Inhibition of Toll-like receptor 2 reduces cardiac fibrosis by attenuating macrophage-mediated inflammation

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Aims
Toll-like receptor 2 (TLR2) is an important player in innate immunity, and recent studies have identified TLR2 as a critical mediator in cardiovascular diseases. Here, we investigated the involvement of TLR2 in angiotensin (Ang) II-induced cardiac fibrosis and the underlying mechanisms.

Methods and results
TLR2 knockout (TLR2 KO) mice (B6.129-Tlr2tm1Kir/) or wild-type (WT) mice (C57BL/6) treated with neutralizing anti-TLR2 antibody (T2.5) were used. The expression of TLR2 mRNA and protein in the heart was significantly up-regulated on days 1, 3, and 7 after Ang II infusion (1500 ng/kg/min). Enhanced expression of TLR2 was mainly detected in macrophages and neutrophils that had infiltrated into the heart. Both knockout of TLR2 and inhibition of TLR2 by neutralizing antibody ameliorated cardiac fibrosis induced by Ang II. This improvement was associated with a reduction in the infiltration of inflammatory cells, especially macrophages, the production of inflammatory cytokines, chemokines, and the activation of nuclear factor-κB. Bone marrow transplantation experiments between WT and TLR2 KO mice revealed that Ang II-induced cardiac fibrosis is mainly mediated by bone marrow-derived inflammatory cells. Mechanically, the deficiency of TLR2 inhibits macrophage-dependent cardiac fibroblast activation through TGFβ/Smad2/3 pathway.

Conclusion
Inhibition of TLR2 protects against Ang II-induced cardiac fibrosis by attenuating macrophage recruitment and the inflammatory response in the heart and may be a novel potential therapeutic target for hypertensive heart disease.

Keywords
Toll-like receptor 2 • Angiotensin II • Cardiac fibrosis • Macrophages

1. Introduction
Hypertensive cardiac remodelling, characterized by left ventricular (LV) hypertrophy, cardiac inflammation, and fibrosis, is a leading cause of chronic heart failure.1 The activation of the renin–angiotensin system plays an important pathophysiological role in hypertensive cardiac remodelling.1 Angiotensin (Ang) II cannot only cause haemodynamic effects but also trigger a series of inflammatory responses.1 We and others have demonstrated that Ang II infusion can cause inflammatory cells, such as macrophages, neutrophils, and T cells, infiltrate into heart tissues.2,11 Accumulating evidences have suggested that myocardial infiltration of pro-inflammatory cells such as macrophages play pivotal roles in the initiation and development of cardiac fibrosis and dysfunction.1 Upon activation, heart-infiltrated macrophages release various pro-inflammatory cytokines and chemokines, which interact with other cells, such as fibroblasts and cardiomyocytes, leading to cardiac remodelling.13,8 However, the early inflammatory events underlying the Ang II infusion-induced inflammatory cells infiltration and activation in the heart have not been fully elucidated.

Toll-like receptors (TLRs) are pattern recognition receptors of innate immune system that recognize pathogen-associated molecular patterns and damage-associated molecular patterns, such as lipopolysaccharide, peptidoglycan, heat shock proteins during inflammatory response.9 Following TLR activation, inflammatory cells produce the diverse cytokines and chemokines required for leucocytes activation and chemotaxis, which are believed to be involved in the pathogenesis of various
diseases. Recently, increasing evidence indicates that TLR2 plays important roles in cardiovascular diseases. Activation of TLR2 contributes to restenosis after stent implantation. Conversely, TLR2 knockout reduces the degree of atherosclerosis in low-density lipoprotein receptor-deficient mice. Moreover, TLR2 knockout mice are protected from Staphylococcus aureus- or doxorubicin-induced myocardial dysfunction. In addition, TLR2 deficiency markedly attenuates LV dilatation and mortality and preserves ventricular function after infarction. In addition, TLR2 has been reported to promote pulmonary inflammation and fibrosis induced by bleomycin. However, the role of TLR2 in hypertensive cardiac remodelling still remains unclear.

In the present study, we examined cardiac inflammation and fibrosis in TLR2 knockout (KO) mice, neutralizing anti-TLR2 antibody-treated mice and bone marrow-transplanted TLR2 chimeric mice before and after Ang II infusion. We found that deficiency or neutralizing antibody inhibition of TLR2 markedly reduced cardiac fibrosis by attenuating macrophage-mediated inflammation. Bone marrow transplantation (BMT) experiments revealed that cardiac fibrosis is determined by bone marrow-derived cells. In vitro studies further demonstrated that TLR2 inhibition suppressed macrophage-mediated cardiac fibroblast (CF) activation. The mechanisms underlying these actions were associated with inhibition of NF-κB and TGFβ1/Smad2/3 signalling pathways.

2. Methods

Refer to the Supplemental material online for more detailed experimental methods.

2.1. Animals and treatment

The TLR2 KO mice (B6.129-Tlr2tm1Kric) and WT mice (C57BL/6) were purchased from the Jackson Laboratory. Hypertensive cardiac fibrosis was induced in 10- to 12-week-old male TLR2 KO mice and matched C57BL/6 WT mice by subcutaneous infusing of Ang II (Sigma-Aldrich, St Louis, MO, USA) at a dose of 1500 ng/kg/min or saline using osmotic mini-pumps (Alzet MODEL 1007D; DURECT, Cupertino, CA, USA) at a dose of 0.1% collagenase II and 2.4 U/mL dispase II (Sigma-Aldrich) in phosphate-buffered saline. Cells were blocked with antibodies described in Supplementary material online.

2.2. Histopathology

The heart sections were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned (5 μm). Then the heart sections were stained with haematoxylin and eosin (H&E) and Mason’s trichrome reagent as described previously. Immunohistochemical staining was performed as described previously. Heart sections were stained with antibodies described in Supplementary material online.

2.3. Flow cytometry

The content of inflammatory cells was quantified by flow cytometry analysis as described. Mice hearts were digested with 0.1% collagenase II and 2.4 U/mL dispase II (Sigma-Aldrich) in phosphate-buffered saline. Cells were blocked with antibodies described in Supplementary material online.

2.4. Quantitative real-time PCR

Total RNA was extracted by the Trizol reagent method (Invitrogen) and first-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Promega, Southampton, UK). Quantitative real-time PCR (qPCR) was performed with an iCycler IQ system (Bio-Rad, USA) as described. The primer sequences were described in Supplementary material online.

2.5. Western blotting

Western blot analysis was performed as described in Supplementary material online. The images were quantified by the use of the Odyssey infrared imaging system (Li-COR Biosciences, Lincoln, NE, USA).

2.6. Cytokines measurement

The mouse heart lysate preparation and the levels of 37 inflammatory factors were measured simultaneously by mouse Procarta Immunoassay Kit according to the manufacturer’s protocol (Affymetrix, USA). For the protein quantification assays, Bio-Plex™ 200 System (Bio-Rad, USA) was used to analyse the concentration of each cytokine in each sample.

2.7. Cell isolation and culture

WT and TLR2 KO mice (8-week-old) were anaesthetized by an overdose of pentobarbitral (100 mg/kg, intraperitoneal injection). Bone marrow-derived macrophages (BMDMs) were isolated from tibias and femurs of mice as described in Supplementary material online. The isolation of CFs was performed using an enzymatic digestion from hearts of 6–8-week-old WT and TLR2 KO mice as described in Supplementary material online.

2.8. Co-culture experiment

Co-culture experiment was performed as described in Supplementary material online.

2.9. Macrophage migration assay

BMDM migration was determined in Boyden chambers (Cell Biolabs, USA) as described in Supplementary material online.

2.10. Statistical analysis

Results are presented as mean ± SEM. The number of experiments in every case is shown in figure legends. Statistical differences between groups were analysed by the non-parametric tests (Kruskal–Wallis or Mann–Whitney) or by the parametric test one-way analysis of variance (ANOVA) followed by the Tukey–Kramer test for group differences. Results are considered significant at P-values of <0.05.

3. Results

3.1. Ang II infusion up-regulates TLR2 expression in the heart

To investigate the role of TLR2 in the hypertensive heart, we first examined TLR2 expression after Ang II infusion. qPCR and western blot analysis showed that Ang II treatment significantly increased TLR2 mRNA and protein expression in the hearts on days 1, 3 and 7 after Ang II infusion (Figure 1A and B). To determine the cell type responsible for the up-regulation of TLR2 in hearts after Ang II infusion, flow cytometry was performed. Ang II infusion significantly increased the number of CD45+ TLR2+ cells, but not markedly affected the number of CD45-.
TLR2+ cells compared with saline control (Figure 1C). Furthermore, the TLR2+ cells were mainly CD45+ F4/80+ macrophages and CD45+ CD11b+ Gr-1+ neutrophils (Figure 1D). Double colour immunofluorescent staining further confirmed that TLR2 was predominantly expressed on Mac-2-positive macrophages and Gr-1-positive neutrophils in cardiac tissues after Ang II infusion (Supplementary material online, Figure S1). Collectively, these results suggest that inflammatory cells are the main source of TLR2 expression after Ang II stimulation and may be involved in cardiac remodelling in response to Ang II infusion.

3.2. TLR2 deficiency reduces Ang II-induced cardiac fibrosis

To determine the role of TLR2 in the development of hypertension and cardiac remodelling induced by Ang II, we tested the effect of TLR2 deficiency or inhibition of endogenous TLR2 activation with neutralizing anti-TLR2 antibody on blood pressure, cardiac function, and hypertrophy using the non-invasive tail-cuff method and echocardiography. Although the systolic blood pressure (SBP), the mean values of heart rate, anterior wall thickness (AWT), posterior wall thickness (PWT), fractional shortening (FS%), and ejection fraction (EF%) in the Ang II-treated WT group was increased compared with saline-treated WT and TLR2 KO mice, but no statistical difference of SBP, AWT, PWT, FS%, and EF% was observed between WT and TLR2 KO mice after Ang II infusion (Supplementary material online, Figure S2A and B). Moreover, there were no differences in cardiac hypertrophy reflected by heart weight to body weight ratio (HW/BW) and the myocyte size between WT and TLR2 KO mice after Ang II infusion (Supplementary material online, Figure S2C and D). Consistent with the data from TLR2 KO mice, blood pressure, cardiac

Figure 1 Angiotensin II infusion increased the expression of TLR2 in the heart tissues. (A) Relative mRNA expression of TLR2 in wide-type (WT) hearts before and after 1, 3, and 7 days of Ang II infusion. (B) Western blot analysis of TLR2 protein expression (top) and quantification (lower) in the heart tissues before and after 1, 3, and 7 days of Ang II infusion. (C and D) Flow cytometry analyses the time course change in the expression of TLR2+ cells in CD45+/CD45− cells, CD45+/F4/80+ macrophages, CD45+/CD11b+ Gr-1+ neutrophils, and CD45+/CD3+ T cells before and after 1, 3, and 7 days of Ang II infusion. Data are expressed as mean ± SEM (n = 3-4 mice per group). *P < 0.05 vs. saline control (A–D: Mann–Whitney U test).
function, and hypertrophy reflected by SBP, FS%, EF%, and HW/BW were also similar between anti-TLR2-treated group and isotype IgG-treated group after Ang II infusion (Supplementary material online, Figure S3). Thus, both knockout of TLR2 and inhibition of TLR2 activation do not affect Ang II induced the change of blood pressure, cardiac function, and hypertrophy at day 7 of Ang II infusion.

To determine whether TLR2 inhibition attenuates Ang II-induced cardiac fibrosis, Masson’s trichrome staining was performed. Ang II-induced increase of collagen deposition in the interstitial and perivascular area of heart tissues was significantly decreased in the TLR2 KO mice compared with WT mice after Ang II infusion (Figure 2A). Moreover, immunohistochemistry and qPCR demonstrated that the expression of fibrotic markers, including collagen I, collagen III, and α-SMA, was also markedly decreased in TLR2 KO mice compared with that in WT mice after Ang II infusion (Figure 2A and B). In addition, the protein level of α-SMA was lower in TLR2 KO mice than in WT mice (Figure 2C). There were no significant differences in the fibrotic area and the expression of fibrotic markers between the two groups of saline treatment (Figure 2). These results were further confirmed in mice treated with anti-TLR2 antibody and isotype IgG (Supplementary material online, Figure S4). Thus, these data demonstrate that TLR2 inhibition suppresses the development of cardiac fibrosis induced by Ang II infusion.

3.3. TLR2 deficiency inhibits Ang II induced the infiltration of inflammatory cells in the heart

To determine whether TLR2 on the accumulation of inflammatory cells in the heart during Ang II infusion, the cardiac tissues of WT and
TLR2 KO mice were examined. On day 7 of Ang II infusion, H&E staining showed that the infiltration of inflammatory cells in the cardiac tissue of TLR2 KO mice was significantly decreased compared with WT mice (Figure 3A). Flow cytometry further revealed that Ang II infusion significantly increased the proportion of CD45+ leucocytes, CD45+ F4/80+ macrophages, CD45+CD11b+Gr-1+ neutrophils, CD45+CD3+ T cells in hearts compared with saline-treated heart, and the most abundant cells in the CD45+ leucocytes were the macrophages (~65%), whereas neutrophils and T cells are only 25 and 10%, respectively (Figure 3B). In contrast, these alterations were markedly attenuated in the TLR2 KO hearts (Figure 3B). Immunohistochemistry further demonstrated that the number of Mac-2-positive macrophages was markedly lower in TLR2 KO hearts than that in WT mice after Ang II infusion (Supplementary material online, Figure S6A). Consistent with the observation from TLR2 KO mice, treatment of mice with neutralizing anti-TLR2 antibody also markedly decrease in the accumulation of inflammatory cells including macrophages in the heart tissues compared with isotype IgG-treated WT mice (Supplementary material online, Figure S6A). These results indicate that TLR2 mainly affects the recruitment of macrophages into cardiac tissues. To further test whether TLR2 affects macrophage migration, a transwell migration assay was performed in vitro using BMDMs isolated from WT and TLR2 KO mice. Ang II treatment significantly increased WT macrophages migration, whereas the migration ability was markedly reduced in TLR2-deficient macrophages (Figure 3C), indicating that TLR2 contributes to Ang II-induced migration of macrophages into the injured heart.

3.4. TLR2 deficiency attenuates Ang II-induced expression of inflammatory factors

To test whether TLR2 deficiency reduces the inflammatory response in the Ang II-induced cardiac injury, we examined the protein levels of a panel of cytokines, chemokine, and other factors, which play critical roles in inflammatory response,26 by using Procarta cytokine profiling kit in the hearts of WT and TLR2 KO mice infused with Ang II for 7 days. The expression of interleukins (IL) (IL-1α, 1β, 6, 9, 10, 12p70, 13, 17A, and 21), chemokine (C-X-C motif) ligand (CXCL) (CXCL1, CXCL2, and CXCL10), chemokine (C-C motif) ligand 5 (CCL5), and growth factors including tumour necrosis factor α (TNF-α), betacellulin, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, TGFβ1, and leukemia inhibitory factor was significantly decreased in the hearts of TLR2 KO mice compared with WT mice (Figure 4A). Furthermore, the level of p65 phosphorylation was markedly lower in TLR2 KO mice than that in WT mice after Ang II infusion (Figure 4B). Consistent with the data from TLR2 KO mice, blockage of TLR2 with anti-TLR2 antibody also decreased the mRNA expression of several pro-inflammatory mediators, including IL-1β, IL-6, and CCL2 (Supplementary material online, Figure S5C).

3.5. TLR2 deficiency in bone marrow-derived cells prevents Ang II-induced cardiac inflammation and fibrosis

To confirm the critical role of inflammatory cells in the development of cardiac fibrosis, we performed BMT experiments to address the question of whether TLR2 deficiency in bone marrow-derived cells alters cardiac inflammation and fibrosis in mice. First, we generated TLR2 bone marrow chimeric mice, in which the bone marrow cells were reconstituted with transplanted bone marrow cells. After 7 days of Ang II infusion, WT mice transplanted with TLR2 KO BM showed definitely decreased inflammation and cardiac fibrosis compared with WT mice transplanted with WT BM as proved by H&E staining, Masson staining and qPCR analysis of related cytokines (IL-1β, IL-6, TNF-α, and MCP-1) and fibrogenic genes (collagen I, collagen III, and α-SMA) (Figure 5A and B). In contrast, TLR2 KO mice transplanted with WT BM exhibited marked increased inflammation and cardiac fibrosis compared with TLR2 KO mice with TLR2 KO BM (Figure 5A and B). Overall, the BMT studies suggest that decreased cardiac fibrosis in TLR2-deficient mice is due mainly to decreased pro-inflammatory response of myeloid cells with TLR2 ablation.

3.6. TLR2-deficient macrophages inhibit cardiac fibrosis through TGF-β/SMAD signal pathway

To determine the molecular mechanisms of the inhibition of cardiac inflammation and fibrosis in TLR2 KO mice after Ang II infusion, we examine several signalling pathways, including TGFβ1/Smad2/3, Stat1, and Stat3, which are known to play critical roles in cardiac fibrosis.21 As shown in Figure 6A, there was a significantly decrease in the levels of TGFβ1 and phosphorylated Smad2/3 in TLR2 KO mice compared with WT mice after Ang II infusion. No significant difference in the levels of Stat1 and Stat3 phosphorylation was observed between two groups after saline or Ang II infusion (Supplementary material online, Figure S6B), indicating an important role of TLR2 in regulating TGFβ1/Smad2/3 pathway.

To determine whether TLR2 deficiency directly affects CF activation, we isolated CFs from WT and TLR2 KO mice, respectively. WT or TLR2 KO CFs were starved for 24 h and then exposed to 100 nM Ang II for 24 h. The levels of α-SMA and TGFβ1 were similar between TLR2 KO CFs and WT CFs after Ang II stimulation (Supplementary material online, Figure S6C), indicating that TLR2 in fibroblasts may not contribute to Ang II-induced cardiac fibrosis.

To further confirm TLR2-deficient macrophages inhibits fibroblast to myofibroblast transition, BMDMs from WT and TLR2 KO mice were co-cultured with isolated WT CFs. Western blot analysis showed that the protein levels of α-SMA, TGFβ1, and Smad2/3 were markedly up-regulated in fibroblasts which were co-cultured WT BMDMs in response to Ang II, whereas these effects were markedly attenuated by TLR2-deficient BMDMs (Figure 6B). Furthermore, the expression levels of collagen I and III mRNA were also markedly lower in fibroblasts co-cultured TLR2 KO BMDMs than that in fibroblasts co-cultured WT BMDMs in response to Ang II (Figure 6C). Together, these results suggest that TLR2 deficiency inhibits macrophage-mediated fibroblast activation through the TGFβ1/Smad2/3 signal pathway.

4. Discussion

In the present study, we investigated the role of TLR2 in regulating Ang II-induced cardiac remodelling by using TLR2 KO mice and anti-TLR2 antibody-treated mice. Our results showed that the expression of TLR2 was markedly increased in the heart after Ang II infusion, and knockout or blockage of TLR2 significantly inhibited Ang II-induced fibrosis and inflammation. BMT experiments confirmed that the Ang II-induced cardiac fibrosis was mainly determined by bone marrow-derived inflammatory cells. Moreover, knockout of TLR2 markedly...
Figure 3  TLR2 deficiency inhibits Ang II induced the infiltration of inflammatory cells in the heart. (A) Heart sections of WT and TLR2 KO mice after saline or Ang II infusion were stained by H&E staining (magnification, ×200). (B) Flow cytometry analysis of CD45+ leucocytes, CD45+F4/80+ macrophages, CD45+CD11b+Gr-1+ neutrophils, and CD45+CD3+ T cells were performed in WT and TLR2 KO hearts with saline or Ang II infusion. Bar graph shows the percentage of cells in 2*10^4 cells. (C) Transwell analysis was performed to examine the migration of macrophages from WT and TLR2 KO mice. Bar graph shows the number of migrated cells. Data are expressed as mean ± SEM (n = 4 per group). *P < 0.05 vs. saline control; **P < 0.05 vs. WT Ang II infusion or WT macrophages after Ang II stimulation (A–C: Kruskal–Wallis test).
attenuated macrophage-mediated fibroblast transition, thereby suppressing cardiac fibrosis induced by Ang II infusion. These effects were associated in part with inhibition of TGFβ1/Smad2/3 signalling pathway. Thus, our study demonstrates that TLR2 plays a critical role in regulating Ang II-induced cardiac fibrosis.

It has been reported that cardiac fibrosis is associated with abnormal cardiac remodelling and increased ventricular stiffness. Cardiac fibrosis disrupts the co-ordination of myocardial excitation–contraction coupling and cardiomyocytes slippage that may cause impairment of systolic and diastolic function, and subsequent LV dilation. Cardiac fibrosis also may result in arrhythmias and sudden cardiac death. However, the mechanisms that regulate cardiac fibrosis are not fully elucidated. Recently, inflammation is reported to play an important role in the initiation and development of hypertensive cardiac fibrosis. TLR2 is a key innate immunity molecule for sensing inflammatory mediators and ligands, and is involved in the activation of NF-κB signalling which is required for the leucocyte recruitment and activation thereby contributing to various fibrotic diseases. For example, TLR2 deficiency can reduce lung and cardiac fibrosis through reversion of suppressive immune microenvironment. TLR2 knockout also attenuates the inflammation and expression of TGF-β, matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9), CCL2, and the amounts of myofibroblasts in kidney after unilateral ureter obstruction injury. Conversely, activation of TLR2-JNK-ERK pathways by surfactant protein A can promote macrophages to secrete TGF-β, and anti-TLR2 or TLR2 deficiency blocks this effect. However, the role of TLR2 signalling in Ang II-induced inflammation and myocardial fibrosis remains unclear. In the present study, we demonstrated that knockout of TLR2 or neutralization by anti-TLR2 antibody significantly reduced Ang II-induced inflammation and cardiac fibrosis (Figures 2–4).

TLR2 has been known to be activated by endogenous ligands that are released from injured tissue cells. Ang II is one of the major humoral factors that trigger apoptosis by stimulating activation of several signalling pathways, including p38 MAPK and p53. Cellular debris produced by apoptosis can bind to TLR2 and lead to leucocytes recruitment, which

Figure 4 TLR2 deficiency attenuates Ang II-induced expression of inflammatory factors. (A) The expression of cytokines in WT and TLR2 KO heart tissues after 7 days of Ang II infusion was analysed by Procarta Immunoassays. (B) Western blot analysis of relative p-P65 protein expression (left panel) and quantification (right panel) in WT and TLR2 KO hearts before and after Ang II infusion. Data are expressed as mean ± SEM (n = 3 mice per group). *P < 0.05 vs. saline control; †P < 0.05 vs. WT Ang II infusion (A: Mann–Whitney U test; B: Kruskal–Wallis test).
produce the diverse cytokines and chemokines thereby contributing to cardiomyopathy and cardiac dysfunction. Leucocytes migration and activation are critical events in the development of hypertensive cardiac fibrosis. Thus, we focused on the effect of TLR2 on the recruitment and activation of pro-inflammatory cells in the heart. Our results demonstrated that Ang II infusion markedly increased the infiltration of CD45^+ leucocytes in WT hearts, particularly F4/80^+ macrophages, whereas this effect was markedly reduced in TLR2 KO mice after Ang II infusion (Figure 3). Moreover, TLR2 KO mice showed decreased expression of 20 cytokines, chemokine and growth factors, and activity of NF-κB signalling pathway compared WT mice after Ang II infusion (Figure 4). Furthermore, chemokine-induced recruitment of peripheral leucocytes to tissues is a critical step in the development of various inflammatory diseases. Overwhelming evidences demonstrate that TLR2 plays a critical role in the expression of chemokine and their receptors, such as CCL2 and CCL3, CXCL1, CCR1, CCR2, and CCR5, in macrophages and other cell types. Here, we found that TLR2 deficiency markedly attenuated Ang II-induced expression of several chemokines, including CXCL1, CXCL2, CCL5, and CXCL10, in the heart tissues (Figure 4A), indicating that TLR2-mediated chemokine expression after Ang II injury may account for the increased macrophages infiltration in the hearts after Ang II infusion. Importantly, BMT studies support the hypothesis that TLR2 deficiency in myeloid cells decreases accumulation of inflammatory cells in the heart and cytokine expression during Ang II

**Figure 5** TLR2 deficiency in bone marrow-derived cells prevents Ang II-induced cardiac inflammation and fibrosis. (A) Masson trichrome staining and H&E staining of heart tissues from TLR2 bone marrow chimeric mice after Ang II infusion (magnification, ×200). (B) qPCR analysis of α-SMA, collagen I, collagen III, IL-1β, IL-6, TNF-α, and MCP-1 mRNA expression in TLR2 bone marrow chimeric mice after Ang II infusion. (C) Immunohistochemistry staining of Mac-2 in TLR2 bone marrow chimeric mice after Ang II infusion. Data are expressed as mean ± SEM (n = 3–5 mice per group). *P < 0.05 vs. WT BM to WT (A–C: Mann–Whitney U test).
Collectively, these results indicate that inhibition of TLR2 activation can attenuate macrophage migration and activation in the hearts after Ang II injury.

Pathological stimuli can stimulate tissue fibroblasts differentiation into myofibroblasts, which has been considered as a major mechanism responsible for fibrosis. TGF-β1/Smad2/3 signalling has an essential role in the development of fibrosis. Ang II treatment is well known to stimulate TGF-β1/Smad2/3 signalling contributing to cardiac fibrosis. It is reported that TLR2 promotes tissue fibrosis through enhancing inflammation and TGF-β expression in lung and kidney. However, the exact mechanism of TLR2 signalling to regulate Ang II-induced myocardial fibrosis is largely unclear. Macrophages are known to trigger the differentiation of fibroblasts into myofibroblasts mainly through TGF-β-dependent signalling in Ang II-infused heart. Our results showed that Ang II infusion significantly increased the levels of TGF-β1 expression and Smad2/3 phosphorylation in WT hearts, which were attenuated in TLR2 KO mice (Figure 6A). By doing BMT, we found that bone marrow-derived cells significantly contribute to TLR2-mediated cardiac fibrosis (Figure 5A and B). To more directly demonstrate the effect of TLR2 macrophages on the transition of fibroblasts to myofibroblasts, we co-cultured WT fibroblast with BMDMs from WT and TLR2 KO mice, and found a marked decrease in myofibroblast activation, collagen expression, and the expression of TGF-β1 and phosphorylated Smad2/3 in the presence of TLR2 KO macrophages in response to Ang II (Figure 6B and C). Thus, our results reveal a novel function of TLR2 in contributing to macrophage-mediated myofibroblast activation and cardiac fibrosis.

In conclusion, this study demonstrates the critical role of TLR2 in the inflammatory process during the development of hypertensive cardiac fibrosis. TLR2 promotes macrophage migration and activation, thereby leading to fibroblast transition and collagen production in the heart after Ang II infusion. Thus, blockage of TLR2 signalling could be an attractive approach for the treatment of hypertensive heart diseases and other inflammatory disorders. Further investigations in other animal models of cardiac fibrosis are needed to determine the clinical use of TLR2 inhibition as a pharmacological therapy.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Conflict of interest: none declared.


References


