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Cellular and Molecular Mechanisms of Angiotensin-II-mediated Fibrosis

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ABSTRACT

Angiotensin-II (Ang-II) is involved in many conditions related to heart failure and pathologic cardiac hypertrophy. Ang-II induces the synthesis of monocyte chemoattractant protein-1 (MCP-1) which mediates the uptake of monocytic $CD34^{+}CD45^{+}$ fibroblast precursors into the heart, that differentiate into collagen-producing fibroblasts. These cells are implicated in the development of Ang-II-mediated cardiac fibrosis. Tumor necrosis factor-alpha (TNF) is also implicated in Ang-II-induced cardiac fibrosis, however it is unknown how TNF is involved. In our current study we demonstrate that mice with genetic deletion of both TNF receptors developed significantly less cardiac fibrosis when subjected to Ang-II than wild-type mice. To investigate the role of each receptor separately, mice lacking only TNF receptor 1 (TNFR1-KO) or receptor 2 (TNFR2-KO) were infused with Ang-II for one week. Collagen deposition was greatly reduced and fewer $CD34^{+}CD45^{+}$ cells were present in TNFR1-KO than in wild-type and TNFR2-KO hearts. Quantitative RT-PCR showed a significantly lower transcriptional upregulation of key fibrosis- and inflammation-related gene expression in TNFR1-KO hearts. In addiction, cardiac hypertrophy was reduced in these mice compared to wild-type and TNFR2-KO. These data suggest that TNF is required for Ang-II-dependent cardiac fibrosis and exerts its effects through TNFR1.

1. INTRODUCTION

In general, the term fibrosis describes the deposition of collagen in the tissue. On one hand, collagen is a ubiquitous, essential protein that keeps organs together and builds up connective tissue. On the other hand, too much or too little collagen can result in either increased or decreased tissue stiffness. Non-adaptive fibrosis (also called interstitial or reactive fibrosis) is the deposition of collagen in the interstitial space between live cells in the absence of cell death [1]. Various studies report on non-adaptive fibrosis in the chronically diseased heart and in the aftermath of myocardial infarct [1-3].

In the setting of chronic cardiac disease, non-adaptive fibrosis results in cardiac remodeling and the stiffening of the ventricle, thereby impairing left ventricular function. It is therefore regarded an important mechanism which contributes to the symptoms and progression of heart failure. Often, the development of cardiac fibrosis is associated with inflammation [4].

Increased levels of Angiotensin-II (Ang-II), an 8 amino acid peptide hormone, are implicated in almost every condition leading to congestive heart failure, starting from hypertension to myocardial infarction. High levels of Ang-II stimulate the development of cardiac fibrosis. The inflammatory response is also promoted by Ang-II and the metabolic regulation in the heart is altered [5-8].

Previous studies using a murine model of continuous Ang-II-infusion developed by our group have shown that Ang-II exposure resulted in interstitial deposition of collagen, which was mediated by the monocyte chemoattractant protein-1 (MCP-1)-dependent influx of $CD34^{+}CD45^{+}$ fibroblast precursors into the heart [9]. These cells were attracted from the bone marrow and differentiated into collagen-producing fibroblast precursors and thus contributed to the development of cardiac fibrosis [10]. Mice deficient in MCP-1 did not develop cardiac fibrosis after Ang-II infusion, despite hypertension and cardiac hypertrophy [9]. Together with various markers of inflammation, Ang-II infusion also stimulated

production of tumor necrosis factor–alpha (TNF) in the heart; genetic deletion of MCP-1 inhibited the Ang-II-induced increase in TNF transcription [9].

TNF is a major regulator of immunity and inflammation, but it also contributes to the regulation of cardiac structure and function, both in health and disease. Short term expression of TNF is a beneficial stress-response, but long-term expression or high levels of TNF are deleterious [11, 12]. TNF acts through two distinct cell surface receptors, a 55-kDa TNFR1 and a 75-kDa TNFR2 [13]. While both receptors are similar in their extracellular domains, they differ in their intracellular regions, suggesting different, distinct modes of signaling and effects for each receptor.

TNFR1 is thought to mostly mediate the deleterious effects of TNF, whereas TNFR2 is thought to be responsible for protective mechanisms. The net effects of both receptors on heart failure may depend on the relative contribution of signaling through each of the two receptors [14].

In our current study we set out to determine whether both or only one of the two TNF receptors is required for the development of cardiac fibrosis.

Our data suggest a synergistic interaction of Ang-II and TNF on cardiac fibrosis and adverse cardiac remodeling, specifically through involvement of TNFR1 signaling.

Parts of these, as well as additional data have been recently published in the Journal of Molecular and Cellular Cardiology [15] (Thomson Reuters Impact Factor 2011: 5.166) [16].

2. METHODS

2.1 Animals

B6;129S-Tnfrsf1a^{tm1Imx}Tnfrsf1b ^{tm1Imx}/ J ("TNFR1R2-KO") and their B6129SF2/J ("B6129") wild-type control mice, as well as C57BL/6-Tnfrsf1a^{tm1Imx}/J ("TNFR1-KO"), B6.129S2-Tnfrsf1b^{tm1Mwm}/J ("TNFR2-KO") and their C57BL/6J ("C57") wild-type control mice were purchased from Jackson Laboratory. Mice were infused with 1.5 µg/kg/min Ang-II via subcutaneously implanted osmotic pumps for 1 week [9]. Control animals were implanted with sterile saline-filled pumps.

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US NIH. All animals were treated in accordance with the guidelines of the Baylor College of Medicine Animal Care and Research Advisory Committee.

2.2 Cardiac fibrosis

Hearts were arrested in diastole by infusion with cardioplegic solution and perfusion fixed using Zinc/Tris, embedded in paraffin and sectioned as described earlier [9, 10]. To measure collagen deposition, deparaffinized sections (4-6 per mouse) were stained with picrosirius red. Images within the left ventricle (4 per section) were scanned and collagen stained areas were calculated as percentages of the total myocardial area using ImagePro software. To evaluate overall macrophage influx, sections were stained with an antibody against Mac-2 and counterstained with eosin as described earlier [9].

2.3 Identification of fibroblast populations

Cardiac fibroblasts were isolated as described previously [9, 10]. Freshly isolated cells were incubated with PE-conjugated anti-CD34, PE/Cy-5-conjugated anti-CD45, and either FITC-conjugated anti collagen type I or calcein^{AM}. Fluorescence intensities were measured on a Beckman Coulter Epics XLMCL.

2.4 mRNA expression

Total RNA was isolated from the whole heart with TRIzol reagent (Life Technologies) and purified via columns. Total cDNA was synthesized using a Verso cDNA synthesis kit (Thermo Scientific). Real-time PCR amplifications were performed with SYBR Green on a C1000 Touch cycler (BioRad). Gene expression was measured by the ΔΔCT method and was normalized to 18S ribosomal RNA levels. The data are presented as the fold expression relative to the saline-treated wild-type group. All primer pairs were verified to comply with MIQE guidelines [17].

2.5 Cell size measurement

Perfusion-fixed heart sections were stained with tetramethylrhodamine-labeled wheat germ agglutinin; cell nuclei were counterstained with DAPI. Images were taken within the left ventricle (4 images per section, 2-4 sections per mouse). Cardiomyocyte size was measured in arbitrary units using ImagePro software.

2.6 Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using InStat software (Graphpad). One-way ANOVA was used to evaluate differences between all groups (control and treated) and post-hoc testing (Tukey-Kramer Method) was performed when appropriate. A P-value <0.05 was considered statistically significant.

3. RESULTS

3.1 Deletion of TNFR1, but not TNFR2, prevented development of interstitial fibrosis after Ang-II infusion

Using mice deficicient in both TNF receptors, we found that TNF signaling was required for Ang-II-induced development of cardiac fibrosis. Specifically, we found that in mice with genetic deletion of TNFR1, infusion of Ang-II did not lead to increased collagen deposition in the heart compared to saline-treated mice (1.6±0.3% vs. 0.8±0.2% collagen area), whereas in mice lacking TNFR2 Ang-II exposure resulted in interstitial cardiac fibrosis. TNFR2-KO mice showed significant deposition of collagen in the heart which was not different from wild-type mice (4.7±0.7 % vs. 4.2±0.6% collagen area, Figure 1, Figure 2). These results demonstrated that the Ang-II-mediated reactive cardiac fibrosis required signaling through TNFR1, but not TNFR2.

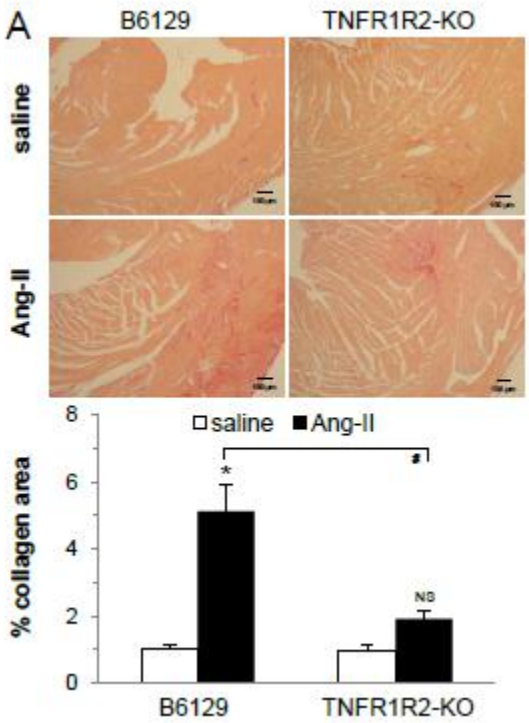

Figure 1: Tissue sections were stained with picrosirius red (image magnification: x100) after 1 week of continuous Ang-II treatment (n=5-6/group) and collagen deposition was evaluated in the left ventricle. Control mice received saline (n=3/group). In contrast to their corresponding wild-type mice (B6129), mice deficient in both TNF receptors (TNFR1R2-KO) were protected from Ang-II exposure, as interstitial collagen deposition was lower than in wild-type mice and comparable to saline values. *P<0.05 between Ang-II- and saline-treated groups (same genetic background). #P<0.05 compared to Ang-II-treated wild-type group. NS = no significant difference (Figure taken from [15]).

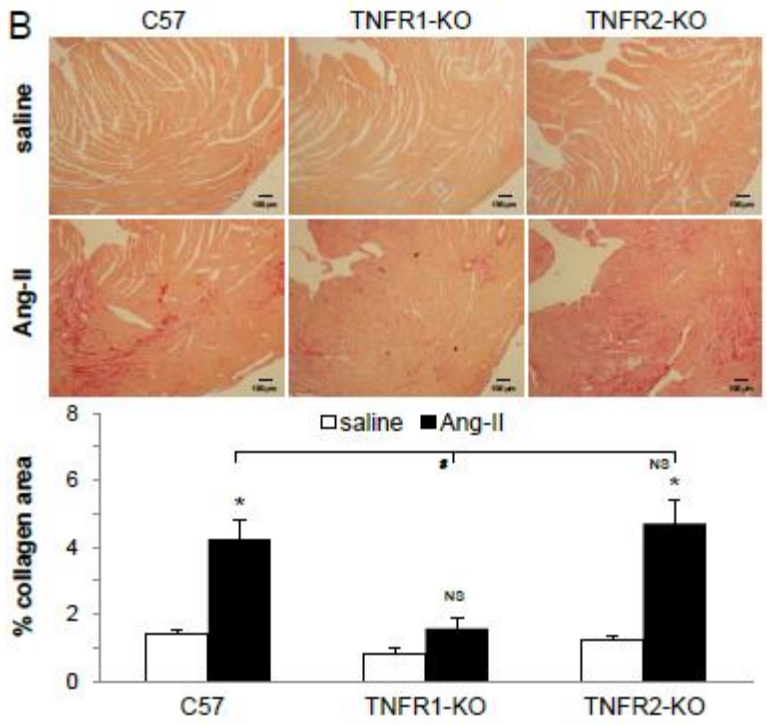

Figure 2: Tissue sections were stained with picrosirius red (image magnification: x100) after 1 week of continuous Ang-II treatment (n=5-6/group) and collagen deposition was evaluated in the left ventricle. Control mice received saline (n=3/group). Mice deficient in TNFR1 (TNFR1-KO) did not develop interstitial collagen deposition in response to Ang-II when compared to their corresponding wild-type group (C57) or to mice deficient in TNFR2 (TNFR2-KO), the latter two not being different from each other. *P<0.05 between Ang-II- and saline-treated groups (same genetic background). #P<0.05 compared to Ang-II-treated wild-type group. NS = no significant difference (Figure taken from [15]).

3.2 In mice deficient in TNFR1 signaling Ang-II-infusion did not induce the presence of myeloid fibroblast precursors in the heart

Our group has previously shown that Ang-II-induced cardiac fibrosis was mediated by myeloid precursor cells that matured into fibroblasts [9]. To ascertain whether these cells were present in mice lacking TNFR1 or TNFR2, we isolated cardiac cells from mice after 1 week of Ang-II infusion and performed flow cytometry. We did not find an increase in monocytic $CD34^{+}CD45^{+}$ fibroblasts in mice deficient in both TNF receptors (0.4±0.1 % [Ang-II-treated] vs. 0.1±0.1 % [saline-treated]; p>0.05, Figure 3 A-B). Specifically, in the absence of TNFR1, no monocytic $CD34^{+}CD45^{+}$ fibroblast precursors were found in the heart, whereas hearts from mice with deletion of TNFR2 showed an increase of viable $CD34^{+}CD45^{+}$ cells, similar to levels in wild-type hearts. Also, the amount of $CD34^{+}CD45^{+}$ cells that also produced

collagen was reduced in TNFR1-KO hearts after Ang-II infusion. These data indicate that Ang-II and TNF together induced the presence of fibroblast precursors of myeloid origin via signaling through TNFR1, but not through TNFR2. Results from a separate experiment, in which we stained cardiac tissue for the presence of macrophages, showed that there was no difference between wild-type and knockout hearts (Figure 3 C), implying that the overall influx of macrophages in response to Ang-II was not affected by TNF signaling.

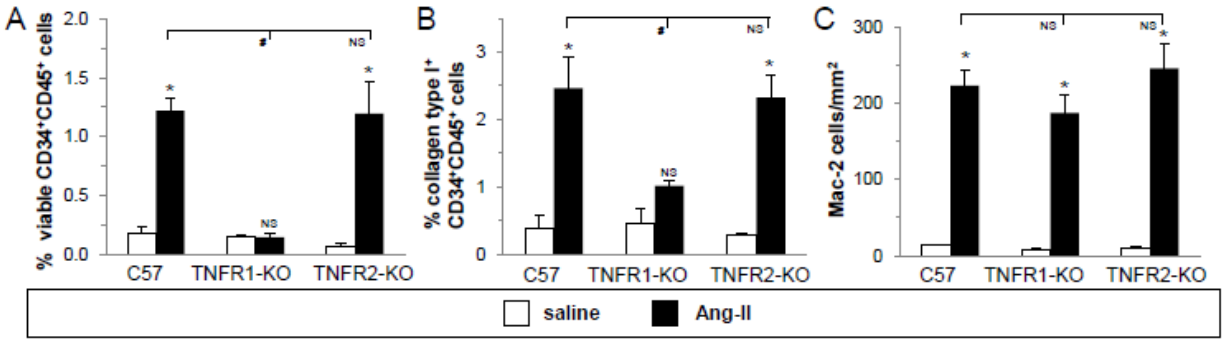

Figure 3: Hearts were removed and non-myocytes isolated. A) Dispersed cells were analyzed for both CD34 and CD45 expression in the presence of calcein (viability marker) by flow cytometry. B) Cells were analyzed for collagen type I, CD34 and CD45 expression. C) Cardiac tissue sections were stained for Mac-2+ cells. Control mice received saline (n=3/group), and treated mice received Ang-II for 1 week (n=5/group). *P<0.05 between Ang-II- and saline-treated groups (same genetic background). #P<0.05 compared to Ang-II-treated wild-type group. NS = no significant difference (Figure taken from [15]).

3.3 In mice deficient in TNFR1 signaling fibrosis- and inflammation- related genes were not upregulated by Ang-II-infusion

To determine which genes were affected by Ang-II we performed Real-Time PCR. In TNFR1-deficient mice we found significantly smaller increases in collagen types I and III, and osteopontin mRNA expression and no increase in alpha-smooth muscle actin (α-SMA) and transforming growth factor beta-1 (TGF-β1) mRNA expression after 1 week of Ang-II exposure. In mice lacking TNFR2, transcriptional activation of these genes was comparable to wild-type. We also found only minimal increases in MCP-1, TNF, CCR2, and IL-6 mRNA in TNFR1-KO hearts, whereas these genes were significantly upregulated

in wild-type and TNFR2-KO hearts after 1 week of Ang-II infusion (Figure 4). We then tested whether either TNF receptor was upregulated in KO mice to compensate for the lack of the other TNF receptor, but no such compensatory increases were found. These data suggested an important role of TNFR1 signaling in Ang-II-induced transcription of fibrotic and inflammatory genes.

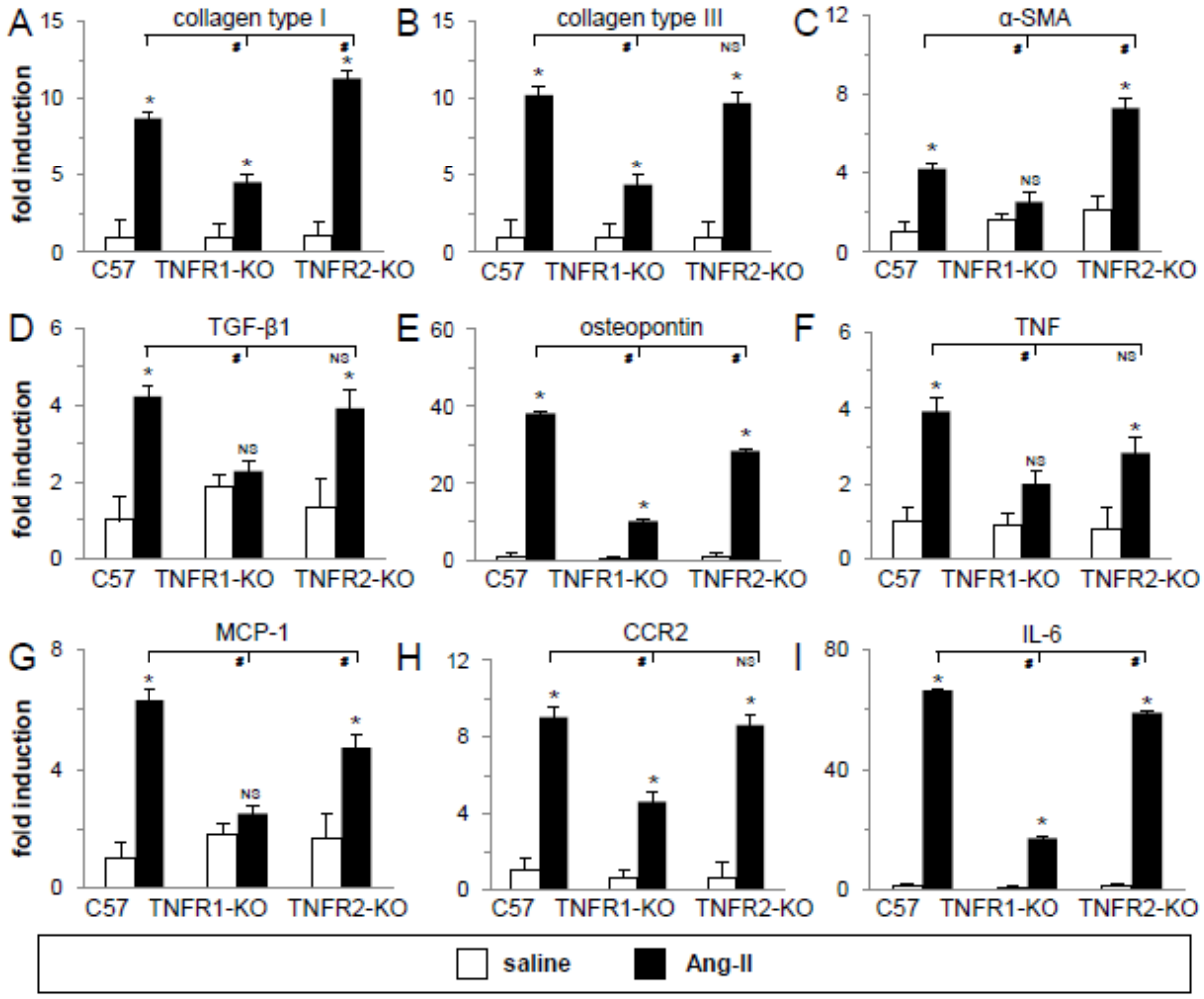

Figure 4: Total myocardial RNA was isolated and subjected to real-time PCR using specific primers for the indicated genes and SYBR Green. mRNA expression for each gene was calculated as fold induction compared to the saline-treated wild-type (C57) group. Control mice received saline (n=3-5/group), treated mice received Ang-II for 1 week (n=8/group). *P<0.05 between Ang-II- and saline-treated groups (same genetic background). #P<0.05 compared to Ang-II-treated wild-type group. NS = no significant difference (Figure taken from [15]).

3.4 Mice deficient in TNFR1 signaling developed less cardiac hypertrophy than wild-type mice in response to Ang-II infusion

Wild-type and both knockout mice lost body weight after one week of Ang-II infusion, which was not different between individual groups. Whole heart weights, however, increased in wild-type and TNFR2-KO mice, but not in TNFR1-KO, in response to Ang-II infusion. As a result, heart weight-to-body weight ratio in wild-type and TNFR2-KO mice was significantly higher than in TNFR1-KO mice (6.1±0.1 vs. 5.9±0.1 vs. 5.2±0.04, Figure 5**Error! No bookmark name given.** A-C). These data show that TNFR1 was involved in Ang-II-mediated cardiac hypertrophy.

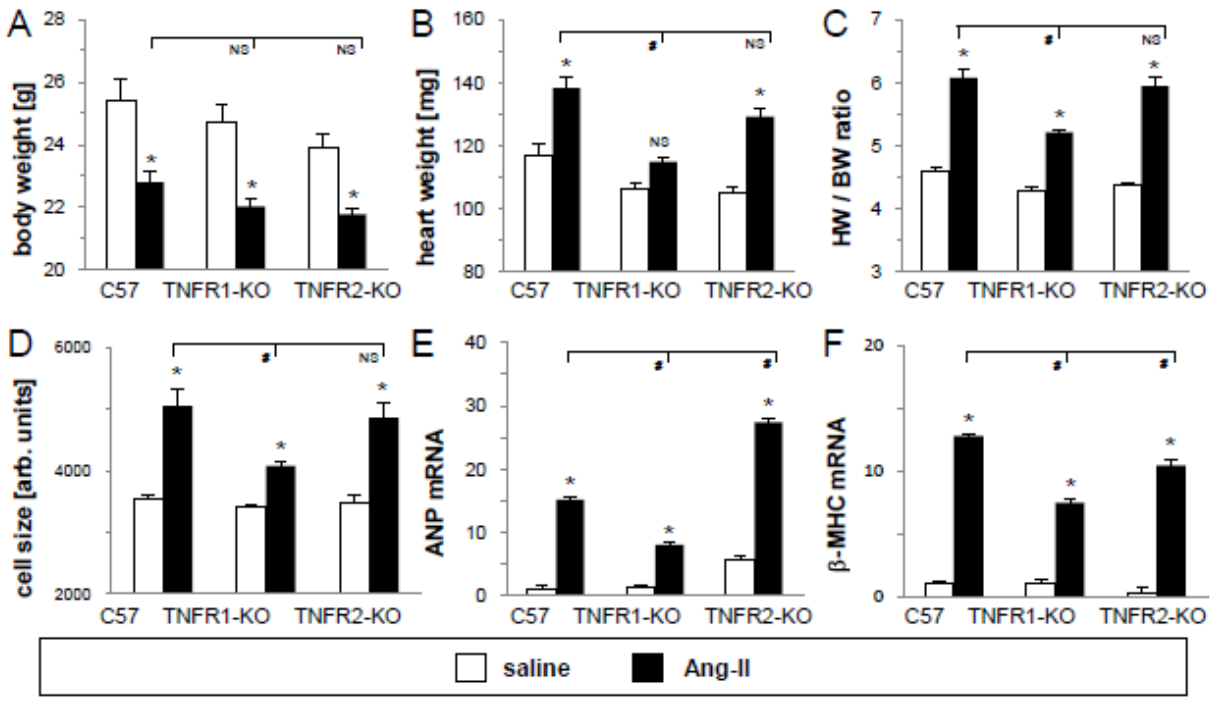

Figure 5: A-C) Body weights and whole heart weights were recorded at time of heart isolation after 1 week Ang-II treatment (n=8/saline, n=16/Ang-II groups). D) The average cardiomyocte size (in arbitrary units) within the left ventricle was measured by staining heart sections with wheat germ agglutinin (n=3/saline, n=5-6/Ang-II groups). Real-time transcriptional regulation of ANP and β-MHC was determined using specific primers and SYBR Green (n=5/saline, n=6-8/Ang-II groups). *P<0.05 between Ang-II- and saline-treated groups (same genetic background). #P<0.05 compared to Ang-II treated wild-type group. NS = no significant difference (Figure taken from [15]).

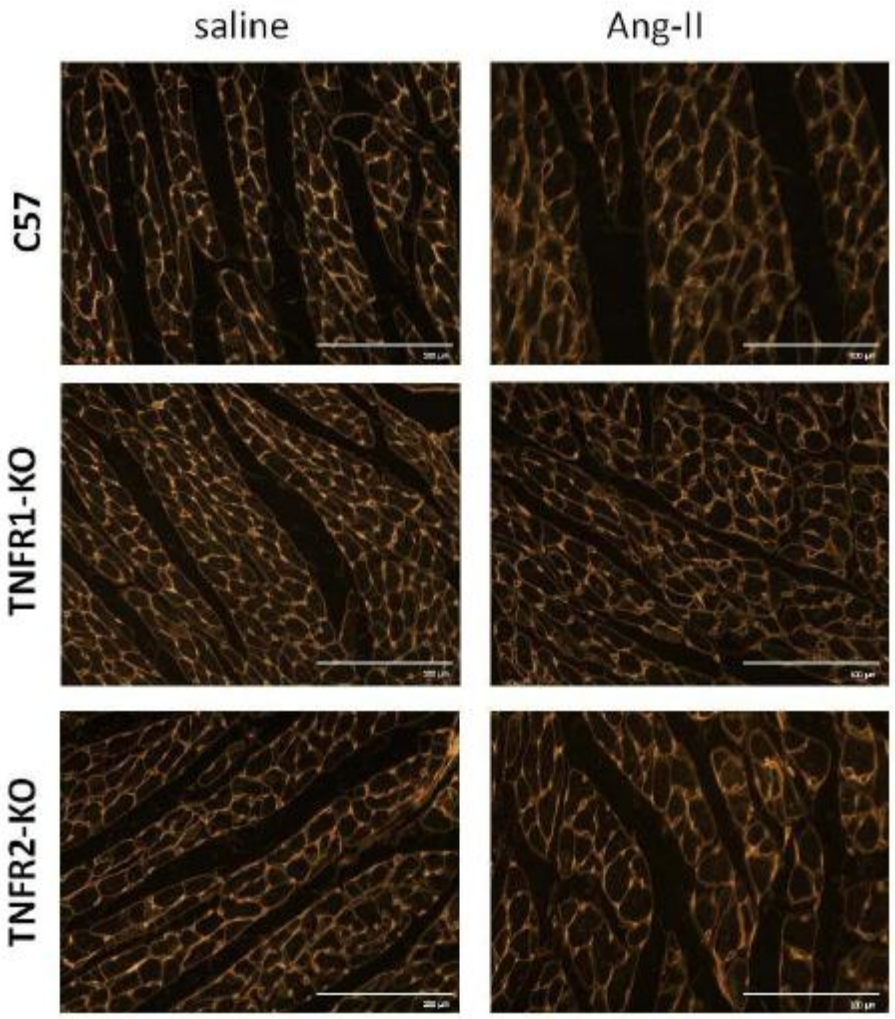

Figure 6: Hearts were arrested in diastole by infusion of cardioplegic solution and perfusion fixed. Deparaffinized heart sections were stained with tetramethylrhodamine-labeled wheat germ agglutinin to visualize cell membranes, and the cell areas were determined in arbitrary units per cell. After 1 week of Ang-II exposure, the average cardiomyocyte size increased in all three mouse types, but this increase was smaller in TNFR1-KO hearts than in C57 wild-type and TNFR2-KO hearts. Control mice received saline (image magnification: x400) (Figure taken from [15], supplementary material).

We also measured the size of cardiomyocytes in wheat germ agglutinin-stained (WGA) heart sections (Figure 5 D, Figure 6). Our measurements show a 43% increase in size in wild-type hearts and a 40% increase in size in TNFR2-KO mice, but a significantly smaller increase of 20% in TNFR1-KO mice. Also, the mRNA level of atrial natriuretic peptide (ANP) and beta-myosin heavy chain (β-MHC), two hypertrophy-related genes, increased in all three mouse groups, but this increase was significantly greater in wild-type and TNFR2-KO hearts than in TNFR1-KO hearts after Ang-II infusion (Figure 5 E-F). These data show that TNFR1 was involved in the development of cardiac hypertrophy during Ang-II exposure.

4. DISCUSSION

Our lab has recently shown that continuous infusion of Ang-II to mice induced the synthesis of MCP-1 and uptake of myeloid $CD34^+CD45^+$ fibroblast precursor cells into the heart [9]. These cells differentiated into fibroblasts that produce collagen and thus mediated cardiac interstitial fibrosis. We also demonstrated that infusion of Ang-II to mice lacking MCP-1 did not induce the presence of this monocytic fibroblast population, nor was cardiac fibrosis observed in these mice. Furthermore, deletion of MCP-1 inhibited Ang-II-driven expression of TNF in the heart [9]. TNF has strong pro-inflammatory and some pro-fibrotic properties [12, 18, 19]. Therefore we were interested if TNF is involved in the Ang-II-mediated initial inflammatory response and resulting cardiac fibrosis.

To tackle this question, we exposed mice deficient in either one or both TNF receptors to Ang-II for one week. In the absence of both TNF receptors, TNFR1 and TNFR2, Ang-II did not induce deposition of collagen in the heart. These results supported the literature in that TNF was able to affect the renin-angiotensin system [20-22]. For instance, one study showed that the deleterious effects of TNF overexpression in the heart were reduced by blocking Angiotensin-II type 1 receptor, AT1 [22]. Other studies implicated an involvement of TNF in the Ang-II-mediated effects on hypertension and hypertrophy [23, 24]. In addition, in vitro, TNF increased the AT1 receptor on cardiac fibroblasts, thereby sensitizing these cells to Ang-II [25]. As inflammation is a key component in Ang-II-mediated cardiac hypertrophy and fibrosis, a direct involvement of TNF was suggested.

We were specifically interested in the roles of the two distinct TNF receptors, TNFR1 and TNFR2 in Ang-II-mediated cardiac fibrosis. Since the receptors differ considerably within their intracellular regions, distinct functions are likely. The literature suggests that TNF exerts its deleterious effects through TNFR1, whereas TNFR2 is thought to be responsible for the protective effects of TNF [26, 27]. In our experiments, mice lacking TNFR1 did not show the presence of monocytic $CD34^+CD45^+$ fibroblast

precursors in the heart after Ang-II infusion. These data implicate a role of TNFR1 signaling in monocyte-to-fibroblast differentiation and/or attraction of monocytic $CD34^{+}CD45^{+}$ fibroblast precursors. These cells are also thought to contribute to scar formation and normal wound repair, as well as in diverse other pathological fibrotic responses [28]. TNF was shown to stimulate the secretion of chemokines and hematopoietic growth factors in peripheral monocytic $CD34^{+}CD45^{+}$ fibroblast precursors in vivo and to affect the differentiation of monocytes into several different cell types in vitro [29, 30]. We suggest that TNF exerts its effects on monocyte uptake and/or differentiation preferably through TNFR1, since no myeloid fibroblast precursors were found in the heart of mice lacking TNFR1 after Ang-II infusion.

In our model, deletion of TNFR1 indeed prevented the transcriptional upregulation of genes related to inflammation [31, 32]. Since Ang-II-induced cardiac fibrosis may be a result of chronic inflammatory injury, suppressed upregulation of inflammation-related proteins may explain the reduced collagen deposition in TNFR1-KO mice. In addition, key fibrosis-related genes [32, 33] were also not upregulated in TNFR1-KO mice after Ang-II exposure. By contrast, both inflammatory and fibrotic genes were upregulated to similar extents in wild-type and mice lacking TNFR2. Thus, our data also argue against a protective role of TNFR2 in Ang-II-mediated cardiac fibrosis, as mice deficient in TNFR2 did not develop worse remodeling than wild-type mice. Based on these results, we suggest that TNF is involved in the development of Ang-II-induced fibrosis via signaling through TNFR1, but not TNFR2, rendering TNFR1 as the main potential target for further investigations.

5. CONCLUSION

TNF signaling via TNFR1 was required to increase MCP-1-driven uptake of $CD34^{+}CD45^{+}$ fibroblast precursor cells due to Ang-II exposure. In addition, TNFR1 was necessary for the Ang-II-induced transcriptional upregulation of key fibrosis- and inflammation- related genes. Genetic deletion of TNFR1, but not TNFR2, prevented the development of cardiac hypertrophy and cardiac fibrosis.

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