension and cardiac hypertrophy in mice. Deletion of MyD88 did not prevent cardiac hypertrophy and the pressor response to Ang II tended to increase. Moreover, the increase in inflammatory gene expression (Tnfa, Nos2, and Agtr1a) was significantly greater in the heart and kidney of MyD88-deficient mice than in wild-type mice. Thus, pathways involving MyD88 may actually restrain the inflammatory responses. However, in mice with nonfunctional TRIF (Trif−/− mice), Ang II–induced hypertension and cardiac hypertrophy were abrogated, and proinflammatory gene expression in heart and kidneys was unchanged or decreased. Our results indicate that Ang II induces activation of a proinflammatory innate immune response, causing hypertension and cardiac hypertrophy. These effects require functional adaptor protein TRIF-mediated pathways. However, the common MyD88-dependent signaling pathway, which is also activated simultaneously by Ang II, paradoxically exerts a negative regulatory influence on these responses. (Hypertension. 2015;66:647-656. DOI: 10.1161/HYPERTENSIONAHA.115.06011.)
on the association of TLRs with one of several adaptor proteins that determine the specificity of signaling. Activation of TLRs often initiates 2 major adaptor-dependent signaling pathways: one is myeloid differentiation protein 88 (MyD88) dependent and the other is toll interleukin receptor domain-containing adaptor molecule 1, also known as toll interleukin receptor domain-containing adaptor–inducing interferon-β (TRIF) dependent. Besides their role in induction of proinflammatory genes, signaling by different TLRs may be complementary, synergistic, or even antagonistic resulting in complex phenotypes and gene expression patterns.

We have previously shown a link between TLR/MyD88 signaling and myocardial infarction–induced cardiac hypertrophy. Mice lacking MyD88 adaptor protein had significantly improved survival and reduced inflammation, cardiac fibrosis, and cardiac hypertrophy after myocardial infarction. Prompted by these results we tested the hypothesis that TLR/MyD88 signaling is essential in Ang II–induced hypertension, cardiac hypertrophy, and end organ inflammation.

Our results revealed a selective and paradoxical dependence of Ang II hypertension responses on the 2 dominant adaptor proteins of the innate immune pathways TRIF and MyD88. Whereas TRIF-dependent pathways are essential for Ang II–mediated hypertension, cardiac hypertrophy, and the inflammatory response, MyD88 pathways, in contrast, seem to be anti-inflammatory and restrain those responses.

### Materials and Methods

#### Animals

All experiments with animals were done in accordance with the regulations put forth by the Institutional Animal Care and Use Committee of University of Iowa. The wild-type C57BL/6J (WT), and Trifmut (C57BL/6J-Ticam1<sup>−/−</sup>) mice were obtained from Jackson Laboratories. MyD88<sup>−/−</sup> mice were obtained from Dr Shizuo Akira’s group and were bred in the University of Iowa animal facility in specific pathogen-free environment. All mice were in C57BL/6 background.

#### Ang II Infusion and Tail-Cuff Recordings of Blood Pressure

Mini-osmotic pumps (Alzet model 1004, 0.11 μL/h, 28 days), containing saline or Ang II (730, 1000, or 3000 ng/kg per minute), were inserted subcutaneously in mice under anesthesia (2.0%–2.5% isoflurane). Male mice aged 8 to 12 weeks were used for the experiments.

Systolic arterial blood pressure was measured by tail cuff using the Visitech-2000 system. A rigorous system of recording tail-cuff pressures has been in effect in our laboratories over decades and found to be reliable for steady-state blood pressure measurements for long periods of time.

Baseline blood pressure was measured before pump insertion. Tail-cuff recordings were performed at the same time of the day (before noon) to avoid diurnal changes in the pressure. To acclimatize the mice to the procedure, 1 week before the start of the experiments, tail-cuff pressures were measured on 2 days. During the experiments, 20 tail-cuff measurements for each mouse were recorded for each session (3x a week). Average measurements for each mouse were then used for the groups (n=3 mice per group). At the end of the experiments (3 weeks), mice were euthanized and organs were obtained for further analyses.

#### Cardiac Hypertrophy

Cardiac hypertrophy was assessed by measurement of heart weight/body weight ratio in hearts from mice after 3 weeks of saline or Ang II infusion. For measurement of cardiomyocyte diameter and cardiac fibrosis, heart tissues were examined from 4 separate cross-sections collected at different levels (each 250 μm apart) from the center of the heart. In each tissue sample, a pathologist using postexamination masking techniques screened each heart on low magnification to identify areas with the largest cardiomyocytes diameter. At these sites, the 5 largest cardiomyocyte diameters were recorded. Thus, for each heart, 20 diameters were collected and the mean of these data was calculated for each animal.

#### RNA Isolation and Reverse Transcriptase Quantitative Polymerase Chain Reaction

Total RNA from mouse heart and kidneys were isolated using mirVana RNA isolation kit (Ambion) or RNeasy RNA Isolation Kit (Qiagen). A 2-μg aliquot of RNA sample was used to synthesize cDNA in 50-μL reactions using Oligo (dT) as primers and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction was performed on quantitative polymerase chain reaction cycler (Applied Biosystems) using SYBR green-based polymerase chain reaction reactions, as described. Quantifications were done using ΔΔC<sub>t</sub> method where Gapdh was used as a reference gene for normalization of RNA expression.

The primers used in this study have been previously described and are listed in Table S1 in the online-only Data Supplement.

#### Statistical Analyses

Statistical analyses were performed using an unpaired t test or ANOVA as shown in respective figures. Post-hoc analyses on multiple group data were done using Tukey test. A P value of ≤0.05 was considered to be statistically significant. All results are presented as mean±SEM.

### Results

#### Ang II Infusion Increases Blood Pressure in MyD88<sup>−/−</sup>, but Not in Trifmut Mice

When compared with saline infusions, which did not alter systolic blood pressure (SBP) for 3 weeks, Ang II infusion (3000 ng/kg per minute) during the same period increased SBP in both WT and in MyD88<sup>−/−</sup> mice (Figure 1A and 1B). In WT, blood pressure peaked at 147±4 mm Hg (Figure 1A), whereas in Ang II–infused MyD88<sup>−/−</sup> mice SBP reached a peak value of 163±6 mm Hg (Figure 1B). The increase in SBP during the third week of infusion was significantly greater in MyD88<sup>−/−</sup> mice than in WT mice (P<0.05). In contrast to the WT and MyD88<sup>−/−</sup> mice, Ang II did not increase blood pressure in Trifmut mice and the response to Ang II was identical to the response to saline during the final 2 weeks of infusion averaging 116±3 mm Hg with saline and 118±4 mm Hg with Ang II (Figure 1C).

In addition, at lower Ang II dosage (730–1000 ng/kg per minute) in a separate group of studies, the average SBP for the final 2 weeks of infusion of Ang II was significantly lower in Trifmut (129±3) versus WT (135±5) and highest in MyD88<sup>−/−</sup> (142±5 mm Hg; Figure S1).

These results indicate that Ang II–induced hypertension is MyD88-independent and that the presence of MyD88 restrains the hypertensive response to Ang II, which is TRIF dependent.

#### Ang II–Induced Cardiac Hypertrophy Is Abrogated in Trifmut Mice

The heart weight/body weight ratio in WT mice infused with saline, low dose (730 ng/kg per minute), and high dose (3000 ng/kg per minute) of Ang II was 4.88±0.16,
5.23±0.15, and 6.83±0.21 mg/g, respectively (Figure 2A). Corresponding values in MyD88−/− mice were 3.93±0.15, 4.70±0.06, and 6.30±0.86 (Figure 2B) and in Trifmut mice they were 4.39±0.19, 4.72±0.11, and 5.10±0.29 (Figure 2C). The increase in heart weight/body weight ratio with the high dose Ang II versus saline was greater in MyD88−/− than in WT but lowest in Trifmut. The high dose of Ang II infusion induced a 60% increase in heart weight/body weight ratio over saline infusions in MyD88−/− mice, a 40% increase in WT, and a 22% increase in Trifmut (Figure 2). ANOVA and linear regressions confirming the differences in the 3 genotypes are provided in Table S2. Cardiomyocyte diameter and fibrosis confirmed Ang II–induced cardiac structural changes (Table S3).

Cardiac Gene Expression in Ang II–Infused Mice

We compared the expression of inflammation-related genes in the hearts from saline and Ang II–infused WT, MyD88−/−, and Trifmut mice. Tnfa expression was significantly increased by Ang II in WT and MyD88−/− mice, but the increase was much greater in the MyD88−/− hearts than in WT hearts (Figure 3A and 3B). However, Ang II infusion had no effect on Tnfa expression in Trifmut hearts (Figure 3C).

Expression of NADPH oxidase-4 (Nox4) was significantly increased to a similar extent in hearts from WT and MyD88−/− mice with Ang II infusions (Figure 3D and 3E). However, Nox4 expression was unaffected in Trifmut hearts on Ang II infusion (Figure 3F).

The angiotensin type 1-receptor (AT1 receptor [Agtr1a]) expression in heart was unaffected by Ang II infusion in WT mice (Figure 3G), was significantly increased by Ang II in MyD88−/− mice (Figure 3H), and was slightly decreased by Ang II in Trifmut mice (Figure 3I). Similarly, the expression of Cxcl10 a gene that is selectively induced by the TRIF/interferon pathway was enhanced in MyD88−/− supporting a negative regulatory effect of MyD88 on the TRIF pathway. Expression of Cxcl10 was actually decreased in Trifmut mice (Figure S2A).

The muted effect of Ang II infusion on cardiac gene expression was not because of a general unresponsiveness of Trifmut mice because Ang II induced significant overexpression of skeletal muscle actin-α1 (Acta1) in all 3 strains of mice and particularly in Trifmut (Figure S2B). Thus, the intact MyD88 pathway in WT mice has a restraining inhibitory effect on Ang II–induced inflammatory gene expression, whereas the intact TRIF pathway is required for the inflammatory effect of Ang II.
Renal Gene Expression in Ang II-Infused Mice

We compared gene expression in the kidneys of saline and Ang II–infused mice. Ang II infusion resulted in an insignificant increase in expression of Tnfa in the kidneys of WT mice (Figure 4A); however, there was a clear and significant increase in Tnfa in kidneys of MyD88−/− mice (Figure 4B), and a much smaller negligible increase in kidneys of Trifmut mice (Figure 4C).

Ang II infusion did not increase expression of Nox4 in the kidneys of WT mice but significantly increased the expression in kidney of MyD88−/− mice (Figure 4D and 4E) and, conversely, significantly decreased Nox4 in kidney of Trifmut mice (Figure 4F).

The renal expression of Agtr1a was unaltered in kidney of Ang II–infused WT (Figure 4G), significantly increased in kidney of MyD88−/− mice (Figure 4H), but significantly decreased in kidney of Trifmut mice (Figure 4I).

Thus, the absence of MyD88 caused a greater magnitude of proinflammatory gene expression with Ang II in both heart and kidney. In contrast, in the absence of TRIF, the inflammatory gene overexpression was eliminated or even reduced when compared with WT, confirming the inhibitory effect of the MyD88 pathway and the TRIF dependence of the inflammatory response.

Changes in Cardiac TLR and Adaptor Protein Expressions in MyD88−/− and Trifmut Mice

Because TLR3 and TLR4 are likely to be involved in signaling pathways that use the 2 adaptor proteins, we measured their cardiac expressions in MyD88−/− and Trifmut. The results in Figure 5 indicate that deletion of MyD88 was associated with an increase in Trif expression, which along with increases in both Tlr3 and Tlr4 expression must have contributed to the enhanced inflammatory gene expressions observed in MyD88−/− mice. In Trifmut mice, expression of MyD88 was increased, whereas Tlr4 expression was decreased and Tlr3 expression was unchanged. Together, the negative regulatory effect of MyD88 and decreased Tlr4 expression must have contributed to the suppressed inflammatory gene expression in Trifmut.

Discussion

We tested the contribution of the innate immune signaling by the 2 major adaptor proteins of TLRs, MyD88 and TRIF,
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Renal Gene Expression

in Ang II–induced hypertension. Consistent with our earlier demonstration that the innate immune system plays a major role in a genetic model of hypertension, our results show that pathways involving both adaptor proteins contributed to Ang II pressor responses in WT mice, albeit in a mutually opposite manner.

Figure 4. Comparison of proinflammatory gene expression in kidneys of (A, D, and G) wild-type (WT), (B, E, and H) MyD88−/−, and (C, F, and I) Trifmut after chronic infusion (3 weeks) of saline or low dose angiotensin (Ang) II. Fold change in RNA expression was measured by ∆∆CT method using Gapdh RNA as a reference. Values in graphs were normalized to measurements in saline-infused mice (n≥3 each group; *P<0.05). Ang II–induced increase in gene expressions was enhanced in MyD88−/− and reduced in Trifmut when compared with WT. Agtr1a indicates angiotensin receptor type 1a; Nox4, NADPH oxidase 4; and Tnfa, tumor necrosis factor-α.

Figure 5. Baseline RNA expression of toll-like receptor (TLR) 3, TLR4, and their adaptor proteins myeloid differentiation protein 88 (MyD88) and toll-interleukin receptor domain–containing adaptor protein–inducing interferon-β (TRIF) in the hearts of wild-type (WT) vs MyD88−/− and WT vs Trifmut mice. Comparisons of RNA expressions between WT and MyD88−/− or WT and Trifmut hearts were done by quantitative polymerase chain reaction and ∆∆CT method. Statistical significance was tested by unpaired t test. Asterisks show significant differences from WT (P<0.05). In MyD88−/− the expressions of Trif, Tlr3, and Tlr4 were increased. In Trifmut, the expression of MyD88 was increased, Tlr4 was decreased, and Tlr3 unchanged.
Our results show that (1) MyD88-dependent innate immune signaling pathways are not responsible for Ang II–induced hypertension, cardiac hypertrophy, and proinflammatory gene expression, (2) TRIF-mediated pathways, however, are the essential determinants of Ang II–induced responses, and (3) the absence of MyD88 unmasks exaggerated Ang II responses indicating that MyD88 signaling is simultaneously activated by Ang II and functions as a negative regulator of proinflammatory pathological responses mediated by TRIF. These results are summarized in the schematic in Figure 6. We arrived at an original and provocative finding that TRIF-dependent pathways are essential and proinflammatory in evoking Ang II hypertension, whereas MyD88-dependent pathways are negative regulators of TRIF pathways and therefore may be considered anti-inflammatory. These results provide novel mechanistic insight in one of the most commonly used models of hypertension and identify putative targets of pathological and therapeutic significance.

**Dominance of the Immune System in Ang II Responses**

Ang II is the major product of the renin–angiotensin system. It plays a major role in cardiovascular regulation of arterial pressure and blood volume, salt retention, and sympathetic activation. It adversely alters the course of several pathological states including hypertension, heart failure, atherosclerosis, and the metabolic syndrome.

The contribution of Ang II to hypertension has long been ascribed to its powerful vasoconstrictor action, its salt retaining properties and its central and peripheral sympathoexcitatory effects. A seminal notion was advanced by the work of Harrison and colleagues that Ang II–induced hypertension is dependent on the immune system. A complementary finding has been our previous results that in a genetic hypertension model, the spontaneously hypertensive rat, Ang II dramatically enhances cytokine release (IL-6 and IL-1β) by splenocytes on their activation by ligands of TLR 7, 8, and 9. That the proinflammatory effect of Ang II was evident in spontaneously hypertensive rat splenocytes before the onset of hypertension suggests a genetic abnormality of the innate immune cells that links their AT1 receptors to the TLR signaling pathways and potentiates proinflammatory responses. More recently, Harrison’s group has also identified an important role of the innate immune system, specifically the dendritic cells, in Ang II hypertension.

The processes that mediate cardiac hypertrophy in response to Ang II have been ascribed to the mechanical afterload of a raised arterial pressure and also to trophic effects on cardiac myocytes with overexpression of contractile proteins by direct activation of AT1 receptors or indirectly by the adrenergic stimulus of excessive sympathetic activation. However, in a manner similar to the Ang II pressor response, the trophic cardiac effect seems to be also mediated by immunologic pathways.

**Ang II Hypertension and Cardiac Hypertrophy Are MyD88 Independent but TRIF Dependent**

An unexpected finding of this study was that MyD88, a widely recognized essential adaptor protein for the activation of proinflammatory TLR pathways, negatively regulated the proinflammatory TRIF-mediated response to Ang II. In fact, we had previously reported a beneficial reduction of cardiac hypertrophy in the MyD88−/− mice after myocardial infarction. Similarly, Wang et al reported a reduction in...
hypertrophy in MyD88−/− mice on Ang II infusion. However, they used a subpressor dose of Ang II (∼400 ng/kg per minute). In our present study, our model was primarily a model of hypertension using Ang II infusions at high pressor rates (3000 ng/kg per minute) and the resulting hypertension, cardiac hypertrophy, and particularly the proinflammatory gene expressions were exaggerated.

These doses of Ang II cause significant vasoconstriction, neurohumoral, and central nervous system activation with increased sympathetic activity in addition to the renal and cardiac effects. Thus, the differences in results may represent the release of different endogenous ligands that may engage different adaptor mediated pathways. Furthermore, a recent abstract42 indicates that the hypertension and hypertrophy caused by Ang II infusions (1000 ng/kg per minute) were preserved in MyD88−/− mice. This study’s conclusion was in accordance with ours that MyD88 does not contribute to hypertension.

Contrasting Effects of Signaling Through TRIF Versus MyD88 Adaptors on Gene Expression in Heart and Kidney

We observed contrasting changes in proinflammatory gene expression in MyD88−/− and Trifmut mice when compared with WT mice. Tnfα is a key cytokine in Ang II–induced increases in blood pressure and cardiac hypertrophy.38,39,43,44 We found that in both heart and kidney the magnitude of induction of Tnfα expression was much higher in MyD88−/− than in WT and Trifmut.

Similarly, reactive oxygen species production resulting from activation of renin–angiotensin system in renal tissue leads to hypertension16 and the expression and activation of Nox4 RNA45,46 promote cardiac hypertrophy.27–49 We found that Nox4 expression was slightly increased in heart of WT mice after 3 weeks of Ang II but significantly enhanced in both kidney and heart of MyD88−/− and, in contrast, markedly downregulated in kidney and unchanged in heart of Trifmut mice.

Increased expression of Agrp1a was also significantly increased during the 3-week infusion of Ang II in both heart and kidney of MyD88−/− and conversely reduced significantly in Trifmut mice, whereas no significant changes were observed in WT mice.

Taken together these expressions of proinflammatory genes correlate with the development of hypertension and cardiac hypertrophy and demonstrate that the TRIF adaptor protein is the essential mediator of those responses, whereas MyD88 is an effective negative regulator.

TLR-Mediated Dual Activation of MyD88-Dependent and TRIF-Dependent Signaling Pathways

Multiple TLRs interact selectively and specifically with ligands to initiate signaling cascades through adaptor protein molecules resulting in pro- or anti-inflammatory responses.50,51 The most common adaptor protein is the MyD88 that was initially recognized as a component of the IL-1 receptor signaling complex.27 All TLRs except TLR3 and a subset of TLR4 signaling events depend on MyD88.27,50,51 MyD88-independent TLR4 pathways are activated by bacterial lipopolysaccharides in macrophages causing induction of several genes, including C-X-C motif chemokine 10 (CXCL10), regulated on activation, normal T cell expressed and secreted (RANTES), and macrophage colony stimulating factor.52 The adaptor molecule of the MyD88-independent signaling events is TRIF.27,51 TRIF induction of interferon regulatory factor-3 activation and interferon-β and interferon-inducible genes are MyD88 independent.33–35

Our results indicate that Ang II hypertension induces simultaneous activation of TLR pathways that are dependent on both adaptor proteins and have opposing effects in the WT mice with a net increase in arterial pressure, cardiac hypertrophy, and modest increases in cardiac and renal proinflammatory gene expression. The hypertension, cardiac hypertrophy, and the proinflammatory gene expression caused by Ang II were predominantly TRIF mediated. The expression of a TRIF-dependent chemokine Cxcl10, which was enhanced in the hearts of MyD88−/− mice when compared with WT but decreased in Trifmut mice, further supports the induction of TRIF-dependent inflammatory signaling, which is negatively regulated by MyD88-dependent signaling.

Because TLRs are selectively activated by a large number of specific ligands of either exogenous or endogenous origin, the determination of which ligands are generated during the prolonged infusion of Ang II is challenging. One might suggest that the early rise in pressure during the first days of Ang II infusion may provoke cellular damage which releases damage-associated molecular patterns into the circulation which then activate TLRs on innate immune cells initiating the inflammatory cascade. An important question is how MyD88 exerts its anti-inflammatory effects.

Negative Regulation of TRIF Responses by MyD88 Is Anti-Inflammatory

TLR signaling via different adaptor proteins may function in context-specific56–57 as well as tissue-specific manner.58 Moreover, multiple mechanisms of negative regulation of these adaptor pathways have been described.59 It is not clear whether the MyD88 anti-inflammatory effect results from direct stimulation of specific inhibitors or from gene repression effects of transcription factor nuclear factor-xB.60–62 Our results, however, suggest that the deletion of MyD88 results in increased cardiac expression of TLR3 and TLR4 as well as TRIF, all of which would contribute to exaggerated responses and support a negative regulatory influence of MyD88. In contrast, the TRIF mutation decreased TLR3 without altering TLR4 expression but increased MyD88 expression. The latter would contribute to the observed suppression of responses to Ang II.

Another conceptually attractive mechanism that would explain the enhanced TRIF-dependent inflammatory responses in MyD88−/− mice is signaling flux redistribution,63 whereby removal of MyD88 enhances the availability and flux of signaling substrates through the alternative TRAM/TRIF-dependent pathway. Enhanced inflammatory response in MyD88-deficiency has also been reported in other models as well.57,64 Indeed, MyD88 functions as a negative regulator of the TRIF-dependent pathway in a corneal inflammation model.65 In this study, TLR3 ligand–induced inflammatory cellular infiltration in cornea was exacerbated in MyD88−/− mice. It is also noteworthy that MyD88 signaling also produces
anti-inflammatory molecules, such as IL-10,\textsuperscript{66} which requires intact MyD88 for its anti-inflammatory action.\textsuperscript{67} The constitutive expression by MyD88 of A20, interleukin-1 receptor associated kinase-M (IRAK-M), and peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)), which result in downstream repression of nuclear factor-\(\kappa\)B transcription factor but not TRIF-dependent interferon regulatory factor 3, would also result in enhancement of proinflammatory responses in MyD88 deficiency.\textsuperscript{68,69}

Our results showing exacerbation of Ang II hypertension and inflammatory gene expression in kidney and heart including Cxcl10 in the MyD88\(\sim\)heart support a similar anti-inflammatory effect of MyD88. Thus, in the context of Ang II--induced hypertension and cardiac hypertrophy, it is clear that MyD88 exerts a novel negative regulatory role.

**Interaction of Ang II With TLRs**

Exactly how the Ang II pathway interacts with the TLR pathway is not understood. Ang II--induced hypertension involves the adaptive immune system,\textsuperscript{14} which in turn is regulated by the activation of the innate immune system by TLRs that detect specific ligands (damage-associated molecular patterns). Cytokines released by the innate immune system, such as proinflammatory cytokine IL-6, are essential for Ang II--induced hypertension in mice.\textsuperscript{70} Moreover, as mentioned above, we have recently shown that Ang II is capable of markedly enhancing specific proinflammatory TLR responses in the immune cells of spontaneously hypertensive rats but not in the immune cells of normotensive Wistar-Kyoto rats.\textsuperscript{71} The components of TLR signaling are widely expressed in multiple tissues and cell types. Although systemic Ang II infusion has wide-ranging effects on various tissues, Ang II is not known to be an agonist of the TLRs. It is possible that a downstream product of Ang II--AT1 receptor binding may generate a TLR agonist or the AT1R signaling pathway might interact with the components of the TLR pathway to activate it.

**Perspective**

Inflammation is a hallmark of hypertension and end organ damage including cardiac hypertrophy. TLRs and their adaptor proteins are integral components of innate immunity whose role in hypertension and end organ damage is now fully recognized. Our finding that Ang II responses result from activation of different pathways with pro- and anti-inflammatory adaptor proteins opens up opportunities for new molecular targets to treat recalcitrant hypertension.

Hypertension represents an abnormal immunologic state that involves the adaptive as well as the innate immune system. Our finding indicates that a hypertensive state may be exacerbated as a result of suppression or impairment of MyD88-dependent pathways. Conversely, targeting the TRIF pathway may be therapeutic by blocking the inflammatory response and enhancing the negative regulatory effects of MyD88.

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**Disclosures**

None.

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What Is New?

• We discovered a dual simultaneous activation by angiotensin (Ang) II of 2 innate immune signaling pathways. One that includes toll-interleukin receptor domain–containing adaptor protein–inducing interferon-β, as its adaptor protein, is essential for Ang II–induced hypertension, cardiac hypertrophy, and proinflammatory gene expression, whereas the other that is dependent on adaptor protein myeloid differentiation protein 88 mediates an anti-inflammatory response that restrains Ang II hypertension, hypertrophy, and inflammatory gene expression.

What Is relevant?

• Ang II–induced hypertension and end organ damage depend on inflammatory immune responses. However, the mechanisms of Ang II–induced inflammatory responses are not fully understood. Now, the knowledge of the specific adaptor proteins that regulate innate immune inflammatory pathways provides opportunities for novel interventions to treat hypertension and end organ damage.

Summary

We have discovered that a toll-interleukin receptor domain–containing adaptor protein–inducing interferon-γ adaptor-dependent inflammatory pathway of the innate immune system rather than the common myeloid differentiation protein 88 adaptor-dependent pathway mediates Ang II–induced hypertension, cardiac hypertrophy, and proinflammatory gene expression. Paradoxically, the myeloid differentiation protein 88 adaptor signaling activated by Ang II is anti-inflammatory. This study provides valuable insight into the mechanisms of hypertension and the potential for developing new therapies.

Novelty and Significance

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<td>For</td>
<td>5′-GCTGCGCTATTTTCTGC-3′</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>Rev</td>
<td>5′-TCTCAGTGCCGCCTAC-3′</td>
</tr>
<tr>
<td>Gapdh</td>
<td>For</td>
<td>5′-CATTTCCTGTATGACAATAGATACG-3′</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Rev</td>
<td>5′-TCCAGGTTTCTTACTCCTTGGA-3′</td>
</tr>
<tr>
<td>MyD88</td>
<td>For</td>
<td>5′-GTGTTTGTGGTGTCGGACCTG-3′</td>
</tr>
<tr>
<td>MyD88</td>
<td>Rev</td>
<td>5′-GTCAGAAACACCACCACCATGC-5′</td>
</tr>
<tr>
<td>Nox4</td>
<td>For</td>
<td>5′-CTCTACTGGATGACTGAAACC-3′</td>
</tr>
<tr>
<td>Nox4</td>
<td>Rev</td>
<td>5′-AGTCAGGTCTGGTTTCTTGCC-3′</td>
</tr>
<tr>
<td>Tlr3</td>
<td>For</td>
<td>5′-CCAGAAAGAATCTAATCAATATAGATTGTC-3′</td>
</tr>
<tr>
<td>Tlr3</td>
<td>Rev</td>
<td>5′-TTTTTCTAAGAGGCATTCTGCTTGGAG-3′</td>
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<tr>
<td>Tlr4</td>
<td>For</td>
<td>5′-GGCAACTTGGACCTGAGGAG-3′</td>
</tr>
<tr>
<td>Tlr4</td>
<td>Rev</td>
<td>5′-CATGGGCTCTCGGTCCATAG-3′</td>
</tr>
<tr>
<td>Tnfa</td>
<td>For</td>
<td>5′-TGCTATGTCTCAGCTCTTC-3′</td>
</tr>
<tr>
<td>Tnfa</td>
<td>Rev</td>
<td>5′-GAGGCCATTTGGGAACTTCT-3′</td>
</tr>
<tr>
<td>Ticam1</td>
<td>For</td>
<td>5′-CAGCTCAAGACCCCTACAGC-3′</td>
</tr>
<tr>
<td>Ticam1</td>
<td>Rev</td>
<td>5′-CTCCACACAGGCTCGTC-3′</td>
</tr>
</tbody>
</table>

Table-S1. Sequences of primers used for QPCR.
Table-S2. Analyses of linear regressions and slopes of heart weight to body weight ratios (mg per gram body weight) in response to saline, low dose Ang II and high dose Ang II.

<table>
<thead>
<tr>
<th>Statistical parameter</th>
<th>WT</th>
<th>MyD88/-</th>
<th>Trif&lt;sup&gt;mut&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Best-fit values</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.00066 ± 0.00008</td>
<td>0.00078 ± 0.00015</td>
<td>0.00022 ± 0.00012</td>
</tr>
<tr>
<td>Y-intercept when X= 0.0</td>
<td>4.845 ± 0.1523</td>
<td>4.039 ± 0.2176</td>
<td>4.636 ± 0.1994</td>
</tr>
<tr>
<td>X-intercept when Y= 0.0</td>
<td>-7366</td>
<td>-5228</td>
<td>-20919</td>
</tr>
<tr>
<td>1/slope</td>
<td>1520</td>
<td>1295</td>
<td>4513</td>
</tr>
<tr>
<td><strong>Is slope significantly non-zero?</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>69.13</td>
<td>25.82</td>
<td>3.293</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.0001</td>
<td>0.0002</td>
<td>0.0996</td>
</tr>
<tr>
<td>Deviation from zero</td>
<td>Significant</td>
<td>Significant</td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>Data</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of X values</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Maximum number of Y replicates</td>
<td>8</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Total number of values</td>
<td>20</td>
<td>15</td>
<td>12</td>
</tr>
</tbody>
</table>

Linear regressions and the slopes of the dose-dependent increases in heart weights to body weight ratio were significant in WT and MyD88/- (P<0.0001 and 0.0002, respectively) but not in Trif<sup>mut</sup> (P= 0.0996). The X-intercepts and 1/slope values indicate a greater weight gain in MyD88/-.
Table-S3. Effect of Ang II (3000 ng/kg/min) vs. saline infusions for 3 weeks.

<table>
<thead>
<tr>
<th>Measurements</th>
<th>WT</th>
<th>MyD88&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Trif&lt;sup&gt;mut&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difference in cardiomyocyte diameter (Ang II vs. Saline, µm)*</td>
<td>Δ 5.05 ± 0.63 µm</td>
<td>Δ 5.21 ± 0.23 µm</td>
<td>Δ 2.629 ± 0.25 µm</td>
</tr>
<tr>
<td>% left ventricular fibrosis after Ang II infusion.†</td>
<td>0.75 ± 0.36 %</td>
<td>0.45 ± 0.10 %</td>
<td>0.54 ± 0.06 %</td>
</tr>
</tbody>
</table>

* The mean ± SE of myocyte diameter in hearts of saline treated control mice was 19.51 ± 0.21 µm (n= 7). Increase in cardiomyocyte diameter was significantly greater in MyD88<sup>−/−</sup> and WT than in Trif<sup>mut</sup>.
† Left ventricular fibrosis was not seen in saline-infused mice and was minimal with Ang II in all three genotypes.
Figure-S1. Systolic blood pressure measured during saline and low-dose Ang II infusion for 3 weeks in (A) WT, (B) MyD88−/− and (C) Trifmut mice. Black lines represent saline-infused mice and Red lines represent values in Ang II-infused mice. Pressor responses were greater in MyD88−/− than in WT and nearly abrogated in Trifmut.
Figure-S2. (A) Expression of the TRIF/IFN-dependent gene Cxcl10 in the hearts of saline-vs. Ang II-infused WT, MyD88$^{-/-}$ and Trif$^{mut}$ mice. (B) Acta1 expression in hearts of saline-vs. Ang II-infused WT mice, MyD88$^{-/-}$ mice, and Trif$^{mut}$ mice. Fold change in RNA expression was measured by ΔΔC$_{t}$ method using Gapdh RNA as a reference. Values in graphs are normalized to measurements in saline-infused mice. Asterisk indicates statistical significance of responses to Ang II vs. saline infusion (P< 0.05). Acta1 expression was uniformly increased by Ang II in all three genotypes, whereas Cxcl10 was reduced in Trif$^{mut}$ and increased in MyD88$^{-/-}$. 